

IMPERIAL

Imperial College London
Department of Bioengineering

Submitted for the Degree of
Doctor of Philosophy

Engineering division of labour in *Yarrowia lipolytica* to relieve the metabolic burden of polysaccharide degradation

Eliza Atkinson

Submitted: 24th January 2025

Supervisor: Dr Rodrigo Ledesma-Amaro
Co-supervisor: Prof. Guy-Bart Stan

Abstract

Agricultural and food wastes are a promising renewable, low-cost feedstock for bioproduction of value-added compounds. Creating a consolidated bioprocess using these complex substrates in a single microorganism is limited by metabolic burden; the overexpression of heterologous genes redirects endogenous resources from essential processes, thus reducing the growth and productivity of the cell. Microbial consortia, employing division of labour (DOL) between cells, can mitigate this burden and achieve waste utilisation beyond that attainable in monocultures. In this thesis I investigate the use of DOL in consortia for the degradation of plant-derived polysaccharides by *Yarrowia lipolytica*, a promising host for a range of biotechnological applications.

Firstly, I explore the relationship between expression, secretion and metabolic burden. I design and engineer a 2-strain consortium for the degradation of starch, with one strain expressing α -amylase and the other expressing glucoamylase. I model this system and identify *in silico* a threshold of expression above which DOL delivers faster growth compared to a monoculture co-expressing both amylases. *In vivo* I identify *Y. lipolytica* consortia which can achieve higher growth rates than their equivalent monoculture on starch as the sole carbon source. The performance of these starch-degrading consortia is determined by the strongest amylase strain.

Secondly, I explore how designing different relationships between the two cells in a consortium alters their behaviour compared to monoculture. I design and engineer 2-strain consortia with one strain expressing α -amylase, for starch degradation, and the other expressing xylanase for xylan degradation. Here, I use the substrate specificities for glucose and xylose to create different population dynamics between the two cells. I observe that the generalist consumer DOL strategy, where both cells can consume both glucose and xylose, provides the greatest growth advantage over a monoculture equivalent and achieves a 14.11% increase in β -carotene production compared to the monoculture strategy.

Declaration

I hereby certify that everything included in this thesis is my own work, and in the instances where work has been used from other sources, it is acknowledged appropriately.

In the preparation of this work, generative AI (Chat GPT-3.5 by OpenAI) was employed exclusively for the purposes of aiding in the search for academic literature and rewording specific sections for enhanced clarity and conciseness, in accordance with Imperial College London AI Guidance. All research, interpretation, and writing were independently conducted by the author.

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC). Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose. When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes. Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Acknowledgements

This thesis would not have been possible without the support and guidance of many individuals, to whom I am sincerely grateful.

First and foremost, I would like to express my deepest gratitude to my supervisors, Rodrigo Ledesma-Amaro and Guy-Bart Stan. Your guidance has not only helped create the contents of this thesis but has also shaped me as a researcher. To Rodrigo, thank you especially for constantly reaffirming that the project is going well, even when I doubted myself, and for creating such a welcoming lab full of inspiring people. To Guy, thank you for always challenging me to think critically and motivating me to embrace coding. Thank you also to the EPSRC Biodesign Engineering CDT for the funding to support this project, and to Geoff and David for keeping the CDT running and supporting me during my time as student representative.

To my parents, thank you so much for the lifetime of love and support you have given me. To Mum, thank you for always being the one I can go to when things are tough, and thank you especially for reading this entire thesis, and almost every piece of work I've ever written. To Dad, thank you for helping to pay all my bills, and for checking up on me when you are bored at lunch, doing this PhD would have been impossible without you. To Isobel, thank you for being a wonderful sister.

I am also deeply grateful to my labmates, both in RLA lab and in the Centre for Synthetic Biology, who created an environment that made even the most stressful of days more manageable. Thank you to all the postdocs, especially Young, Razieh, Piotr, Cinzia and Jing, who have offered their advice throughout the project. Thank you to Piotr and Jing for running the HPLC, to David Bell for running the GC-MS, and to Javiera and Gosia for keeping the entire lab running. And thank you to the lunchtime safe-space crew for all the laughs and free food.

Finally, to my friends. To Ellie, Hannah and Dany, thank you for all the pictures of your furry family members, I love them more than I love you. To Tom, Brad, Ed and Fio, thank you for hanging out with me every week; your insistence on roleplaying Every. Single. Meal. in D&D has really helped hone both my patience and creativity. To all my other friends, I'm sorry I can't thank all of you but know that I love and appreciate you.

This thesis is as much a product of my work as it is a testament to the incredible people I have been privileged to know and learn from during this chapter of my life.

Contents

Contents	4
List of Figures	9
List of Tables.....	12
List of Equations	12
List of Abbreviations.....	13
1. Introduction	14
1.1. Next generation biotechnology: Synthetic biology enables the use of renewable feedstocks	14
1.2. The burden of expressing heterologous genes	17
1.3. Creating microbial consortia for biotechnology	21
1.4. The benefits of microbial consortia for biotechnology	24
1.5. Challenges in microbial consortia and how synthetic biology can help	27
1.5.1. Dynamic control of populations	27
1.5.2. Cross-feeding and syntrophy	28
1.5.3. Modelling tools	30
1.6. How microbial consortia can benefit alternative feedstock degradation	30
1.6.1. Synergistic interactions of hydrolysis enzymes	32
1.6.2. Constructing complex substrate hydrolysis in bioproduction strains.....	33
1.6.3. Expanding substrate specificities.....	34
1.6.4. Combining specialised hydrolysis species with specialised bioproduction species	36
1.6.5. Detoxification of lignocellulose and food waste feedstocks for bioproduction hosts.....	37
1.6.6. Predicting emergent properties in substrate degradation.....	37
1.7. Consolidated bioprocessing in <i>Yarrowia lipolytica</i>	39
1.8. Aims and objectives	42
1.9. Publications resulting from this work	44
2. Chapter 2: Resource-aware whole-cell model of division of labour in a 2-strain <i>Escherichia coli</i> consortium for starch degradation.....	46
2.1. Introduction to this chapter	46
2.2. Creating the model.....	47
2.3. DOL reduces resource redistribution to heterologous genes	50
2.4. Applying the model to DOL in starch-degradation	53

2.5. DOL can achieve higher growth rate on starch than an equivalent monoculture system.....	55
2.6. Adapting the model to yeast	59
2.7. Limitations of this model	60
2.8. Conclusions for this chapter	61
3. Chapter 3: 2-strain <i>Yarrowia lipolytica</i> consortia for division of labour in the degradation of a polysaccharide substrate.	63
3.1. Introduction to this chapter	63
3.2. Comparing the growth of strains expressing amylases with different promoter and signal peptide (SP) combinations.....	64
3.3. Comparing growth of single- and double-amylase strains on glucose.....	70
3.4. Comparing growth on starch of single-cell double-amylase strains and 2-strain consortia of single-amylase strains	74
3.5. Flask cultures for the consortia which achieved higher growth rates than their equivalent monocultures.....	82
3.6. Observing the changes in population of the two amylase strains	85
3.7. Activity of glucoamylase	88
3.8. Multi-copy amylase strains do not increase the advantage of DOL	89
3.9. Using a stronger glucoamylase	90
3.10. Xylan degradation	93
3.11. Conclusions for this chapter	98
4. Chapter 4: DOL between two different substrate degradation pathways	100
4.1. Introduction to Chapter 4	100
4.2. Combining a starch-degrader and a xylan-degrader.....	101
4.3. Designing different DOL strategies	104
4.4. Consortia of a starch-degrading strain and a xylan-degrading strain can achieve a higher growth than either a starch-degrader or xylan-degrader alone	107
4.5. How growth and population dynamics change over time for each DOL strategy	109
4.6. How different DOL strategies grow on different concentrations of starch + xylan media	116
4.7. The population dynamics observed for 10% xylan can be changed by first culturing with the WT strain	122
4.8. How different DOL strategies respond to a change in the polysaccharide content midway through a culture	127

4.9. How culture temperature affects the DOL strategies	129
4.10. How cells respond to oxidative stress caused by formate	130
4.11. Using a stronger amylase may allow the strains to consume more starch, thus allowing similar growth in starch as in xylan	131
4.12. Reducing catabolite repression may improve the growth of the orthogonal and cross-feeding strains	134
4.13. Conclusions for this chapter	136
5. Chapter 5: Substrate degradation DOL applied to bioproduction of terpenoids	139
5.1. Introduction to this chapter	139
5.2. Production of patchoulol on starch	140
5.3. Consortia of one or multiple starch-degrading strains combined with an optimised β -carotene-producer strain.....	144
5.4. Comparing a degrader + producer consortium to an equivalent monoculture	145
5.5. Applying the DOL strategy of a 2-strain starch-degrading consortium to bioproduction of β -carotene	147
5.6. Applying the DOL strategy of a starch-specialist and xylan-specialist consortium to bioproduction of β -carotene	149
5.7. Conclusions for this chapter	150
6. Conclusions and Future work	152
6.1. Summary of results	152
6.1.1. Result of Objective 1: Modelling of metabolic burden in <i>E. coli</i> predicted the range of expression within which a 2-strain consortium could achieve a higher growth rate on starch than an equivalent monoculture	152
6.1.2. Result of Objective 2: DOL in <i>Y. lipolytica</i> between two cells that both contribute to the degradation of the same substrate can achieve a higher growth rate and final OD600 than an equivalent monoculture	153
6.1.3. Result of Objective 3: DOL between two <i>Y. lipolytica</i> cells that degrade different substrates achieves a higher growth when both cells are generalists for both substrates	153
6.1.4. Result of Objective 4: DOL between two specialist degrader and generalist consumer <i>Y. lipolytica</i> cells could achieve higher bioproduction of β -carotene than a monoculture.....	154
6.2. Reflections and limitations	156

6.2.1.	Matching the <i>in silico</i> and <i>in vivo</i> data would have improved the accuracy of the model to predict the behaviour of the starch-degrading consortia.....	156
6.2.2.	Limitations to the quantification of metabolic burden in <i>Y. lipolytica</i>	156
6.2.3.	Competition for substrate limited the ability of a consortia to outperform its component strains.....	157
6.2.4.	Extracellular hydrolysis limited the mutual dependence that could be achieved.....	158
6.2.5.	Single-organism consortia limit the adaptability to environmental fluctuations	158
6.2.6.	Catabolite repression limited the simultaneous consumption of glucose and xylose	159
6.3.	Future opportunities.....	159
6.3.1.	Building up the consortia toolbox for alternative feedstock utilisation	159
6.3.2.	Self-regulating or externally controlled cell populations.....	161
6.3.3.	Impact on the cost and sustainability of bioproduction.....	162
	Concluding statement	164
7.	Materials and Methods.....	165
7.1.	Computational methods	165
7.1.1.	ODE solver and simulation methods	165
7.1.2.	ODEs used for the basis of the single-cell model	165
7.1.3.	ODEs for consortia and monoculture model.....	166
7.1.4.	Parameters	167
7.1.5.	Use of generative AI for Figures	169
7.1.6.	Statistical analysis	169
7.2.	Materials.....	169
7.2.1.	<i>Yarrowia lipolytica</i> strains.....	169
7.2.2.	Plasmids.....	173
7.2.3.	Genes.....	176
7.3.	Media and buffers	180
7.3.1.	<i>E. coli</i> Media (LB) and antibiotics	180
7.3.2.	Yeast Rich/Complex Media (YPD).....	180
7.3.3.	Yeast Minimal Media (YNB)	180
7.4.	DNA assembly methods	180
7.4.1.	YaTK toolkit.....	180

7.4.2.	Gibson Assembly	181
7.5.	Preparation of competent cells.....	182
7.5.1.	Turbo Competent E. coli (NEB).....	182
7.5.2.	One Shot™ TOP10 Chemically Competent E. coli (Thermo).....	182
7.6.	Escherichia coli transformation	183
7.6.1.	Turbo Competent E. coli (NEB).....	183
7.6.2.	One Shot™ TOP10 Chemically Competent E. coli (Thermo).....	183
7.7.	Plasmid verification.....	183
7.8.	<i>Y. lipolytica</i> transformation	184
7.9.	<i>Y. lipolytica</i> colony PCR	184
7.10.	Amylase activity assays	185
7.10.1.	Micro-SIT	185
7.10.2.	Micro-GOPOD.....	186
7.11.	Growth assays	186
7.11.1.	Growth assays in 96-well plates.....	186
7.11.2.	Predicting desirability of each promoter and signal peptide (SP) for growth....	186
7.11.3.	Growth assays in different formats	187
7.11.4.	Growth assays in flasks - timepoints	187
7.11.5.	Testing different inoculation ratios.....	187
7.11.6.	Growth curves in flasks	187
7.11.7.	Fitting growth parameters.....	188
7.12.	Population tracking	188
7.12.1.	Flow cytometry measurements.....	188
7.12.2.	Microscopy measurements	189
7.13.	Product extraction and downstream analysis methods.....	189
7.13.1.	Carotene production, extraction and analysis	189
7.13.2.	Patchouliol production, extraction and analysis	190
8.	Bibliography	192
9.	Appendices	216

List of Figures

Figure 1.1 Metabolic burden	18
Figure 1.2 Approaches to using microbial consortia for bioproduction of value-added compounds	22
Figure 1.3 Benefits of division of labour (DOL) for the degradation of complex feedstocks.....	31
Figure 1.4 Summary of overall project objectives.....	45
Figure 2.1 Weiße's whole-cell modelling framework	49
Figure 2.2 Simulation results for division of labour (DOL) compared to single-cell co-expression	52
Figure 2.3 Model for starch utilisation with division of labour in a two-strain consortium.....	55
Figure 2.4 The effect on growth rate of changing the values of N_a (the number of Cell A) and N_b (the number of Cell B) in the consortium	56
Figure 2.5 Changes in the steady state growth rate with varying transcription rates for α -amylase (ea) and glucoamylase (eb), in monoculture and division of labour (DOL) systems of expression.	57
Figure 2.6 Changes in the steady state glucose with varying transcription rates for α -amylase (ea) and glucoamylase (eb), in monoculture and division of labour (DOL) systems of expression. ..	59
Figure 3.1 Monoculture and consortium systems for polysaccharide degradation	63
Figure 3.2 Micro-SIT for the supernatants of OsAA strains	66
Figure 3.3 Comparison of growth parameters for single-amylase strains grown on glucose.....	68
Figure 3.4 The desirability of each promoter-SP combination for OsAA and TIGAMY.....	70
Figure 3.5 Growth rate and inflection point of pTEF-controlled single- and double-amylase strains on glucose	72
Figure 3.6 Growth rate and inflection point of 8UASpTEF-controlled single- and double-amylase strains on glucose.....	73
Figure 3.7 Growth parameters for pTEF-controlled double-amylase monoculture strains and 2-strain consortia on 1% soluble starch YNB.....	76
Figure 3.8 Growth parameters for 8UASpTEF-controlled double-amylase monoculture strains and 2-strain consortia on 1% soluble starch YNB	77
Figure 3.9 Growth rate for double-amylase monoculture strains and 2-strain consortia with different preparations of 1% potato starch YNB.....	79
Figure 3.10 Inflection point and growth rate for double-amylase monoculture strains and 2-strain consortia on 1% soluble starch YNB with different inoculation Abs600s.....	80

Figure 3.11 Testing different culture formats for growth of <i>Y. lipolytica</i> in 1% potato starch YNB	82
Figure 3.12 Growth of starch-degrading consortia and monocultures in flasks	84
Figure 3.13 Growth of the YTT3-GFP+YTO1-RFP consortium	86
Figure 3.14 Growth and relative populations of the cell types in Y8TT1-GFP+Y8TO1-RFP consortium	88
Figure 3.15 Micro-GOPOD assay of 8UASpTEF-TlGAMY strains	89
Figure 3.16 Growth of strains with two copies of the OsAA and TlGAMY genes	90
Figure 3.17 Starch degradation and growth of AnGAMY-expressing strains	92
Figure 3.18 Growth parameters of xylanase strains on 2% glucose YNB	95
Figure 3.19 Growth parameters of xylanase strains on 1% xylan YNB	96
Figure 3.20 Growth of a xylanase consortium and monocultures on 1% xylan YNB	97
Figure 4.1 Growth of YTO1+TA1XC consortia on a mixture of starch and xylan	103
Figure 4.2 Design of division of labour (DOL) between a starch-degrader and a xylan-degrader	105
Figure 4.3 Growth of different division of labour (DOL) strategies on a mixture of starch and xylan, with different inoculation ratios	108
Figure 4.4 Growth and population dynamics for different division of labour (DOL) strategies for a consortium with a starch-degrading strain and a xylan-degrading strain.....	111
Figure 4.5 Population dynamics of division of labour (DOL) strategies observed under the microscope	115
Figure 4.6 Growth and population dynamics for a 2-strain consortium of a glucose-consumer and a xylose-consumer.....	116
Figure 4.7 Growth curves for division of labour (DOL) strategies on different media compositions	118
Figure 4.8 Population dynamics of different division of labour (DOL) strategies on different media compositions.....	121
Figure 4.9 Growth of different division of labour (DOL) strategies on starch and pre-treated xylan media	123
Figure 4.10 Population dynamics of different DOL strategies on starch and pre-treated xylan media	126
Figure 4.11 Changes in population dynamics after fresh polysaccharide medium is added ...	128
Figure 4.12 Different division of labour (DOL) strategies compared at different incubation temperatures.....	130

Figure 4.13 Different division of labour (DOL) strategies when under formate stress	131
Figure 4.14 Division of labour (DOL) strategies for AnGAMY + AnXlnD consortia.....	132
Figure 4.15 Growth of generalist consumer and orthogonal specialist consortia using AnGAMY for starch degradation.....	133
Figure 5.1 Metabolic pathways for patchoulol and β -carotene biosynthesis in engineered cells.	140
Figure 5.2 Patchoulol production.....	143
Figure 5.3 Bioproduction from consortia of amylase-expressing strains and a β -carotene overproducer.....	145
Figure 5.4 Division of labour (DOL) strategies between starch-degraders and β -carotene- producers.....	148
Figure 5.5 Production of β -carotene from a consortium of xylanase strain YTA1XC and amylase strain Y8TO1XC.....	150
Figure 6.1 Summary of results	155

List of Tables

Table 3.1 Amylase strains engineered for this chapter	65
Table 3.2 Growth parameters for strains grown on glucose with and without RFP and GFP markers	86
Table 3.3 Xylanase strains engineered for this chapter.....	93
Table 4.1 Strains engineered for this chapter.....	106
Table 4.2 Growth parameters for different division of labour (DOL) strategies grown on different media compositions	124
Table 5.1 β -carotene-producing strains engineered for this chapter	146
Table 7.1 Modelling framework from Weiße et al. (2015)	165
Table 7.2 ODEs for starch degradation and glucose consumption in a 2-strain consortium expressing one amylase (ea or eb) per cell, or a monoculture expressing both amylases	166
Table 7.3 Parameters for an <i>E. coli</i> host cell	167
Table 7.4 Parameters for a yeast host cell	168
Table 7.5 Full list of strains	169
Table 7.6 Full list of plasmids	173
Table 7.7 Gene sequences	176
Table 7.8 Antibiotic preparation	180
Table 7.9 Primers used for Gibson assembly of TRP22 level 1 plasmid	182
Table 7.10 Primers used for sequencing level 0 and level 1 plasmids.....	184
Table 7.11 Primers used for colony PCR.....	185

List of Equations

Equation 2.1 Rate of uptake of glucose for consortia	50
Equation 2.2 Rate of uptake of glucose for monoculture	50
Equation 2.3 ODE for glucose	50
Equation 2.4 ODE for starch	54
Equation 2.5 ODE for intermediate	54
Equation 2.6 ODE for glucose when starch is the carbon source	54
Equation 3.1 Gompertz model	67
Equation 4.1 Biphasic growth model.....	101

List of Abbreviations

Abs600	absorbance at 600 nm measured in the plate reader
AnGAMY	<i>Aspergillus niger</i> glucoamylase
AnXlnD	<i>Aspergillus niger</i> exoxylanase (XlnD)
ATP	adenosine triphosphate
CBP	consolidated bioprocess
DNA	deoxyribonucleic acid
DOL	division of labour
ER	endoplasmic reticulum
FBA	flux balance analysis
GC-MS	gas chromatography-mass spectrometry
GFP	green fluorescent protein
GOPOD	glucose oxidase peroxidase
GRAS	generally recognised as safe
GSMM	genome-scale metabolic model
HLXyn2	<i>Hypocrea lixii</i> endoxylanase (Xyn2)
HPLC	high performance liquid chromatography
HXK	hexokinase
OD600	optical density at 600 nm measured with a spectrophotometer
ODE	ordinary differential equation
OsAA	<i>Oryza sativa</i> α -amylase
PTS	patchoulol synthase
RFP	red fluorescent protein
RNA	ribonucleic acid
SP	signal peptide
TlGAMY	<i>Thermomyces lanuginosus</i> glucoamylase
WT	wild-type
XDH	xylitol dehydrogenase
XK	xylulose kinase
XR	xylose reductase
YNB	yeast nitrogen base

1. Introduction

1.1. Next generation biotechnology: Synthetic biology enables the use of renewable feedstocks

Biotechnology harnesses the specificity and scalability of microorganisms for the creation of pharmaceuticals, fuels, food products, and other high-value compounds (Gupta et al., 2016). It offers a range of advantages over traditional production methods, notably in terms of resource efficiency and sustainability. By utilizing renewable feedstocks, minimizing dependence on fossil fuels and harsh chemicals, and by reducing waste and greenhouse gas emissions, biotechnology emerges as a more environmentally friendly and economically viable choice for production processes (Gavrilescu & Chisti, 2005).

Synthetic biology acts as the ideal partner to empower biotechnology. The design and construction of customised biological parts for novel synthesis pathways allows microorganisms to produce almost any biological molecule found in nature. The synthetic biology principles of standardisation and modularity, as well as the application of engineering design-build-test-learn cycles, allows biotechnology to rapidly prototype and optimise a production pathway (Garner, 2021). As such, synthetic biology is poised to enable further advancement towards ever more sustainable bioproduction.

One essential area of biotechnological advancement is the use of alternative sustainable feedstocks. Current biotechnology is over-reliant on monosaccharide feedstocks, in particular glucose, which requires numerous costly and energy-intensive steps to produce. There is increasing interest in, and demand for, biotechnology that uses feedstocks derived from the waste products of the agricultural and food industries (Ezeorba et al., 2024; Bayer et al., 2023; Sufficiency et al., 2022). Utilising waste, so that it does not end up being incinerated or dumped in landfill, is a primary sustainability goal for many countries around the world, including the UK (UK Department for Business, 2015).

Agricultural waste describes the non-commercially-viable and non-edible parts of crops or plants and byproducts that are generated during agricultural processing, including crop stems, roots, leaves, and husks (Ezeorba et al., 2024). Annual global agricultural waste is estimated at around 3.8 billion metric tonnes, of which 74% is from cereals, 8% from legumes, 3% from oil crops, 10% from sugar crops and 5% from tubers (Lal, 2005). Agricultural waste comes mainly in the form of lignocellulose, made up of cellulose, hemicellulose and lignin. These polymers can be broken down into sugar feedstocks for biotechnology. In traditional processing, lignocellulosic biomass is pretreated, usually physically and/or chemically to remove the lignin,

followed by hydrolysis of cellulose and hemicellulose polymers to fermentable sugars (Kumar et al., 2020). Processes such as acid/alkali and thermal hydrolysis are energy-intensive and expensive processes. Enzymatic hydrolysis has gained favour as it is cheaper, creates fewer toxic byproducts and can result in higher yields (Li et al., 2024b; Bayer et al., 2023).

Food waste describes edible items which are discarded by businesses and homes; this includes avoidable food waste, such as leftovers and expired food, and inevitable food waste such as bones and eggshells (Wang et al., 2022). According to the Food and Agriculture Organization of the United Nations, 1.3 billion tonnes of food is lost or wasted per year globally, which is almost a third of the total food produced (High Level Panel of Experts on Food Security and Nutrition (HLPE), 2014). In the UK, 10.7 million tonnes of food waste was produced in 2021 (Malik et al., 2024). Of this, 29% was inedible, and could be repurposed for biorefinery feedstocks without competing with potential food supplies. Food waste can contain a wide range of macronutrients, including lignocellulose; lignin can be found in stems and shells, cellulose and hemicellulose can be found in vegetable peels and fruit skins. But in addition, food waste can contain other components such as starch found in foods like potatoes and grains, pectins found in fruits, chitin found in shellfish, proteins found in meat scraps, and fats found in kitchen grease, cooking oil and dairy (Moonsamy et al., 2024; Słopiecka et al., 2022). Food waste at the consumer-level, from households and the hospitality sector, accounts for 46% of the total waste in the UK (Jeswani, Figueroa-Torres & Azapagic, 2021). However, a significant amount of edible food is also discarded before it reaches the consumer. This can be due to fluctuating market prices making harvesting not cost-effective, spoilage in transit, quality control standards and throwing away food because of its aesthetics (Jeswani, Figueroa-Torres & Azapagic, 2021). Food waste has been traditionally dealt with by incineration, composting, using as animal feed or dumping in landfill. The latter has further consequences in the destruction of land, soil pollution and production of greenhouse gases. In the UK food waste generates 18 million tonnes of CO₂ equivalent emissions annually (Malik et al., 2024) and overall contributes 17% to the total greenhouse gas emissions from the UK food chain (Jeswani, Figueroa-Torres & Azapagic, 2021). However, as the mantra of recycling and composting has become more publicly popular, food waste valorisation has gained traction in the biotechnology industry as well (Sufficiency et al., 2022). Like lignocellulose, food waste usually requires chemical, hydrothermal, or physical pre-treatment in order to release the macromolecular substances and promote the breakdown of fats, proteins and carbohydrates into small molecules (Wang et al., 2022).

Agriculture and food waste is typically valorised at biorefineries using one of two methods. The first is separated hydrolysis and fermentation (SHF), where the feedstock is first hydrolysed by

commercial enzymes, such as cellulases, amylases, or proteases, and the resulting sugar purified before being added to a separate bioreactor for fermentation to product. This strategy allows the two steps to be carried out in their individual optimal conditions. However, the disadvantages of SHF are the high cost of commercial enzymes and the difficulty in recycling them, as well as the increased infrastructure required to run both processes (Liu et al., 2021b). The second method is simultaneous saccharification and fermentation (SSF), where both steps can be carried out in a single bioreactor. This reduces infrastructure costs and streamlines the overall process, as the end-product of degradation is immediately used in fermentation and product formation (Olofsson, Bertilsson & Lidén, 2008). However, both SHF and SSF rely on the hydrolytic enzyme cocktails being prepared in a separate industrial process, and are therefore limited by the cost of commercial enzymes (Liu, Zhang & Bao, 2016).

Consolidated bioprocesses (CBP) are the gold standard of bioproduction, integrating enzyme production, degradation and product formation into a single bioreactor setup, thereby eliminating the need to buy commercial enzyme cocktails. Bioengineering approaches to CBP have historically focussed on creating a single cell, or monoculture, that can both produce hydrolytic enzymes and produce a desirable product. This CBP microorganism can be created using one of two approaches, depending on the natural abilities of the initial parental microorganism. In the first approach, a naturally-hydrolytic microorganism is engineered to either improve or introduce *de novo* the production of a value-added product. In the second approach, recombinant enzymes for degrading feedstocks are introduced into either well-studied model organisms, with developed genetic tools, such as *Escherichia coli* or *Saccharomyces cerevisiae*, or into species which have specific bioproduction capabilities. The advantages and disadvantages of these two strategies have been reviewed in detail (Li et al., 2024b; Beig et al., 2021; Liu et al., 2021b). In short, both strategies have significant challenges that hinder the development of single cells or monocultures that can do everything. The first approach is limited by a lack of genetic tools for the most promising naturally-degrading species, complicated transformation protocols for these species, a mismatch in the optimal conditions for hydrolysis versus fermentation, and the inhibition of hydrolysis by the end-products. The second approach is challenging due to the many diverse enzymes and cryptic protein-protein or protein-microbe interactions which are required for optimal hydrolysis, and thus the combinatorial challenge of expressing these enzymes in a new host. A significant limitation for both approaches is they require many new heterologous proteins being expressed in a non-native host, which can lead to metabolic burden.

1.2. The burden of expressing heterologous genes

Overexpression of heterologous genes in host cells can lead to decreased growth rates and reduced yields of product (Borkowski et al., 2016). This negative effect is generally known as “metabolic burden”, and is the result of energy and resources being diverted away from essential endogenous bioprocesses (Boo, Ellis & Stan, 2019; Wu et al., 2016; Glick, 1995). As shown in Figure 1.1, metabolic burden can occur at the transcriptional or translational level, redirecting ATP, ribosomes or polymerases towards heterologous genes and depleting nucleotides and amino acids (Ellis, 2019; Kafri et al., 2016). It can also occur at the pathway level, where ATP, redox cofactors, or specific precursor metabolites are redirected towards a desired bioproduction pathway (Jiang et al., 2020). The depletion of chaperone proteins for secretion and proteases for degradation can consequently lead to harmful aggregation of proteins within the cytoplasm (Snoeck, Guidi & De Mey, 2024). In host cells burden also contributes to the instability of recombinant expression plasmids, as it acts as a selection pressure towards downregulating or silencing the heterologous genes (Silva, Queiroz & Domingues, 2012).

While the term burden most often reflects the reallocation of endogenous resources, it can also refer to other negative interactions between host and heterologous components, such as off-target effects, toxicity and cross-talk (Ellis, 2019). When introduced to a new proteome and metabolome, heterologous proteins may be promiscuous, causing off-target effects with any number of impacts throughout the cell (Cardinale & Arkin, 2012). In addition, whether heterologous pathways behave as expected or not, they can create toxic intermediates and byproducts which the host cell has no way to deal with (Jiang et al., 2020). Cross-talk, unintended interactions between signalling components, is a significant issue when creating programmable cellular responses (Perrino et al., 2021; Boo, Ellis & Stan, 2019). Additionally, resource competition creates an indirect dependency between pathways that are supposed to be independent; this can lead to unexpected activation or inactivation of rationally designed genetic circuits (Zhang et al., 2021).

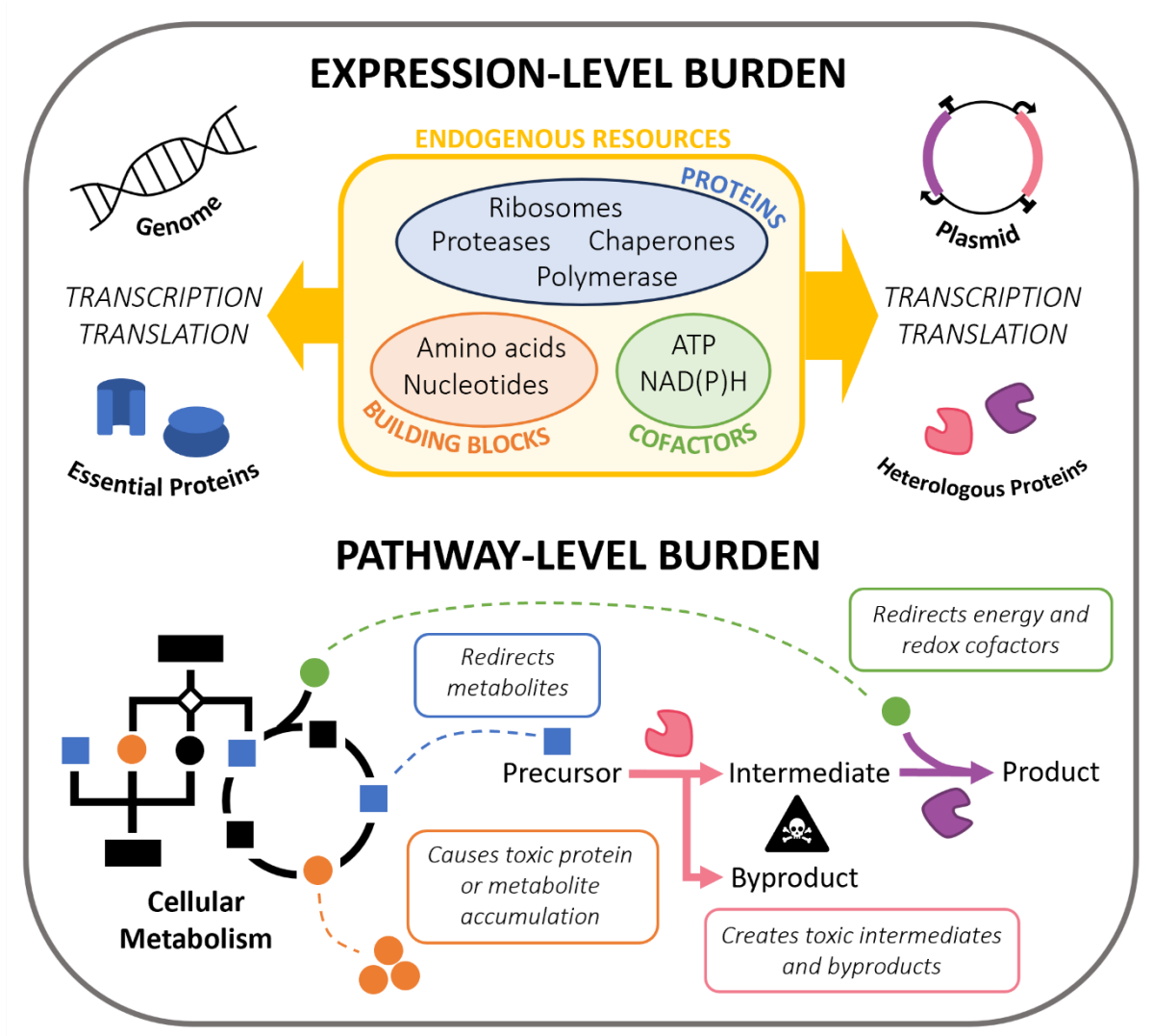


Figure 1.1 Metabolic burden

Metabolic burden refers to the stress placed on a cell when it is engineered to produce heterologous or non-native compounds. This burden is caused by the additional demand for endogenous resources, such as energy, cofactors, transcription and translational machinery and building blocks, as well as precursor metabolites, which are diverted away from essential cellular processes like biomass production, cellular maintenance, and division. In addition, heterologous bioproduction pathways can lead to toxicity through the creation of new intermediates and byproducts, and through the redirection of folding chaperones, transporters and proteases, leading to the aggregation of proteins.

Metabolic burden has been extensively studied in *Escherichia coli*. Rouches *et al.* constructed a plasmid system with tunable copy number and single-cell analysis was used to show that *E. coli* plasmid copy number and metabolic burden are linearly related. Each plasmid resulted in a 0.063% decrease in the host's metabolic efficiency (Rouches *et al.*, 2022). In *E. coli*, metabolic burden is usually linked to the energy demands of translation elongation and the limited availability of ribosomes (Scott & Hwa, 2011; Scott *et al.*, 2010a). Other research suggests that

the metabolic burden does not come from the resource demands of translation itself, but from the interaction of what is described as “toxic” mRNA with cellular components (Singh, Verma & Gaur, 2013), and from errors in protein folding (Li & Rinas, 2020). Codon optimisation, which is used extensively in synthetic biology to improve expression of a heterologous gene, is often highlighted as a burden-causing process. While codon optimisation does not change the amino acid sequence, it will alter the secondary structure of the mRNA, which has an important role in its function (de la Torre & Chin, 2020). Certain synonymous codon sequences cause significant metabolic burden, either through an unknown mechanism that relies on specific “toxic” RNA sequences (Mittal et al., 2018), or through altering the co-translational protein folding (Walsh et al., 2020). In addition codon optimisation has been shown to lead to excessive depletion of the most common tRNA, which stalls the expression of endogenous genes and activates stress responses (Snoeck, Guidi & De Mey, 2024; Bange et al., 2021).

While prokaryotes, like *E. coli*, have often been the focus of metabolic burden research, many of the underlying principles, such as resource limitations, cellular stress, and energetic costs, are also highly relevant to eukaryotes. Increasing heterologous gene expression, or increasing gene length, leads to decreased cell growth and productivity in *Saccharomyces cerevisiae* (Tomala & Korona, 2013; Görgens et al., 2001; Gopal, Broad & Lloyd, 1989). This is attributed to energy limitations and reduced endogenous proteins (Van Hoek et al., 1998). Supplementing growth media with amino acids has been able to rescue the growth in some burdensome processes (Görgens et al., 2005; Ramírez & Bentley, 1993). This suggests that in yeast, as in bacteria, translation is a significant contributor to the cost of heterologous gene expression. However, yeast have also shown a greater ability to adapt to overexpression, as their ribosome pool is less limited, they can increase their physical size and they can upregulate essential endogenous genes in response to burden (Kafri et al., 2016). For yeast, metabolic burden is often seen when large heterologous proteins need to be secreted from the host cell, such as during the production of antibodies (de Ruijter et al., 2018) or insulin (Kazemi Seresht et al., 2013). Protein folding, post-translational modification and secretion require a lot of energy, redox cofactors, enzymes and chaperones. In addition, when heterologous proteins are overexpressed, the endoplasmic reticulum (ER) can become overloaded, leading to oxidative ER stress and activation of the unfolded protein response, which downregulates protein production in order to relieve the ER. Prolonged ER stress can reduce cell growth and lower yields in biotechnological applications (Zahl et al., 2019; Idiris et al., 2010). This is particularly relevant to CBP as hydrolytic enzymes must be secreted into the environment to carry out their action. In yeast, metabolic burden has

been an issue for the secretion of cellulases (Wei et al., 2018) and xylanases (Görgens et al., 2001).

In synthetic biology managing metabolic burden in all its forms is crucial. If the burden is too high, the engineered system can fail, or its performance may decrease significantly over time. As such, synthetic biologists have come up with several techniques to measure burden. Host-aware or constraints-based models have been developed which explicitly account for resource reallocations and other interactions between synthetic pathways and the host (Kerkhoven, 2022). Flux balance analysis (FBA) models have made several strides toward fully host-aware models: incorporating transcriptional regulation (Chandrasekaran & Price, 2010), macromolecular synthesis and its related costs (Dahal, Zhao & Yang, 2020; Goelzer et al., 2015; O'Brien et al., 2013) and other intracellular resource allocations (Regueira et al., 2021; Zeng & Yang, 2020; Jeanne et al., 2018). Coupling these models with ^{13}C -based metabolic flux analysis allows a more quantitative measurement of how heterologous protein expression affects endogenous metabolic pathways (Guo, Sheng & Feng, 2016; Klein et al., 2014). Coarse-grained models have also incorporated the mechanisms of metabolic burden, explicitly modelling the finite pools of energy and ribosome allocations (Nikolados, Weiße & Oyarzún, 2021; Darlington et al., 2018; Weiße et al., 2015). These modelling tools will likely be essential for the future of consortia for substrate utilization as the links between resource allocation, burden, growth and cooperation are intrinsic but challenging to unravel experimentally.

Better understanding of burden leads to new ways to engineer host cells to moderate it (Perrino et al., 2021; Jiang et al., 2020). *In vivo* tools for measuring and mitigating burden include real-time fluorescence measurements of burden (Ceroni et al., 2015), and feedback-regulation which adjusts the expression of a heterologous protein in response to various forms of burden (Ceroni et al., 2018; Siu et al., 2018; Dahl et al., 2013). Dynamic pathway engineering, combining biosensors with bioproduction, allows cells to autonomously adjust their flux in response to their metabolic state (Han, Kim & Seo, 2023; Hartline et al., 2021). These techniques have proven effective to improve the production of heterologous proteins and may prove effective in reducing the burden of CBP. However, even the best engineered cells still maintain intrinsic limitations to the number of heterologous proteins they can express. One solution to overcoming metabolic burden is using division of labour (DOL) between different host cells in a consortium.

1.3. Creating microbial consortia for biotechnology

In nature, microorganisms live symbiotically in communities of multiple species. Mirroring this concept by employing microbial groups, or consortia, could be the solution to many limitations of engineered monocultures, including the limitations on CBP. The consortia applied in biotechnology can fall within a range based on the changes made by scientists: one end of the scale uses isolates taken directly from nature, referred to as the top-down approach, while the other end, the bottom-up approach, uses fully-synthetic consortia with rationally designed interactions (Rafieenia, Atkinson & Ledesma-Amaro, 2022).

In the top-down approach the goal is to exploit and refine the inherent capabilities of natural microbial consortia (Figure 1.2A). Natural consortia have always had a role in industrial processes including biogas formation, bioremediation and food production (Rafieenia, Atkinson & Ledesma-Amaro, 2022). These complex mixtures of microbial species already perform a specific desired function without the necessity of further genetic changes. These consortia are isolated from nature with their full native complexity intact, preserving their natural interactions which are often critical to their function. However, the disadvantage of using natural consortia is that they are poorly characterised, making specific characteristics difficult to adjust (Lyu et al., 2024; Cao et al., 2022). The conventional method to improve the performance of a natural consortium towards a specific purpose is to artificially enrich the culture. This promotes the growth of specific groups of microorganisms using selective environmental conditions to direct the evolution of the culture towards the bioengineering goal (Lin, 2022; Lawson et al., 2019). Systems biology approaches, such as genomics, transcriptomics, proteomics and metabolomics can be used to identify the groups of microorganisms responsible for the desired characteristics (Lyu et al., 2024). Metabolic and mathematical modelling can treat the microbial consortia a bit like a black box: considering inputs like feedstocks and environmental inducers, and outputs such as microbial growth and product yields to predict how the consortia will behave (Lawson et al., 2019). These models can help identify the environmental conditions needed to enrich the culture towards a desired goal.

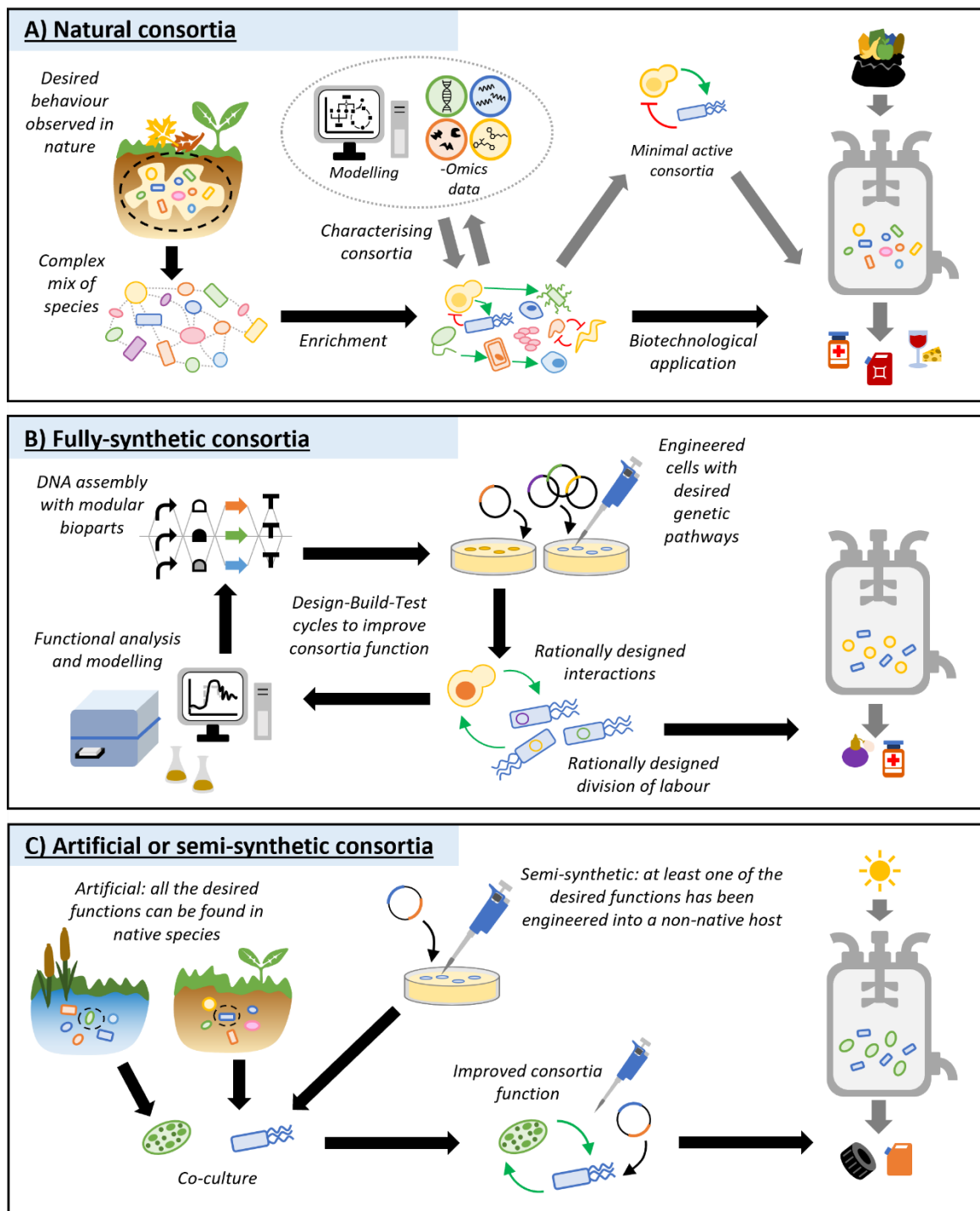


Figure 1.2 Approaches to using microbial consortia for bioproduction of value-added compounds

A) The use of natural consortia for bioproduction relies on existing microbial communities that have evolved to interact and perform specific functions in their natural environments, often making them complex, unpredictable, and less optimized for industrial purposes. Natural consortia are used commonly in bioremediation and conversion of waste feedstocks into value-added compounds. **B)** In contrast, synthetic consortia are engineered *de novo*, allowing for precise control over species composition, metabolic pathways, and interactions to tailor them for specific bioproduction goals. Synthetic consortia combining prokaryotes and eukaryotes are common in pharmaceutical and aroma production. **C)** Artificial and semi-synthetic consortia combine natural and synthetic microorganisms, such as combining a naturally photosynthetic host with a bioproduction host.

On the other hand, the bottom-up approach creates fully-synthetic consortia (Figure 1.2B) using well-characterised organisms with tractable heterologous functions. The bottom-up design of consortia applies the key principles of synthetic biology: standardization, modularity, and rational design (McCarty & Ledesma-Amaro, 2019). In synthetic consortia, all functions and interactions are strategically engineered, allowing full customisation and tunability, as new species, strains or functions can be added or removed as needed without reliance on the microorganisms' natural abilities. This flexibility allows for rapid optimization in a design-build-test-learn cycle to create microbial consortia for specific production goals (Lawson et al., 2019). Because the microorganisms employed are well-characterised, models of these consortia can be more comprehensive, allowing them to predict more precisely how metabolic products are exchanged between different microbes and how they collectively contribute to the overall system's function (Colarusso et al., 2021; Thommes et al., 2019). Importantly, compared to organisms from natural consortia, which may require unusual environmental conditions which are problematic to replicate and maintain in industry, synthetic consortia can be designed with model organisms that grow in well-defined and controlled environments. On the other hand, due to their comparative simplicity compared to natural consortia, synthetic consortia may lack the natural stability and resilience of complex communities, making them more vulnerable to environmental variation or disturbance (Lyu et al., 2024).

By working with natural communities, top-down approaches may capture unpredictable functional capabilities that arise from complex microbial interactions, which might not be easily replicated in a bottom-up approach. However, the complexity of these microbial interactions can make it difficult to pinpoint the exact contributions of individual species or metabolic pathways to the consortium's overall functionality. By designing consortia *de novo*, the bottom-up approach allows precise control over the composition, interactions and behaviour of the community. However, since current synthetic consortia are limited in size, they still cannot always capture the intricate interactions and functions evolved in natural systems.

Top-down and bottom-up approaches can overlap. For example, enrichment of a natural consortia can lead to the identification of minimal active consortia. By determining the key functions in a complex consortium and distinguishing individual microorganisms with those functions, a minimal consortium can be created with fewer species that maintains the original consortium's function. By reducing complexity, minimal consortia allow for easier characterisation and the application of bottom-up approaches to adjust how the individuals and overall consortium behaves. For example, Puentes-Téllez & Falcao Salles, 2018 isolated a natural consortium from soil in a sugarcane plantation, which was then enriched and screened,

to select eighteen lignocellulosic microorganisms. These microbes were then combined in sixty-five different 5-species minimal active consortia, each with different functional diversity. This allowed them to identify the best 5-species consortia that maintained 95% functionality of the whole 18-strain consortium (Puentes-Téllez & Falcao Salles, 2018).

The top-down and bottom-up approaches can also be combined to make artificial or semi-synthetic consortia (Figure 1.2C). Here, I refer to artificial consortia as those designed by picking and choosing from organisms found in nature and combining them in novel ways. This is not a true top-down approach, as these organisms are not found together in nature, but their primary desirable function is not genetically altered, so it is not a complete bottom-up build either. Alternatively, if a bioprocess does not exist in microorganisms naturally, for example many value-added compounds are only naturally synthesised in plants, then engineered microorganisms might be used in combination with natural microorganisms. I label these as semi-synthetic consortia, where at least one member is designed with a fully bottom-up approach. Choosing to use species which already perform desirable functions can be time-saving in the design process, as natural selection has likely accomplished a large amount of optimisation already. However, artificial consortia often suffer from an incompatibility between species that have evolved to prefer very different environments (Qian et al., 2020; Shahab et al., 2020) Once again, synthetic biology can be used to improve the consortia's ability to grow in new defined environments and to engineer specific interactions between the microorganisms (Atkinson et al., 2022a; McCarty & Ledesma-Amaro, 2019). This can be rational bottom-up design, or it can use a more top-down approach of adaptive laboratory evolution which, like enrichment, uses selective environments to enhance desirable traits. This can result in nonintuitive changes to the consortia which would be difficult to design entirely bottom-up (Ben-David et al., 2020; Niu et al., 2018).

1.4. The benefits of microbial consortia for biotechnology

A significant attribute of consortia, of particular interest to synthetic biologists, is division of labour (DOL). In nature, consortia can achieve complex bioprocesses beyond the capability of single cells because individual species within the group can occupy distinct roles and act cooperatively with other members of the community.

One of the main advantages of DOL is the reduction of expression-level metabolic burden. Since fewer proteins need to be expressed in each component host cell, competition between heterologous and endogenous genes for essential resources is decreased (Tsoi et al., 2018a). For example, the largest single-cell genetic circuit to date contains eleven regulatory proteins (Moon

et al., 2012), which is limited by metabolic burden. But more complex circuits or pathways may be achieved by dividing the workload between multiple cells, with cell-to-cell communication pathways to coordinate the component parts (Regot et al., 2011; Tamsir, Tabor & Voigt, 2011).

By reducing burden, DOL can also reduce instability and cell-to-cell variation of non-native genes. In single cells, over time the cell downregulates or silences a pathway, which can lead to variation, or heterogeneity, between cells and an overall decrease in production (Rugbjerg et al., 2018). In consortia the pressure to silence the heterologous pathway is diminished, therefore cell-to-cell variation is reduced. On the other hand heterogeneity in microbial consortia can be advantageous, as the existence of subpopulations can lead to the emergence of new functions (Wang & Dunlop, 2019).

DOL fits effectively within the synthetic biology ideals of modularity; in a consortium each cell can be used as a modular unit in combinatorial designs (Jawed, Yazdani & Koffas, 2019; Zhang & Wang, 2016a; Zhou et al., 2015). Each cell can be optimised in parallel, and by focusing on fewer proteins in each cell, the time and intricacy involved in refining a pathway are significantly reduced (Zhou et al., 2015; Alper & Stephanopoulos, 2004). The overall pathway or circuit can also be fine-tuned by adjusting the population ratio of different cell strains or species involved, either by adjusting their inoculation ratios or creating dynamic adjustments using synthetic biology tools such as quorum sensing or toxin/antitoxin systems (Duncker, Holmes & You, 2021). This approach allows for greater flexibility and control in achieving the desired outcome without the need to manipulate every individual component. For example, rosmarinic acid biosynthesis requires diverging and converging pathways and such non-linear pathways are difficult to achieve in single cells. The modularity of consortia allowed these branches to be more easily balanced, creating a 38-fold increase in yield compared to monoculture (Li, Wang & Zhang, 2019).

Consortia are also able to reduce the pathway-level burden caused by unwanted interactions between subunits of a pathway. Each part of the pathway can be made orthogonal to the others by separation into different cells, thereby reducing the amount of cross-talk that occurs both within the pathway and with the host (Zhang & Wang, 2016a; Teague & Weiss, 2015; Shong, Jimenez Diaz & Collins, 2012). For example, in the design of synthetic cascading bistable switches, Zhang *et al.* found that resource competition prevented the expected coactivation of both switches. To avoid this, they used DOL in a 2-strain consortium that allowed them to achieve the stable coactivation they required (Zhang et al., 2021). DOL can also be used to spatially segregate promiscuous enzymes, thereby avoiding unwanted byproducts. This approach was

shown to increase monolignol production in *E. coli* up to 12-times that of the monoculture (Chen et al., 2017).

Another advantage of DOL is the enhanced specialisation that can be achieved. Using artificial, semi-synthetic or fully-synthetic consortia allows the microbial hosts to be chosen based on pre-existing functional backgrounds. This is particularly important for very complex bioprocesses. For example, photosynthesis is a highly evolved process that would be very difficult to mimic *de novo*, and photosynthetic organisms can be recalcitrant to engineering (Meng, Liu & Chen, 2022). However, semi-synthetic consortia have been designed with photosynthetic bacteria that have been engineered to produce sucrose, allowing them to feed heterotrophic microorganisms for the production of a range of value-added compounds from CO₂ (Sirohi et al., 2021; Hays et al., 2017). In fully-synthetic consortia, host cells with the correct expression machinery, codon-usage, protein folding, and compartment localization can be specifically chosen for each heterogenous protein. For example, yeast is capable of protein folding and post-translational modifications needed for eukaryotic enzymes, but it has limited capacity for key precursors such as aromatic amino acids. On the other hand, *E. coli* aromatic amino acid titres are currently “an order of magnitude higher than that in *S. cerevisiae*” (Shen et al., 2020). Dividing a pathway between bacteria, which produce high yields of precursors and yeast, which create more complex enzymes, has increased yields of value-added compounds (Yuan et al., 2020; Zhang et al., 2017a; Zhou et al., 2015; Minami et al., 2008).

This DOL strategy can also be used to avoid toxic and inhibitory intermediates; the first strain or species, for which an intermediate is inhibitory, can pass it onto a second cell type capable of metabolising the inhibitory compound (Yuan et al., 2020). For example, this strategy was used by Liu, Yang and Jia in a synthetic consortia of *E. coli* and *Pseudomonas putida* for medium-chain-length hydroxyalkanoic acid (mcl-PHA) biosynthesis. *E. coli* was engineered to preferentially consume xylose and to secrete high concentrations of fatty acids and acetate, the latter of which is toxic to *E. coli* in high concentrations. *P. putida* was engineered to produce mcl-PHA from glucose, fatty acids and acetate, thus removing the toxic compound from the environment (Liu, Yang & Jia, 2020). This consortium demonstrates multiple benefits of DOL: reducing resource competition, reducing toxicity and creating mutually-beneficial cross-feeding that enhances production.

In nature, consortia also tend to have high functional redundancy, meaning different species can perform similar functions, which increases the community's overall resilience to environmental perturbations. If one species is inhibited or experiences a decrease in activity due to adverse

conditions, another species, that performs the same function but has different environmental preferences, can take over, ensuring the overall stability of the community.

1.5. Challenges in microbial consortia and how synthetic biology can help

However, DOL is not guaranteed to be advantageous, because consortia have their own set of challenges. Firstly, despite rational design, some DOL pathways end up with more enzymes overall than would be needed in a single cell. For example, in Minami *et al.*, (2008) it required seven enzymes to complete a biosynthetic pathway in an *E. coli*/*S. cerevisiae* consortium that only requires four enzymes in *S. cerevisiae* alone. Therefore, the benefits of reduced burden and increased specialisation must be carefully weighed against the potential of excess engineering. Secondly, when metabolic pathways are split between multiple cells, there is an additional cost of transporting metabolites between them, both a cost of expressing new transport proteins and a cost of efficiency as the metabolites have to diffuse across the space between cells (Tsoi *et al.*, 2018a). Thirdly, when more than one host species is used, they must all be able to grow efficiently with the same growth media, pH, temperature and oxygen requirements (Che & Men, 2019). Fourthly, while complexity within the host cell is reduced, DOL creates a new level of complexity with the relationships and communication between the different cells. The third and fourth issues can make it difficult to control the populations of cells in a synthetic consortium. Cells can have competitive interactions, such as competition for the feedstock available, or antagonistic, such as production of toxins that harm other community members. Differences in species growth rate and fitness may result in the most competitive species dominating the community due to competitive exclusion (Jawed, Yazdani & Koffas, 2019). This can be exacerbated by DOL, as the cells are producing different heterologous proteins, and therefore have unequal metabolic burden. Cells which have a less burdensome pathway will grow better and thus end up dominating the consortium.

1.5.1. Dynamic control of populations

Synthetic biology has already begun to tackle the challenge of controlling populations in synthetic consortia. Synthetic biology tools have expanded to include bacterial acyl-homoserine lactone molecules (Kylilis *et al.*, 2018), yeast mating signals (Williams, Nielsen & Vickers, 2013), and plant hormones (Chen & Weiss, 2005), which can all be used to create novel communication between cells. Kylilis *et al.* used three orthogonal acyl-homoserine lactone systems to precisely control the populations of three *E. coli* strains in a consortium (Kylilis *et al.*, 2018). Antagonistic

interactions to control populations, such as predator-prey relationships, can be designed bottom-up using antimicrobials and toxin/antitoxin systems (Liu et al., 2019a; Kong et al., 2018).

When DOL is implemented between strains it is particularly useful to be able to control their growth rate and populations dynamically to change the flux through different components. The use of re-engineered cell-to-cell signalling has enabled regulation by extracellular signals or autoregulation of population sizes and thus control of different parts of divided labour (Fedorec et al., 2021; Zou et al., 2021; Liu et al., 2020; Miano, Liao & Hasty, 2020). For example, Honjo *et al.* created a synthetic *E. coli* consortium with a genetically programmed two-step process. In the first step the degradation makes intracellular beta-glucosidase enzymes and secretes quorum-sensing molecules. When a specific cell density is reached, detected by quorum-sensing, the degradation strain lyses, releasing glucosidase, and the production strain begins to express the bioproduction genes for isopropanol (Honjo et al., 2019). In this case the consortia autonomously switch between the two states with no external control. Alternatively, Aditya *et al.* developed a system in *S. cerevisiae* for light-dependent regulation of component subpopulations in a consortium (Aditya et al., 2021), which allows for external *in silico* control.

Synthetic control of populations can also be used to reduce heterogeneity. In populations with low-efficiency and high-efficiency producers, lower production usually favours growth, resulting in declining production over time. Synthetic biology tools have been created to reduce this effect, including biosensors which will selectively kill low-efficiency producers and support the growth of high-producers (Wang et al., 2019b). Guo *et al.* used biosensor-controlled cell selection in an *E. coli* consortium producing phenol (Guo et al., 2019), and in an *E. coli* consortium producing protocatechuic acid and hydroquinone (Guo et al., 2020). In both cases a biosynthesis pathway was divided between multiple strains; with the upstream strains providing precursors for downstream ones. The upstream strains were also engineered with the relevant precursor-mediated biosensors, to ensure production of these precursors was continually controlled at a high level and to reduce resource competition with low-efficiency producers.

1.5.2. Cross-feeding and syntrophy

An alternative method of population control is cross-feeding, where metabolites are mutually traded between species or strains. Evolutionary approaches can create new exchanges between native species that don't naturally cooperate. For example, when grown on lactose a methionine-auxotrophic *E. coli* strain will create waste products to feed *Salmonella enterica*; co-evolution of these species resulted in *E. coli* secreting larger amounts of galactose, and *S. enterica* adapting to secrete methionine for *E. coli* (Harcombe et al., 2018). This demonstrates

how cooperation can be favoured even if it may cost metabolically more to sustain. Such evolved syntrophy can also introduce unexpected functions. In the prolonged cultivation of *Clostridium acetobutylicum* with *Clostridium ljungdahlii*, these organisms not only exchanged secondary metabolites, but their synergism altered gene expression in both organisms and led to production of metabolites which could not be synthesized by their monocultures (Charubin & Papoutsakis, 2019).

Mutual dependence between strains, or syntrophy, can be created using rationally designed cross-feeding of metabolites between auxotrophs (Fritts, McCully & McKinlay, 2021). For an auxotrophic organism in a consortium, which lacks the ability to synthesise an essential metabolite, survival is dependent on other consortium members releasing that metabolite (Morris et al., 2013). Species or strains with different auxotrophies can be designed to be co-dependent, complementing each other by cross-feeding, creating a syntrophic consortium. However, rationally designing cross-feeding is not always simple; within these syntrophic consortia not all members survive equally. Mee et al. created fully-synthetic consortia of *E. coli* using strains auxotrophic for different amino acids. They found that auxotrophs for energetically-expensive amino acids, like arginine, lysine, methionine and threonine, dominate a consortium after only 2-3 days (Mee et al., 2014). This demonstrates that the cell fitness of individual component members can be asymmetrically affected by cross-feeding because of the varied costs of different metabolites.

In yeast, syntrophic pairs can be identified with genome-scale high-throughput screens, testing pairwise combinations of auxotrophs in a wide range of metabolites (Peng et al., 2024; Aulakh et al., 2023). Aulakh et al. applied syntrophic pairs to the production of malonic semialdehyde (MSA) in *S. cerevisiae*. They expressed two heterologous genes for MSA production in a single-strain monoculture and used DOL in both non-syntrophic and syntrophic 2-strain consortia, where one strain expresses a single gene. They found the non-syntrophic consortia did reduce the metabolic burden, but did not increase bioproduction. Whereas the syntrophic consortia were all able to produce more MSA per unit biomass, and the best could achieve a titre 2-fold higher than the monoculture (Aulakh et al., 2023). Peng et al. further applied these *S. cerevisiae* syntrophic consortia to DOL in the resveratrol production pathway, where a syntrophic consortia achieved production 3.16-fold higher than a monoculture, and 1.21-fold higher than a non-syntrophic consortia (Peng et al., 2024). Similarly, Park et al. used DOL in syntrophic consortia of *S. cerevisiae* and *Yarrowia lipolytica* to improve production of 3-hydroxypropionic acid (Park et al., 2024).

1.5.3. Modelling tools

Another method to overcome the challenges of DOL in synthetic biology is the use of modelling tools. By far the most used computational tools for consortia design are genome-scale metabolic models (GSMMs) (Ibrahim, Raajaraam & Raman, 2021). To construct a GSMM for consortia a stoichiometric matrix is made for each species and additional exchange reactions are added to include the movement of metabolites between species. Flux balance analysis (FBA) can then be used to optimise community functions such as growth rate or desired production (Khandelwal et al., 2013; Zomorodi & Maranas, 2012). FBA has been used to predict optimal co-culturing conditions (Hanly, Urello & Henson, 2012; Klitgord & Segrè, 2010), to predict stability of consortia populations (Zomorodi & Segrè, 2017; Koch et al., 2016), to measure effects of metabolite cross-feeding (Henson & Hanly, 2014; Zhang & Reed, 2014), and to identify non-intuitive divisions of labour in central carbon metabolism (Thommes et al., 2019). Other than GSMMs, coarse-grained phenomenological models have been used to identify the range of burden within which the consortium is stable (Mauri et al., 2020; Harvey, Heys & Gedeon, 2014). Tsoi *et al.*, found that DOL is only beneficial when mitigating metabolic burden overcomes both the host cell's resilience to burden and the cost of transporting intermediates between cells (Tsoi et al., 2018a).

1.6. How microbial consortia can benefit alternative feedstock degradation

Natural consortia which can degrade lignocellulose are common in forest and agricultural soil (Gladkov et al., 2022). They can also be isolated from the gut of ruminant animals, such as cattle, sheep, deer and camels, which eat foods high in lignocellulose (Li et al., 2021; Srivastava et al., 2021). Food waste is commonly valorised with natural microbial consortia in anaerobic fermenters to make biogas (Wang et al., 2022). Specific species for the degradation of agriculture and food waste can be enriched from these natural consortia. Carlos *et al.* enriched a natural consortium for growth on lignin or xylan, then used metagenomics to identify the species composition. They found that while the genetic diversity was maintained, certain species became more abundant on the different substrates. They found that *Proteobacteria* became dominant in consortia grown on lignin, with *Pseudomonas* being the dominate genus, while *Bacteroidetes* became more prevalent in consortia grown on xylan, with *Sphingobacterium* as the dominant genus (Carlos, Fan & Currie, 2018). This approach allows specific species with the desired degradation functions to be identified for use in artificial and semi-synthetic consortia, and it can help identify the genes needed for fully-synthetic consortia.

DOL means consortia can degrade and utilise complex feedstocks more effectively than monocultures. As stated previously, a monoculture can be limited by the number of enzymes it produces and its inability to efficiently process the variety of substrates in agriculture and food waste, while microbial consortia bring together diverse species and strains that specialise in different enzymatic functions. The benefits of DOL for complex substrate degradation are summarised in Figure 1.3.

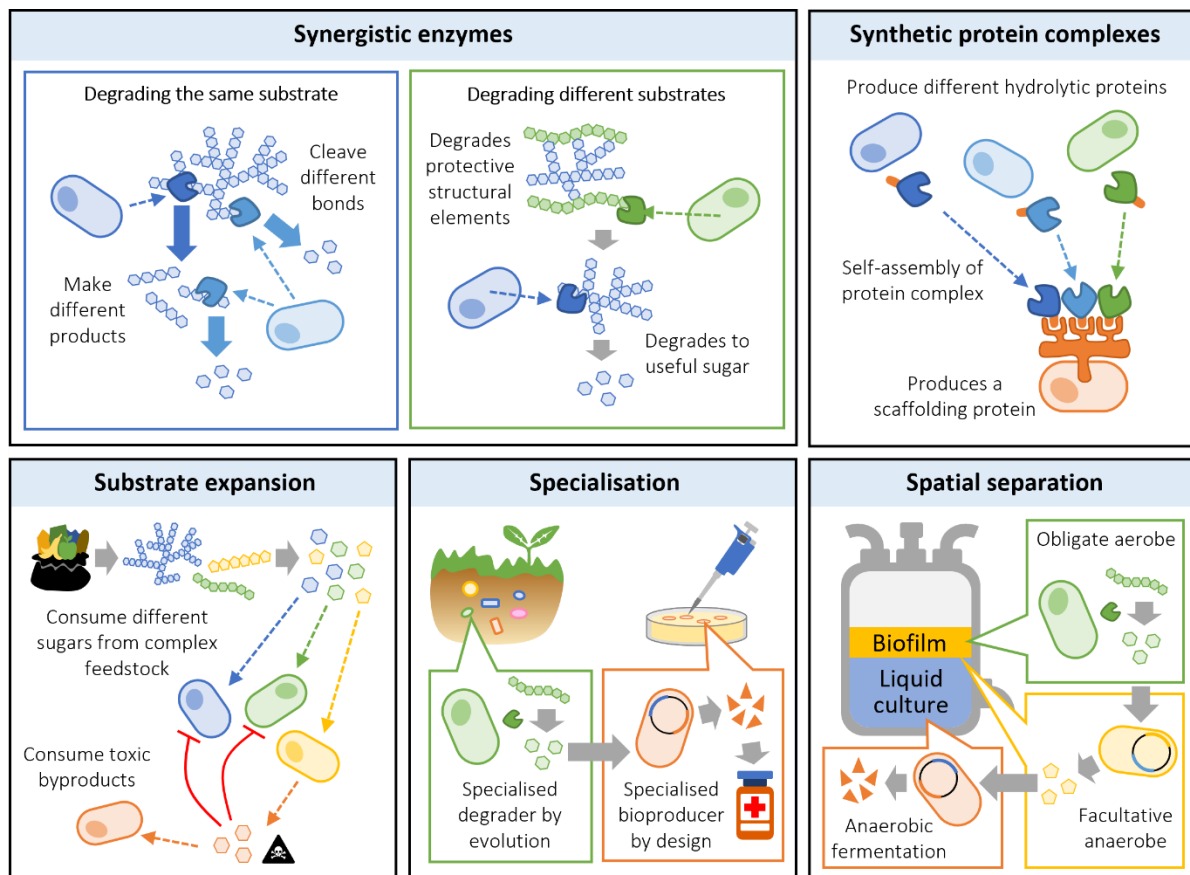


Figure 1.3 Benefits of division of labour (DOL) for the degradation of complex feedstocks

Synergistic enzymes: DOL enables different hydrolytic enzymes to be expressed in separate cells, which reduces metabolic burden on each individual cell while maintaining the enzymes' combined effectiveness. **Synthetic protein complexes:** Using synthetic biology, enzyme–substrate–microbe interactions, similar to native systems, can be mimicked by expressing synthetic protein complexes in non-native host cells. In a DOL system, one strain can carry a scaffolding protein while other strains express the hydrolytic enzymes. **Substrate expansion:** Because complex substrates provide a diverse array of carbon sources, different cells can break down distinct parts of the feedstock; this specificity helps to prevent catabolite repression and allows for the removal of toxic byproducts. **Specialisation:** Each cell type can be selected for traits that optimise its role, such as species that are naturally suited to specific bioprocesses. **Spatial separation:** When the ideal environmental conditions differ among species, bioreactors can be designed with immobilized cells, providing distinct environments tailored for each cell type.

1.6.1. Synergistic interactions of hydrolysis enzymes

Different microbes within a consortium can produce all the complementary enzymes that are required to break down complex substrates. For example, some microbes may specialise in producing cellulases that degrade cellulose, while others may produce hemicellulases for hemicellulose breakdown. In addition, while two species might both produce cellulases, those cellulases will have different enzyme specificities and activities, which may provide enhanced degradation when used together. The combined enzymatic action leads to a more complete and efficient degradation process than that achievable by a single cell.

Synergy can be rationally designed between two species which degrade the same part of agriculture and food waste. For example, Ruhong *et al.* identified two white-rot fungi species which had different expression patterns for lignin-degrading enzymes. Combining them in an artificial consortium showed that the increased enzyme diversity led to improved degradation efficiency compared to monoculture. (Ruhong *et al.*, 2020). Increased ligninolytic enzyme production compared to monoculture has been found in other artificial consortia of white-rot fungi and other fungal and bacterial species from soil (Chen *et al.*, 2019; Ijoma & Tekere, 2017; Qi-he *et al.*, 2011; Bader *et al.*, 2010; Freitag & Morrell, 1992). For cellulose degradation, *Trichoderma reesei* and *Aspergillus niger* in combination can provide different enzymes for cellulose degradation; *T. reesei* expresses cellobiohydrolase activity, while *A. niger* expresses β -glucosidase and endoglucanase activity (Ahamed & Vermette, 2008). Similarly, higher hydrogen production was observed for an artificial consortium of *Bacillus cereus* and *Brevundimonas naejangsanensis*, which express α -amylase and glucoamylase respectively for degradation of starch, compared to monoculture (Wang *et al.*, 2019a).

Synergy can also be designed between two species which degrade different elements of agricultural and food waste. For example, ligninolytic species are often very poor at saccharification, and vice versa for cellulolytic species. Therefore, artificial consortia of ligninolytic and cellulolytic or hemicellulolytic species can be used for lignin pre-treatment and saccharification simultaneously (Chen *et al.*, 2019; Copete-Pertuz *et al.*, 2019; Mewada, Albert & Pandya, 2017; Ma & Ruan, 2015). Artificial consortia can also combine species specialised for production of proteases and lipases in order to degrade the diverse substrates found in food waste (Baiano, 2014). Enriched natural consortia and artificial consortia have shown promise in valorising waste cooking oil (Ke *et al.*, 2021a, 2021b), pectin (Vemparala *et al.*, 2023) and chitin (Vortmann *et al.*, 2021).

Fully-synthetic consortia can also be made with other diverse degradation pathways. Moraïs *et al.* engineered *Lactobacillus plantarum* to express either an endoglucanase, to degrade cellulose, or an endoxylanase, to degrade xylan. When combined to form a two-strain consortium they demonstrated synergistic activity, significantly increasing the release of soluble sugars from wheat straw (Moraïs *et al.*, 2013). Zhang *et al.* created a fully-synthetic consortia for ethanol production from wheat straw using *S. cerevisiae* with recombinant cellulases and *Pichia pastoris* with recombinant xylanases (Zhang *et al.*, 2017b).

1.6.2. Constructing complex substrate hydrolysis in bioproduction strains

In nature cellulose and hemicellulose degradation is often carried out by large multiprotein complexes called cellulosomes. This organised structure holding enzymes together in proximity to the cell enables enzyme-enzyme synergy and enzyme-substrate-microbe synergy. Engineering a complete CBP for cellulose into a non-native host using a “designer cellulosome” has been unsuccessful, likely due to limited energy and high interference between the components and with the host (Brenner, You & Arnold, 2008; Verbeke *et al.*, 2013; Tsai *et al.*, 2009). Microbial consortia offer a way to minimise metabolic burden while maintaining the complexity which defines a cellulosome’s efficiency.

Tsai, Goyal & Chen engineered a consortium of four strains of *S. cerevisiae*, each expressing a different subunit of the cellulosome complex. Three strains expressed a different cellulase enzyme, which could then be assembled upon a scaffolding protein displayed on the surface of the fourth strain to create a minicellulosome. Altering the ratio of the four strains in this consortium allowed them to optimise the processes of cellulose hydrolysis and ethanol production, resulting in higher yields and titres than the equivalent enzymes expressed by a single cell (Tsai, Goyal & Chen, 2010).

Minicellulosomes have also been created in non-model, but more industrially relevant microorganisms. Moraïs *et al.* expanded on their work with xylanase and cellulase synergies (Section 1.6.1) to create designer minicellulosomes in *L. plantarum* with a consortium of three distinct engineered strains of the bacterium: one strain expressed cellulase, one expressed xylanase and one produced the anchored scaffoldin protein (Moraïs *et al.*, 2014). They further engineered this consortium to produce more cellulosic enzymes, and utilised the flexibility of adjusting the populations in the consortium to investigate the best stoichiometric ratio of the components (Stern *et al.*, 2018).

Anandharaj *et al.* engineered a 63-enzyme cellulosome into *Kluyveromyces marxianus*, which has a higher secretory capacity than *S. cerevisiae*. To achieve this large complex, they used a consortium of two engineered *K. marxianus* strains. They designed one strain to express the scaffold genes responsible for forming the backbone of the cellulosome complex, and the second host to express dockerin-fused enzymes, which are the cellulases and hemicellulases. These enzymes attach to the scaffold via dockerin-cohesin interactions, assembling the cellulosome. The ratio of each strain was controlled according to the complexity of the cellulosic substrates to achieve a higher ethanol titre (Anandharaj *et al.*, 2020).

Introduction of a full chitinolytic pathway has been attempted in *E. coli*, with mixed results. Vortmann *et al.* used a bottom-up approach to design a fully-synthetic consortium of *E. coli* and *Corynebacterium glutamicum* for conversion of chitin (a polysaccharide found in shellfish and fungi) to lysine. The *E. coli* strain was designed to express three chitinolytic enzymes that degrade chitin into acetate, which it consumes, and glucosamine. The *C. glutamicum* consumes this glucosamine and produces lysine, which it cross-feeds to the *E. coli*. However, they found that the metabolic burden on the *E. coli* was too much, so they attempted to use a 4-strain consortium, with a separate *E. coli* strain for each of the three chitinolytic enzymes. Unfortunately this still did not provide enough substrate for the *C. glutamicum* strain, and the authors concluded that better secretion or enzymatic activity was needed (Vortmann *et al.*, 2021).

1.6.3. Expanding substrate specificities

While efforts have been made to increase the degradation of complex plant biomass feedstocks, such as agricultural and food waste, the second step of CBP is to enable value-added compound production from the hydrolysed substrates. Most industrial microorganisms preferentially consume hexose sugars. Catabolite repression, a regulatory mechanism where the presence of a preferred carbon source inhibits the metabolism of alternative carbon sources, often prevents efficient utilization of all available substrates, even in engineered microorganisms. For consortia, this means the component species or strains will all be competing for the same preferred substrate over other substrates. The hydrolysis of hemicellulose provides abundant pentose sugars, and the degradation of lignin produces heterogeneous low-molecular weight aromatics. Consortia of microbes that have preferences for these diverse substrates will minimize the adverse effects of resource competition and result in more efficient utilisation of the carbon in plant biomass towards desirable products (Zhang *et al.*, 2021).

One of the best-known natural pentose fermenters is *Scheffersomyces stipitis*, and much research has been done in the consortium of *S. stipitis* and *S. cerevisiae* for ethanol production (Naseeruddin, Desai & Venkateswar Rao, 2021; Pathania, Sharma & Handa, 2017; Hanly & Henson, 2013, 2014; Karagöz & Özkan, 2014; Srilekha Yadav et al., 2011). Other organisms used in consortia as native pentose-metabolisers include *Candida tropicalis* (Liu et al., 2019b), *Candida shehatae* (Hickert et al., 2013) and *Bacillus coagulans* (Demiray, González-Fernández & Tomás-Pejó, 2024). These consortia allow simultaneous conversion of both hexose and pentose sugars, maximizing substrate utilisation rates and increasing production.

Fully-synthetic consortia of model organisms have been engineered to have specificity for different sugars to improve the efficiency of substrate utilisation in mixed sugar media. Xie, Eiteman & Altman engineered three strains of *E. coli* to solely consume one of either glucose, xylose or arabinose (Xia, Eiteman & Altman, 2012) and this consortium was further engineered to increase activity and orthogonality for production of ethanol (Flores et al., 2019). A similar strategy was used to make a synthetic consortia of three *E. coli* strains for glucose-galactose-mannose mixtures (Chappell & Nair, 2017) and three *S. cerevisiae* strains for glucose-xylose-arabinose mixtures (Verhoeven et al., 2018). These consortia show more stable bioproduction in mixed sugars compared to monocultures which lose performance over time (Verhoeven et al., 2018) and have been used to reduce wasted sugars in the production of value-added compounds (Rajpurohit & Eiteman, 2020; Maleki, Safari & Eiteman, 2018; Saini et al., 2017). Using strains of the same species in fully-synthetic consortia has an advantage over artificial or semi-synthetic consortia as it guarantees the strains have compatible environmental conditions for growth.

When DOL is applied to sugar-specificity it can also be applied to bioproduction pathways in parallel. Each cell type can be engineered to consume a sugar which is favourable for a different biosynthesis pathway, for steps which require intermediates via the pentose phosphate pathway xylose is preferable over glucose (Zhang & Wang, 2016b).

Modelling in the form of dynamic FBA (dFBA) has been used to predict optimal co-culture strategies in mixed sugar media. Hanly et al. utilized a dFBA set-up in which intracellular reactions were calculated using FBA for each time step, while kinetic equations were used for substrate uptake, product secretion and other exchanges between members of the consortium. Thus, the model can account for dynamic community growth conditions and interactions between the organisms. This dFBA set up was used to obtain conditions for simultaneous consumption of glucose and xylose in yeast-yeast or yeast-*E. coli* consortia (Henson & Hanly,

2014; Hanly, 2013; Hanly & Henson, 2011, 2013; Hanly, Urello & Henson, 2012), and for yeast-yeast consortia in the presence of toxic products of hydrolysis (Hanly & Henson, 2014).

1.6.4. Combining specialised hydrolysis species with specialised bioproduction species

The final step of CBP is to use the sugars provided by agriculture and food waste degradation for production of value-added compounds. Artificial and semi-synthetic consortia can also combine a naturally-degrading species with a natural or synthetic high producer of a desirable product.

Trichoderma reesei is commonly used as a cellulolytic partner. It has been paired with a range of different native and non-native bioproduction hosts, including with *E. coli* to produce isobutanol (Minty et al., 2013), with *Ustilago maydis* to produce itaconic acid (Schlembach et al., 2020) and with *Rhizopus delemar* to produce fumaric acid (Scholz et al., 2018). Consortia of a naturally-degrading species and a bioproduction host species have also been used for production of butanol from xylan (Jiang et al., 2018) polyhydroxyalkanoates from inulin (Corrado et al., 2021) and acetone–butanol–ethanol from cassava starch (Tran et al., 2010), to name just a few.

Optimising these consortia can be challenging, as previously stated native hydrolytic species may have limited genetic characterisation and few available engineering tools. In addition, these hydrolytic species may be intolerant to the high yields of desired product produced by the industrial strains (Fan, 2014). One of the biggest challenges is the difference in abiotic conditions required by native cellulolytic species compared to most industrially-relevant bioproduction hosts. When these species with different environmental preferences are forced to coexist, their growth and metabolic activity can be imbalanced, making it difficult to maintain a stable and efficient consortium where all species perform their intended roles.

Many of the most optimal lignocellulolytic species are obligate aerobic fungi, while many desirable bioproduction pathways are carried out during anaerobic fermentation. Maintaining optimal environments, in particular aeration, for both lignocellulolytic species and bioproduction species is assisted by DOL in novel bioreactor set-ups. An artificial consortium of cellulolytic obligate anaerobe *Clostridium phytofermentans* and facultative anaerobe *E. coli* showed a 250% increase in biomass over monocultures when grown in a biofilm environment which allowed aerobic/anaerobic cycling (Park et al., 2020). Biofilm reactors have also been used to create an oxygen gradient between obligate aerobe *T. reesei*, which carries out cellulose degradation, and obligate anaerobic *Clostridium tyrobutyricum*, which consumes lactic acid and produces butyric acid. Bridging the gap between the two was *Lactobacillus pentosus*, which consumes fermentable sugars and produces lactic acid (Shahab et al., 2020).

1.6.5. Detoxification of lignocellulose and food waste feedstocks for bioproduction hosts

The degradation of lignocellulose results in several toxic byproducts, such as phenolic aldehydes, aromatic acids, formic acid, acetic acid, furfural, and 5-hydroxymethylfurfural (5-HMF), which can hinder microbial growth and fermentation. Microbial consortia are often better at detoxifying these inhibitors because different species can possess various mechanisms for resistance. The expansion of substrate specificity discussed in Section 1.6.3 includes substrates which are considered inhibitors: a species which can consume toxic metabolites can be used to remove them from the feedstock. For example, Millán Acosta *et al.* created a semi-synthetic consortium of *Fusarium striatum* and a xylose-consuming strain of *S. cerevisiae*. *F. striatum* was capable of metabolising harmful compounds like furfural and 5-HMF, while *S. cerevisiae* fermented sugar to ethanol (Millán Acosta *et al.*, 2021).

Many of the inhibitory aromatic compounds come from lignin degradation. Co-cultures of *Amycolatopsis sp.*, *Pseudomonas putida*, *Acinetobacter baylyi*, and *Rhodococcus jostii* have shown promise in assimilating aromatic compounds for both valorisation and detoxification (Xu *et al.*, 2019). These inhibitory compounds can also exhibit the same catabolite repression described in Section 1.6.3, and therefore benefit from substrate-specificity. For example, *A. baylyi* represses the consumption of 4-hydroxybenzoic (4-HB) acid until all benzoate in the media has been depleted; both these compounds are inhibitory to bioproduction. Singh *et al.* overcame this repression by using two strains, one which would consume only benzoate and another that would consume only 4-HB. When grown together they removed these two inhibitors simultaneously, and then the detoxified feedstock was consequently used by *K. marxianus* to make ethanol (Singh *et al.*, 2019).

Recently, Rafieenia *et al.* evolved a *Y. lipolytica* strain which could consume citric acid, another byproduct of lignocellulose pretreatment, but was unable to consume glucose (Rafieenia *et al.*, 2024). In this strategy, this citric acid consumer strain was grown in a consortium with a wild-type (WT) strain. The WT consumed glucose in the lignocellulose hydrolysate and produced further citric acid as a byproduct of its metabolism. This consortium was able to achieve higher growth, lower citric acid accumulation and higher β -carotene production than any of the component monocultures (Rafieenia *et al.*, 2024).

1.6.6. Predicting emergent properties in substrate degradation

Emergent properties are functions that arise from the collective and synergistic activities of different consortia members working together. They are difficult to predict from the

characteristics of individual component species alone, because they rely on high-order interactions, beyond simple cell-to-cell interactions or additive functions (Sanchez-Gorostiaga et al., 2019). The synergism of degradation enzymes, cross-feeding, cell-to-cell communication, spatial structuring and toxic byproduct removal are all consortia functions that can contribute to emergent properties that improve degradation of agricultural and food waste. Being able to predict these emergent properties enables rational design of artificial and synthetic consortia.

GSMMs, FBA and flux variability analysis (FVA) have been applied to predicting emergent properties in consortia for CBP. Chiu et al. used GSMM to show that metabolites are produced in consortia that are not produced in monocultures of either species alone. They also demonstrated that species which produce emergent properties must lie within a “Goldilocks zone” of species similarity, not too similar as to overlap in their niches, but not too different that they can’t share metabolites (Chiu, Levy & Borenstein, 2014). Perisin & Sund used GSMM from 773 gut microorganisms to model all pairwise combinations of the species specifically for valorisation of food waste to high-value products. They found that simulated 2-species consortia could produce higher biomass and higher production than monocultures of the same strains, and that these consortia could generate products that monocultures were not expected to produce. They also found that mutualistic and commensal interactions were more common in consortia with emergent properties, and that mutualists had higher cross-fed metabolites (Perisin & Sund, 2018). Their simulations were then validated *in vivo* by Schwalm et al., who created one of the 2-species consortia of *Clostridium beijerinckii* and *Yokenella regensburgei*, and demonstrated that they could achieve higher productivity of butyrate, and this was driven by cross-feeding of lactate from *Y. regensburgei* to *C. beijerinckii* (Schwalm et al., 2019). Similar models can be built for any species that has a GSMM.

In addition to GSMM, coarse-grained ecological models and individual agent-based models have also been used for predicting emergent properties (van den Berg et al., 2022). Many of these models require *a priori* assumptions about the interactions between species, which works well with consortia with few component members where the pairwise interactions are decipherable but is less helpful for higher-order interactions. Sanchez-Gorostiaga et al. demonstrated the importance of higher-order interactions in lignocellulose degradation when they created artificial consortia of two to six *Bacillus* or *Paenibacillus* species which expressed endoamylase for starch degradation. In an additive system, the overall hydrolytic rate is the sum of the hydrolytic rate of each enzyme. For 2-strain pairwise interactions of *Bacillus* and *Paenibacillus* the effects exceeded the predicted additive outcomes. However, introducing a third *Bacillus* strain produced a lower hydrolytic rate than expected based on addition of the pairwise interactions.

As more species were added to the consortia, the additive system became less predictive, indicating that higher-order interactions lead to further emergent properties (Sanchez-Gorostiaga et al., 2019).

1.7. Consolidated bioprocessing in *Yarrowia lipolytica*

Yarrowia lipolytica is a GRAS-certified, non-conventional, oleaginous yeast and a promising candidate host for next-generation biotechnology. It has been shown to be a good producer of heterologous proteins (Madzak, 2015; Madzak, Gaillardin & Beckerich, 2004) organic acids (Cavallo et al., 2017), aromas (Czajka et al., 2018; Braga & Belo, 2016), carotenoids (Larroude et al., 2018a), and biofuels (Yu et al., 2020; Darvishi et al., 2017; Ledesma-Amaro & Nicaud, 2016a).

As a promising industrial chassis, *Y. lipolytica* also has a range of synthetic biology tools available for use in its engineering (Cao et al., 2023). This includes variable genetic bioparts (Sun et al., 2022; Zhao et al., 2021; Xiong & Chen, 2020; Celińska et al., 2018; Dulermo et al., 2017), DNA assembly techniques (Cao et al., 2023; Celińska et al., 2017) and gene editing protocols (Ganesan et al., 2019; Larroude et al., 2018c). A well-annotated genome sequence (Dujon et al., 2004) and multiple metabolic models (Kerkhoven et al., 2016; Kavšček et al., 2015; Loira et al., 2012; Pan & Hua, 2012) are also available to enable bioinformatic and computational analysis.

In addition to being an effective host for biosynthesis pathways *Y. lipolytica* is also a promising organism for CBP. *Y. lipolytica* has a higher secretory capacity than many alternative yeasts (Theron et al., 2020; Celińska & Nicaud, 2019; Madzak, Gaillardin & Beckerich, 2004) and it naturally produces high levels of proteases and lipases, allowing it to digest and ferment a range of substrates such as glycerol, tallow waste, waste water, and chemically-processed hydrolysates of cassava starch and inulin (Ledesma-Amaro & Nicaud, 2016b). However, *Y. lipolytica*'s capacity for growth on lignocellulose is limited and the pathways for metabolism of a number of simple polysaccharides or monosaccharides, such as sucrose, maltose, xylose or cellobiose, are cryptic or absent (Spagnuolo et al., 2018). Therefore, effort has been made in recent years to introduce degradation of complex feedstocks to (Celińska, Nicaud & Białas, 2021), and expand substrate specificities for (Spagnuolo et al., 2018; Lazar et al., 2015; Papanikolaou & Aggelis, 2010) *Y. lipolytica*.

Creating a CBP for *Y. lipolytica* in a single cell has also been limited by the expression and secretion of hydrolytic proteins. *Y. lipolytica* has some endogenous β -galactosidase genes (Ryu, Hipp & Trinha, 2016; Guo et al., 2015), and can grow on cellobiose after either evolution or rational overexpression of these genes (Ryu, Hipp & Trinha, 2016). *Y. lipolytica* has also been

engineered toward cellulose degradation by expressing endoglucanases and cellobiohydrolases from *T. reesi* (Xu et al., 2018; Boonvitthya et al., 2013; Park, Chang & Ryu, 2000). However, Wei et al. found that overexpression of three of these genes in a high-lipid-producing strain of *Y. lipolytica* resulted in a two-fold reduction in lipid production as a result of metabolic burden. This was specifically due to competition for resources within the endoplasmic reticulum (ER), where both protein folding and lipid production take place (Wei et al., 2018). Guo et al. overexpressed six cellulolytic genes, both endogenous and heterologous, to create a strain which could grow on cellulose pulp (Guo et al., 2017). While the maximum growth rate of *Y. lipolytica* was impaired by the overexpression, the growth was still sufficient for bioproduction of lipids, lipase and ricinoleic acid (Guo et al., 2018).

Of specific interest to this thesis is the growth of *Y. lipolytica* on raw starch (Ledesma-Amaro, Dulermo & Nicaud, 2015) and on xylan (Wang et al., 2014). Starch is a polysaccharide of glucose monomers, joined by α -(1,4) glycosidic bonds to form straight-chain amylose, and with the addition of α -(1,6) glycosidic bonds to form the branched amylopectin. *Y. lipolytica* has been engineered to grow on starch as the sole carbon source by co-expression of glucoamylase from *Aspergillus niger* and an α -amylase from either rice *Oryza sativa* (Ledesma-Amaro, Dulermo & Nicaud, 2015) or rice pest *Sitophilus oryzae* (Celińska et al., 2020). These enzymes act synergistically: α -amylase is an endoamylase which binds randomly in the sugar chain to catalyse hydrolysis of α -(1,4) glycosidic bonds and glucoamylase is an exoamylase, which cleaves α -(1,4) glycosidic bond at the non-reducing end of starch, as well as α -(1,6) glycosidic bonds at branch points of amylopectin (El-Fallal et al., 2012).

Xylan is one of the main components of hemicellulose and is made up mainly of xylose monomers with β -(1,4)-glycosidic bonds, but it can be branched with other monomers, like arabinose, galactose, glucuronic acid and galacturonic acid through β -(1,3)- or β -(1,3, 1,4)-glycosidic bonds. There are mixed reports on whether *Y. lipolytica* can naturally grow on xylose (Spagnuolo et al., 2018), however it does contain a cryptic pathway for xylose metabolism. Growth on xylose can be achieved with overexpression of endogenous genes for xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulose kinase (XK) (Niehus et al., 2018) or with overexpression of endogenous XK with heterologous expression of XDH and XR from *Scheffersomyces stipitis* (Ledesma-Amaro et al., 2016; Li & Alper, 2016). *Y. lipolytica* has also been engineered to consume other monomers of xylan such as galactose (Lazar et al., 2015) and arabinose (Papanikolaou & Aggelis, 2010). For the degradation of the xylan polysaccharide, *Y. lipolytica* was engineered to grow on birchwood xylan by expression of endoxylanase from *Trichoderma harzianum* (Wang et al., 2014). Co-culturing this endoxylanase strain with another

Y. lipolytica strain engineered with exoxylanase from *A. niger* resulted in higher degree of xylan degradation (Wang et al., 2014).

With the exception of the last example from Wang et al., most of these strategies aim to achieve a monoculture CBP. However, as previously noted, the work by Wei et al. on cellulase genes found that expression and secretion of these hydrolytic enzymes can result in metabolic burden (Wei et al., 2018). This is consistent with other observations of decreased biomass yields due to metabolic burden in *Y. lipolytica* (YaPing et al., 2017; Roth, Moodley & Van Zyl, 2009). Korpys-Woźniak et al. have done significant research in the last few years in characterising the mechanisms of metabolic burden in *Y. lipolytica*. They expressed four different heterologous reporter proteins, which differed in size and post-translational modifications, under the control of different strength promoters. They found that bigger, more modified proteins led to bigger reductions in growth and higher induction of stress responses (Korpys-Woźniak et al., 2020). To further elucidate the underlying mechanisms, they looked at the global changes in transcription for secreting different proteins. They found that highly-secreted proteins resulted in upregulation of oxidative stress genes, probably due to protein folding in preparation for secretion. The transcriptomics also revealed that *Y. lipolytica* enters a growth arrest phase in order to withstand increased metabolic burden (Korpys-Woźniak & Celińska, 2021). In a similar experiment, Swietalski et al. found that large secreted proteins were limited at a translational level, while small secreted proteins were limited at the secretion level (Swietalski et al., 2020). When working on the cellulase genes, Wei et al. found that adjusting the C/N ratio of the media to reduce protein synthesis and increase lipid production helped relieve the metabolic burden, as did using a chemical chaperone, and deleting an inhibitor of lipid production (Wei et al., 2018, 2021). However, another approach to reduce metabolic burden is using DOL.

Artificial consortia with *Y. lipolytica* as the bioproduction host have been used for CBP. Lubbe et al. used *Y. lipolytica* in combination with cellulolytic bacteria *Cellulomonas fimi*. Here the bacteria degraded cellulose to provide substrate for *Y. lipolytica*, as well as exchanging a number of intracellular metabolites by cross-feeding (Lubbe, Bowen & Northen, 2017). Lee et al. created a semi-synthetic consortium of *Y. lipolytica* with *Pseudomonas putida*, a lignin degrader, *C. fimi*, a cellulose degrader, and *Methylobacterium extorquens*, a methylotroph. *M. extorquens* consumed the toxic formaldehyde created as a byproduct of *P. putida* metabolism, and it was also engineered to provide methionine to both *P. putida* and *C. fimi* by cross-feeding. The *Y. lipolytica* was able to grow on the products of degradation provided by *P. putida* and *C. fimi* (Lee et al., 2021). Intasit et al. used a consortia of *Aspergillus tubingensis*, which has cellulases and

xylanases, and *Y. lipolytica* to produce biodiesel lipids from palm empty fruit bunch waste (Intasit et al., 2020).

In summary, *Y. lipolytica* is a desirable host for CBP due to its industrial relevance and its high secretory capacity necessary for hydrolytic enzymes, however its effectiveness in degrading alternative feedstocks is limited by metabolic burden. In this thesis I hypothesise that *Y. lipolytica* CBP would benefit from DOL in a consortium, thus reducing metabolic burden and increasing growth on the polysaccharides found in agricultural and food waste feedstocks.

1.8. Aims and objectives

Objective 1: Create a mechanistic model of DOL in substrate degradation to identify the range of expression within which DOL may be beneficial to growth on starch

Building upon existing host-aware mechanistic models, and the work from the previous MRes, the first objective of this project is to create a model of shared cellular resource distribution in monoculture versus consortia. This model will be used to simulate DOL in the degradation of a polysaccharide feedstock.

1. Characterise the effect of heterologous protein expression on the resource pools of ribosomes and energy: The single-cell model previously created by Weiße et al. (2015) will be adapted to a 2-strain consortium, where each cell expresses one of two heterologous proteins, and compared to a model where the single cell co-expresses both proteins. By explicitly modelling the intracellular molecules, this model will predict if DOL enables a higher growth rate and/or higher production of heterologous proteins.
2. Identify whether DOL can theoretically enable higher growth rate on starch compared to monoculture: The 2-strain consortium model will be adapted specifically to express two diverse amylases, and ODEs added for the degradation of starch to glucose by these enzymes. The model will predict the range of expression (if any) within which DOL provides a higher growth rate than a monoculture co-expressing both amylases.

This work was published in Atkinson, E. et al. (2022) Resource-aware whole-cell model of division of labour in a microbial consortium for complex-substrate degradation. *Microbial Cell Factories*. 21 (1), 1–12.

Objective 2: Build novel 2-strain consortia for cooperative degradation of a single alternative complex feedstock in the industrially-relevant organism *Yarrowia lipolytica*

The second objective of this project is to develop a novel fully-synthetic consortium of two strains of *Y. lipolytica*, where one strain expresses α -amylase from *Oryza sativa* and a second strain expresses glucoamylase from *Thermomyces lanuginosus*. Cultured together this 2-strain consortium will be able to grow on starch as the sole carbon source. Here, the focus is to look at how expression levels affect metabolic burden, and how metabolic burden influences the potential benefit of DOL.

1. Characterise the effect of expression and secretion level on burden: Standardised genetic parts will be alternated with combinatorial assembly to create variably expressing strains. These strains will then be assessed for growth parameters reflecting burden to see if expression and burden are related in a way predicted by Objective 1.
2. Assess the combination of two strains for growth on an individual complex feedstock: Each 2-strain consortium will be compared to its component strains and to a control single strain that co-expresses both enzymes using the same bioparts.
3. Characterise the benefit of DOL: Where DOL does achieve better growth than a single strain monoculture equivalent, these 2-strain consortia will be scaled up and further investigated to observe their population dynamics.

Objective 3: Rational design of substrate specificities to create different population dynamics in the cooperative degradation of two alternative complex feedstocks

The third objective of this project is to develop a novel fully-synthetic consortium of two strains of *Y. lipolytica*, where one strain expresses an endoamylase from *Oryza sativa* and a second strain expresses an exoxylanase from *Aspergillus niger*. Cultured together this two-strain consortium will be able to grow on mixtures of starch and xylan. Here, the focus is to look at how combining different degradation capacity and different substrate specificity can achieve different dynamics in the consortia.

1. Assess different consortia designs for growth on mixtures of complex polysaccharides: Degradation and substrate specificities can be varied between strains, creating orthogonality and cross-feeding. 2-strain consortia will be grown on mixtures of starch and xylan to assess the preferred relationships for growth on mixed complex substrates.

2. Characterise the dynamics of different relationships: Population dynamics will be monitored over time for different ratios of the two strains and different concentrations of the two substrates to observe their stability or variability.
3. Assess further benefits of DOL: The different consortia designs will also be measured for their robustness to environmental instability and toxic byproducts.

Objective 4: Bioproduction in *Y. lipolytica* utilising DOL for degradation of complex feedstocks

The final objective applies the second half of a CBP: the bioproduction. Either a degradation host can be combined with a bioproduction host, or the DOL strategies implemented in Objectives 2 and 3 can be applied, with both cells making product but differing in their hydrolytic capacity or substrate specificity. Here, the focus will be on whether DOL in substrate degradation allows higher production of value-added products.

The overall objectives of the project are summarised in Figure 1.4.

1.9. Publications resulting from this work

1. In preparation: “Division of labour between two amylases enables *Yarrowia lipolytica* to achieve higher growth rates using starch as the sole carbon source” (Expected submission early 2025)
2. In preparation: “Enhanced growth through division of labour: insights into substrate-generalist and substrate-specialist dynamics in synthetic microbial consortia reveals strategies to improve growth and production from mixed polysaccharide feedstocks.” (Expected submission early 2025)
3. Atkinson, E. *et al.* (2022) Resource-aware whole-cell model of division of labour in a microbial consortium for complex-substrate degradation. *Microbial Cell Factories*. 21 (1), 1–12.
4. Rafieenia, R.¹, Atkinson, E.¹ & Ledesma-Amaro, R. (2022) Division of labor for substrate utilization in natural and synthetic microbial communities. *Current Opinion In Biotechnology*. 75, 102706. (¹These authors contributed equally to this work).
5. Atkinson, E. *et al.* (2022) Principles, Tools, and Applications of Synthetic Consortia Toward Microbiome Engineering. *Principles in Microbiome Engineering*. 195–218.
6. Darvishi, F., Rafatiyan, S., Abbaspour Motlagh Moghaddam, M.H., Atkinson, E., *et al.* (2022) Applications of synthetic yeast consortia for the production of native and non-native chemicals. *Critical Reviews in Biotechnology*.

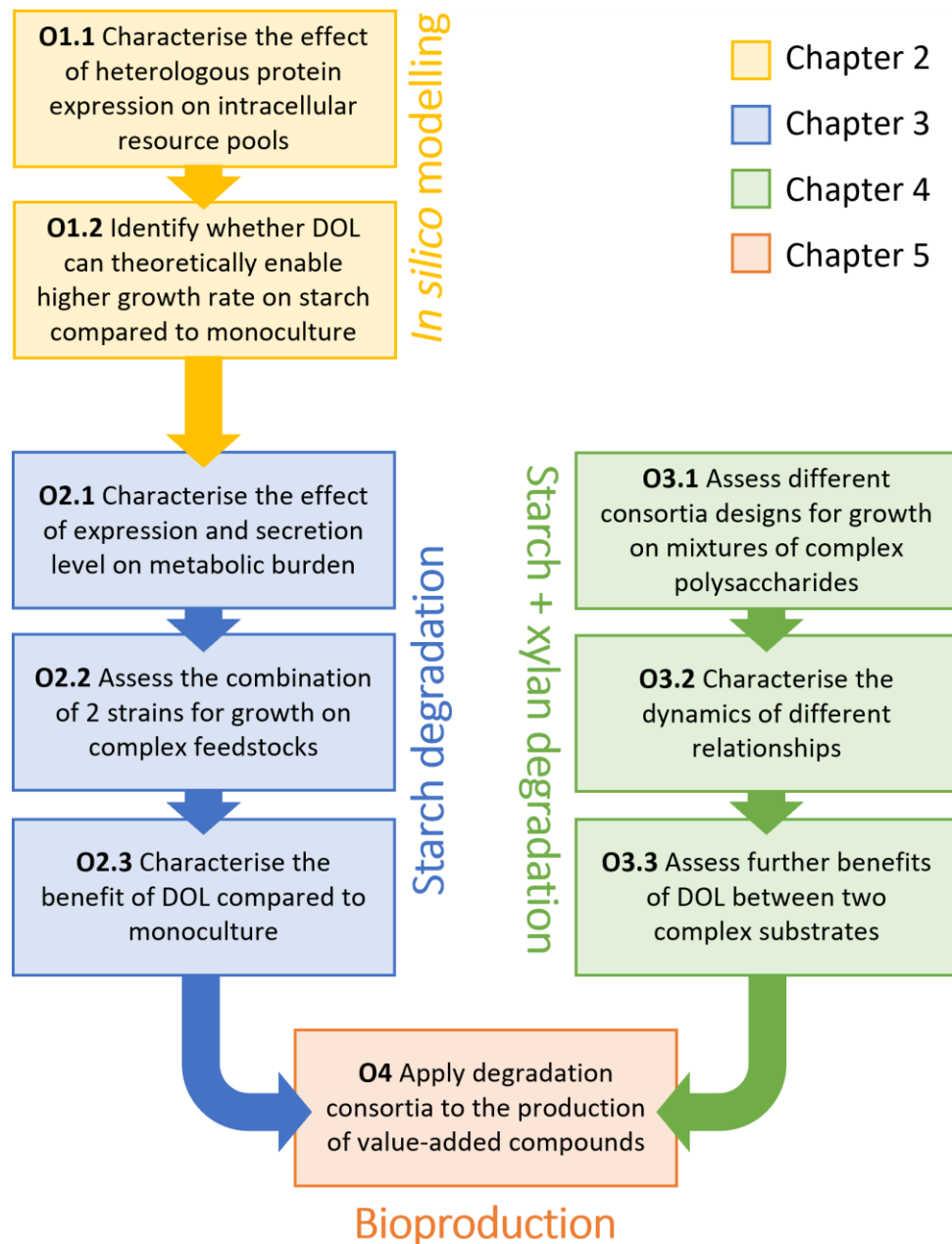


Figure 1.4 Summary of overall project objectives

2. Chapter 2: Resource-aware whole-cell model of division of labour in a 2-strain Escherichia coli consortium for starch degradation

The information and figures presented in this chapter were previously published in Atkinson *et al.*, (2022). A previous iteration of this model was presented as part of Eliza Atkinson, Systems and Synthetic Biology MRes Thesis (2020).

2.1. Introduction to this chapter

The mechanisms influencing metabolic burden and hence the benefits of division of labour (DOL) can be complex and difficult to decipher in their *in vivo* context. “Host-aware” is a phrase used to describe modelling approaches which consider the relationship between heterologous protein expression and host cell growth. Modelling such a relationship *in silico* allows systematic parameter exploration to explore a wide range of different variables that may influence the effect of DOL on the host cells.

A broad range of modelling approaches have been used to gain an understanding of how consortia function, including GSMMs (Thommes *et al.*, 2019) agent-based models (Gutiérrez *et al.*, 2017; Matyjaszkiewicz *et al.*, 2017) and course-grained models, which describe the minimal fundamental relationships and reactions within and between cells using ordinary differential equations. These models have been used to identify key factors enabling co-culture to outperform monoculture (Tsoi *et al.*, 2018b; Harvey, Heys & Gedeon, 2014; Rollié, Mangold & Sundmacher, 2012) and to identify the range of burden within which the consortium is stable (Mauri *et al.*, 2020; Harvey, Heys & Gedeon, 2014). Many of these models are not implicitly host-aware, or they use overly simplified phenomenological observations to account for the effects of burden on consortia function. However, models which explicitly model the intracellular trade-offs and resource allocations that characterise burden have been made for investigating single cells. This includes the dynamics for shared cellular resource pools such as ribosomes (Gorochoowski *et al.*, 2016), global cellular feedback to regulate protein production (Liao, Blanchard & Lu, 2017), and external regulation from varying environments (Sickle *et al.*, 2020).

In 2015, Weiße *et al.* published a host-aware whole-cell model for *E. coli* which links transcription and translation to the allocation of cellular energy, ribosomes, and the proteome (Weiße *et al.*, 2015). In this chapter I will describe the creation of a new host-aware model which integrates the Weiße *et al.* mechanistic whole-cell approach to modelling burden and applies it to investigating the effects of DOL in consortia.

To act as a proof-of-concept for DOL, I chose to use the degradation of a polysaccharide. Specifically, I apply the novel model to DOL in a 2-strain consortium for the degradation of starch. To break down starch into glucose multiple amylases work synergistically to break down different types of glycosidic bonds. This synergism is an important feature for DOL, as it supports cooperation between strains. In addition, the necessary secretion of these amylases into the extracellular environment may induce additional metabolic burden. This is especially important in yeast (as introduced in Section 1.2), which was the target host of my future experimental work. I hypothesised that expressing these amylases in separate cells in a consortium would reduce the burden compared to expressing them in a single cell, and therefore allow higher growth on starch. This chapter aims to create a model which will support or disprove this hypothesis on the bases of the underlying trade-offs which mitigate metabolic burden during DOL. This will allow the DOL system to be investigated at the molecular level and allow predictions of where the consortium can achieve higher growth rates than its monoculture equivalent.

In a previous thesis I created the first iteration of this model. The aim of this chapter was to improve upon the previous model and address concerns raised during the first examination. Firstly, that the previous iteration of the model did not calculate the total amount of heterologous protein production from the entire population. Secondly, that the parameters required in the ODEs used for starch degradation by amylases were incredibly difficult to obtain using experimental means. Thirdly, that the starch input parameters were set at a very high level, thereby reducing any effect of competition between the two strains for sugar.

2.2. Creating the model

Firstly, I adapted a whole-cell model for burden to predict cellular resource allocation and growth rate when expressing heterologous proteins in a DOL system. The ordinary differential equations (ODEs) describing this whole-cell model are derived from those published by Weiße *et al.* (Weiße *et al.*, 2015) (Figure 2.1, full ODEs given in Materials and Methods 7.1.2). The full details of the assumptions made and how the model equations are obtained can be found in Weiße *et al.* (Weiße *et al.*, 2015), but the key features of the model are:

The proteome can be broadly categorized into four groups: ribosomal proteins (r), transport proteins (et), metabolic proteins (em), and housekeeping proteins (q), as illustrated in Figure 2.1A. Each of these protein categories is characterized by ODEs governing three intracellular species: mRNA (mx), mRNA-ribosomal complexes (cx), and proteins (x), where $x \in \{r, et, em, q\}$.

The model assumes that translation constitutes the entire energy expenditure in the cell. Translation is represented by the binding of free mRNA to ribosomes from a finite pool, where each amino acid addition necessitates one unit of energy (a) (Figure 2.1B). The generation of the energy pool from the substrate is also explicitly modelled via two ODEs. The first ODE represents transport proteins importing an extracellular substrate (s), which is supplied at a constant rate, into the cell to make intracellular substrate (s_i). The second ODE represents metabolic proteins converting the imported substrate into units of energy (a). The overall energy pool available in the cell is therefore represented by an ODE which accounts for both the generation from substrate and the loss via translation (Figure 2.1D)

At each time point, growth rate is calculated based on a set of linear growth laws described by Scott *et al.* (Scott *et al.*, 2010b) (Figure 2.1C). During my previous thesis (Eliza Atkinson, Systems and Synthetic Biology MRes Thesis, 2020), this model was adapted to a two-strain consortium; where one cell (Cell A) expresses enzyme A (ea) and the other (Cell B) expresses enzyme B (eb). Essentially, I made two copies of the Weiße model, each containing the fourteen intracellular molecules considered in Weiße's framework, plus three new ODEs capturing the dynamics of the mRNA, the mRNA:ribosome complex and the protein for each heterologous gene. The equations for ribosomes and growth rate were adjusted to account for the new heterologous proteins as outlined in Nikolados *et al.*, (2020). To enable comparison with the monoculture case, a model was similarly built by adding six ODEs to the model of Weiße *et al.* to capture co-expression of both enzymes A (ea) and B (eb) in a single cell. The same parameters employed by Weiße *et al.* for an *E. coli* host cell were used (Materials & Methods 7.1.4).

In the previous thesis the model did not account for an imbalance in the populations of the consortia versus the monoculture. Since the original Weiße model was intended as a single-cell model for cells in constant exponential phase, there was no need to account for the division of substrate between different cells. However, an important consideration when adapting the model to the consortium was the necessity to provide substrate for both Cell A and Cell B. In the previous iteration of the model the rate of uptake of glucose into the cell, $v_{uptake}(et, s)$, was calculated as if there was only one of each cell in the consortia (Equation Z in Appendix 9.1), and only one monoculture cell (Equation Z' in Appendix 9.1). However the use of these equations caused problems when you consider that overall this means there are two cells in the consortium system compared to only one cell in the monoculture system, creating an unfair comparison.

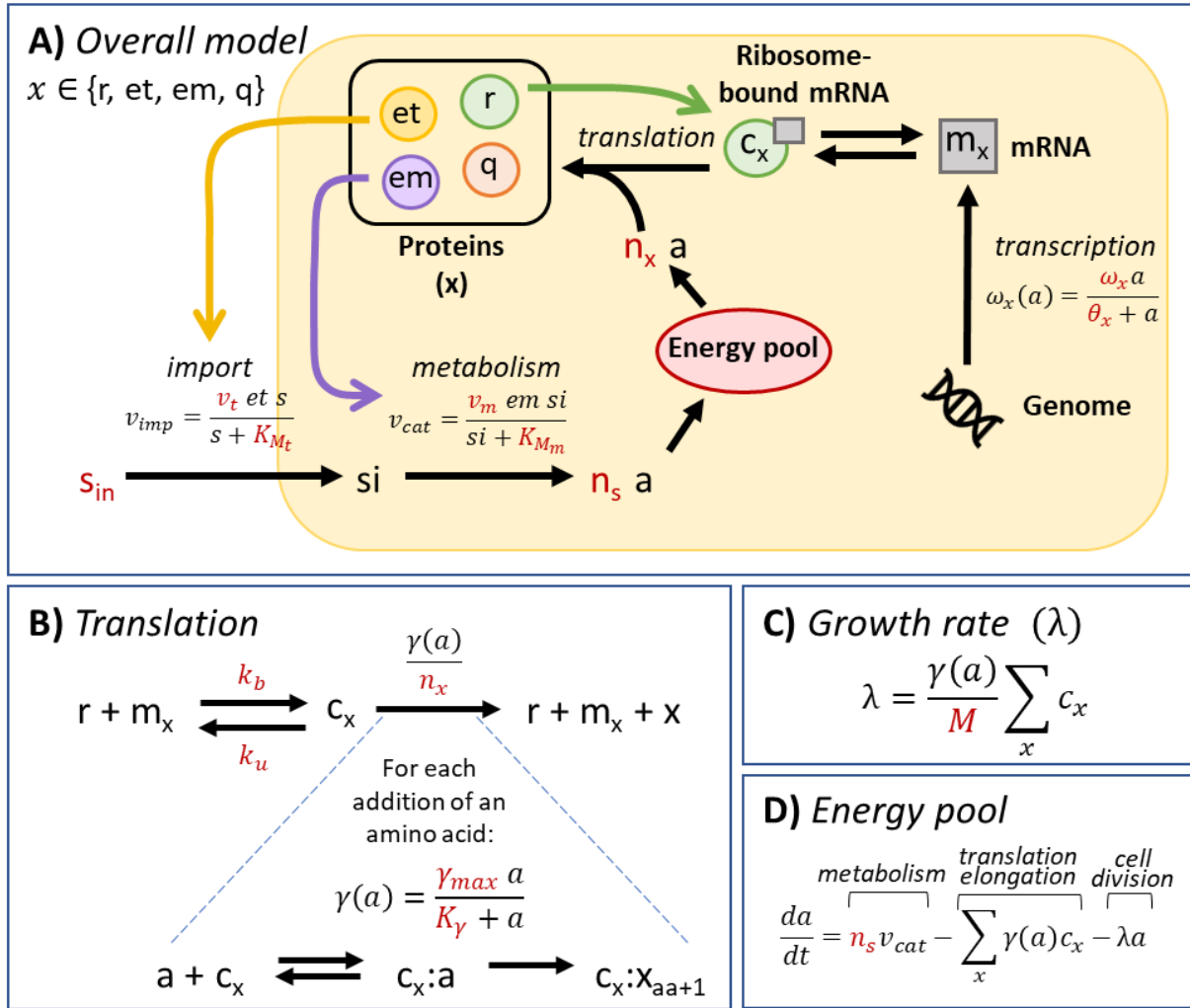


Figure 2.1 Weiße's whole-cell modelling framework

A) Expression of proteins is carried out in 3 steps: transcription, ribosome binding and translation. Free mRNA, ribosome-bound mRNA and protein are denoted by m_x , c_x and x respectively, with x being r for ribosomal, q for housekeeping, em for metabolic or et for transport. The substrate, s , is supplied to the cell at a constant rate (s_{in}) controlled by a chemostat and imported into the cell by transport proteins. The imported substrate, si , is converted into energy units, a , by metabolic proteins. **B)** A simplified translation model accounts for ribosome binding and unbinding to mRNA and then an irreversible elongation reaction. Energy units are consumed in translation elongation, with one energy unit consumed for each addition of an amino acid (aa). **C)** Growth rate, λ , is calculated as a function of the rate of translation of all proteins in the cell and the total proteome content of the cell. All intracellular molecules will be lost at a dilution rate equal to the growth rate. **D)** The finite energy pool available to the cell is determined by the balance between energy produced by metabolism of the substrate and consumption of energy required for protein translation. For a full description of these equations, see Weiße *et al.* (2015).

Parameters (in red): $x \in \{r: \text{ribosomal}, et: \text{transport}, em: \text{metabolic}, q: \text{housekeeping}\}$; s_{in} : rate of glucose input; v_t : k_{cat} of transport reaction; K_{Mt} : Michaelis constant for transport reaction; v_m : k_{cat} of metabolic reaction; K_{Mm} : Michaelis constant for metabolic reaction; n_s : nutrient efficiency; ω_x : maximum transcription rate of protein x ; θ_x : "transcription threshold" for protein x ; γ_{max} : maximum translation rate; K_γ : "translation threshold"; k_b : rate of ribosome binding; k_u : rate of ribosome unbinding; n_x : length of protein x in amino acids; M : all amino acids in the proteome.

This Figure was previously published in Atkinson *et al.* 2022b.

Therefore in the new model, presented in this thesis, the rate of uptake of glucose from the environment into the cells, $v_{uptake}(et, s)$, was adjusted to account for the number of each cell type in the consortia, (Equation 2.1), or in the monoculture (Equation 2.2). In this framework, et is the number of transport proteins per cell, which may vary across Cell A and Cell B, while v_{et} is the k_{cat} of the transport reaction catalysed by all the transport proteins in the cell, and K_{met} is the Michaelis constant for the same transport reaction.

$$v_{uptake}(et, s) = \frac{v_{et} s (N_a et_{cell_a} + N_b et_{cell_b})}{s + K_{met}} \quad \text{Equation 2.1 Rate of uptake of glucose for consortia}$$

$$v_{uptake}(et, s) = \frac{v_{et} s N et}{s + K_{met}} \quad \text{Equation 2.2 Rate of uptake of glucose for monoculture}$$

Here N_a and N_b are the number of Cell A and Cell B respectively, and N is the number of cells in the monoculture population. This framework assumes that the population of both cell types can be tightly controlled. For all the results presented in this chapter I also assume that the populations can be normalised so the ratio of Cell A to Cell B is 1:1; although the ratios of the two cells can be adjusted by changing the values of N_a and N_b . For a fair comparison between the consortium and monoculture cases, we assume there is an equal total number of cells in both cases such that $N = N_a + N_b$.

The amount of glucose available to the cells, s , will depend on the glucose input, s_{in} , the amount of glucose consumed by the all the cells in the population, $v_{uptake}(et, s)$, and the dilution rate, d_s , of the chemostat, modelled using Equation 2.3. All parameter values used in the model are summarised in the Materials and Methods Section 7.1.4.

$$\frac{ds}{dt} = s_{in} - v_{uptake}(et, s) - d_s s \quad \text{Equation 2.3 ODE for glucose}$$

2.3. DOL reduces resource redistribution to heterologous genes

Running a simulation (Materials & Methods 7.1.1) with glucose as the primary substrate shows that DOL in a two-strain consortium composed of Cells A and B achieves faster growth rates than the monoculture case (Figure 2.2A & B). As the maximum transcription rate (ω) of the expressed

heterologous proteins increases, the growth rate decreases. In the monoculture, growth rate is thus dependent on the transcription rate of both enzyme A and enzyme B (Figure 2.2C), highlighting competition for resources between heterologous proteins and host processes. In the consortia, however, the growth rate of each cell (Cell A or Cell B) depends on the transcription rate of the single heterologous protein it expresses. Beyond just the benefit of improving growth rate, this data also demonstrates another benefit of DOL. Because the monoculture is dependent on both genes' expression level this can be a design challenge when trying to optimise either of the two genes in a one-factor-at-a-time approach. In the DOL case both cells could be optimised independently and in parallel.

The model predicts that DOL will improve the amount of heterologous protein that can be produced per cell. Cell A produces 20130 molecules/cell of enzyme A (7.95% of total protein in Cell A) (Appendix 9.2), while Cell B produces 15661 molecules/cell of enzyme B (6.34% of total protein in Cell B) (Appendix 9.2). In contrast a single cell in the monoculture produces 16381 enzyme A and 13394 enzyme B (11.94% of total protein). While the total number of proteins in the Weisse model may be underestimated (Soufi et al., 2015; Milo, 2013), the values for heterologous gene expression as a percentage of total protein lie within an acceptable range as *E. coli* has been shown to tolerate up to 30% of their proteome dedicated to heterologous expression (Dong, Nilsson & Kurland, 1995).

While in the consortia, each cell type, A or B, can produce more of its respective heterologous protein per cell than the monoculture, this may not result in higher heterologous protein yield overall. In a monoculture of 1000 cells, all 1000 will express both proteins. In a consortium with 1000 cells, 500 will express protein A, and 500 will express protein B. Assuming that each cell in these populations produces the amount of protein predicted by the whole-cell model; a 1000-cell monoculture will produce around 16 million enzyme A and 13 million enzyme B. While a 1000-cell consortium will only produce around 10 million enzyme A and 8 million enzyme B. Therefore, while the monoculture case has slower growth and produces fewer heterologous proteins per cell, it may produce more heterologous proteins in total than the equivalent consortia case.

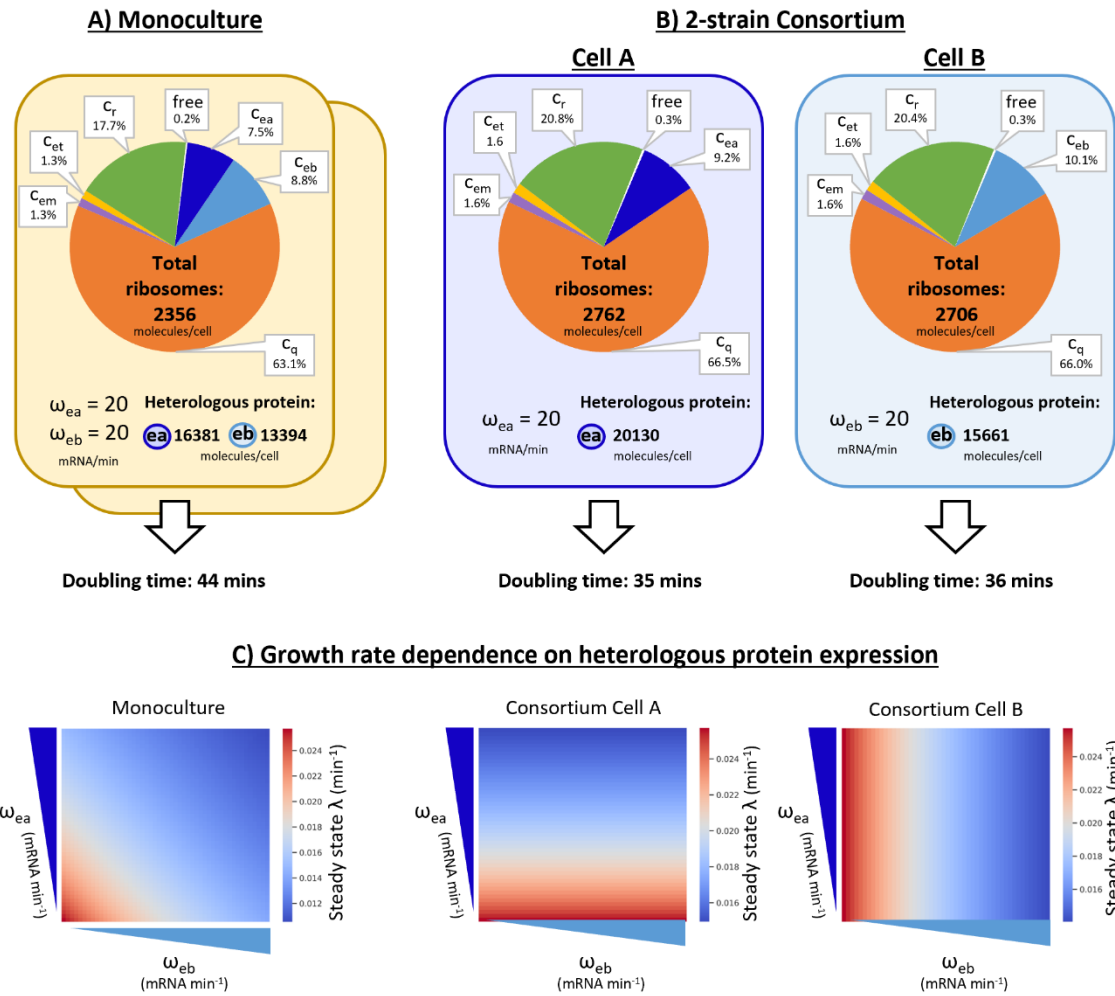


Figure 2.2 Simulation results for division of labour (DOL) compared to single-cell co-expression

Our proposed model accounts for the number of free ribosomes (**free**), and the number of ribosomes engaged in translation (**c_x**) to produce different types of protein (**x**, where **x** is **ea**, heterologous protein A; **eb**, heterologous protein B; **q**, housekeeping protein; **em**, metabolic enzyme; **et**, transport enzyme; **r**, ribosomal protein). Two heterologous proteins, **ea** and **eb**, can be expressed in a monoculture or consortium. Considering maximum transcription rates (ω) of 20 mRNAmin⁻¹ for both proteins, we use this model to predict the steady-state amount of ribosomes dedicated to the translation of these proteins and the associated cell growth rate that results from the reallocation of these ribosomes. **A)** In monoculture, at steady state, 16.3% of ribosomes are diverted to heterologous expression with an associated doubling time of 44 mins. **B)** DOL in a two-strain consortium means the resources of each cell are only diverted to the expression of a single protein. The overall reallocation of ribosomes to heterologous expression is thus reduced. However, the fraction of ribosomes allocated to the translation of each protein type is higher in the consortium case (ea: 9.2%, eb: 10.1% in the consortium case, compared to 7.5% and 8.8%, respectively in the monoculture case). The doubling time is faster compared to the monoculture case, while the number of heterologous proteins of each type produced per cell is higher. **C)** In monoculture, the growth rate (λ) is dependent on the maximum transcription rates (ω) of both heterologous proteins. In the consortium, the growth rate is dependent on only one of the two transcription rates.

Ea & eb: two generic heterologous proteins, **c_x**: ribosome:Mrna complexes for protein **x** ($x \in \{ea, eb, q, em, et, r\}$), λ : growth rate, ω_{ea} : maximum transcription rate of ea, ω_{eb} : maximum transcription rate of eb.

This Figure was previously published in Atkinson *et al.* 2022b.

2.4. Applying the model to DOL in starch-degradation

Next, I wanted to apply the 2-strain consortium to degradation of a complex substrate. The degradation of starch, the second most abundant carbon source in plants, was ideal as it requires two enzyme actions: endo- and exo-amylase. α -Amylase (EC 3.2.1.1) is an endo-amylase breaking α -1,4 glycosidic bonds randomly, yielding maltodextrins. Glucoamylase (EC 3.2.1.3) acts as an exo-amylase, releasing glucose from non-reducing ends through cleaving α -1,4 or α -1,6 bonds. These enzymes are well-characterized across various starch sources and maltodextrins (El-Fallal et al., 2012).

In the previous iteration of this model, I used an original schema for starch degradation, whereby each enzyme reaction produced an unknown number of products at an unknown rate, with the intention of measuring these unknowns in the lab. However this parametrisation would have required calculating the precise length of the starch chain over time, which was not achievable. Therefore instead, for the updated version of the model I have utilized a simplified set of equations proposed by Fujii & Kawamura (Fujii & Kawamura, 1985). When starch is abundant, its degradation is modelled using a two-step process. The α -amylase-dependent first reaction produces an intermediate which can act as a substrate for the glucoamylase-dependent second reaction. The reasoning for this schema is that at the beginning of starch hydrolysis there are relatively few non-reducing ends within the whole molecule that glucoamylase can act on, so the dominant reaction is the α -amylase hydrolysis reaction. Similarly, once a chain is degraded to a certain length, or limited by branches, α -amylase affinity for the substrate decreases. If a large proportion of starch is degraded to short chains, then the α -amylase reaction becomes negligible and the glucoamylase rate parameters will change based on the average chain length of the polysaccharide, as described in Fujii & Kawamura (Fujii & Kawamura, 1985). Both α -amylase and glucoamylase show Michaelis-Menten kinetics in terms of the rate of hydrolysis. Equation 2.4 and Equation 2.5 show the rate of change for starch, s_0 , and the intermediate, s_1 , respectively. When starch is externally supplied as the primary substrate, glucose, s , will vary based on Equation 2.6, replacing the previously stated Equation 2.3 which is only applicable when glucose is supplied as the primary substrate.

$$\frac{ds_0}{dt} = s_{0in} - N_a \frac{v_{ea} ea s_0}{s_0 + K_{m_{ea}}} - d_{s_0} s_0$$

Equation 2.4 ODE for starch

$$\frac{ds_1}{dt} = N_a \frac{v_{ea} ea s_0}{s_0 + K_{m_{ea}}} - N_b \frac{v_{eb} eb s_1}{s_1 + K_{m_{eb}}} - d_{s_1} s_1$$

Equation 2.5 ODE for intermediate

$$\frac{ds}{dt} = N_b \frac{v_{eb} eb s_1}{s_1 + K_{m_{eb}}} - v_{uptake}(et, s) - d_s s$$

Equation 2.6 ODE for glucose when starch is the carbon source

Here ea represents the concentration of α -amylase, while eb denotes that of glucoamylase, v_{ea} and v_{eb} represent the k_{cat} rates for ea and eb , respectively, and $K_{m_{ea}}$ and $K_{m_{eb}}$ are the Michaelis constant for each enzyme. The rate of each step is also dependent on the number of cells expressing α -amylase (i.e. the population of Cell A (N_a)) and the number of cells expressing glucoamylase (i.e. the population of Cell B (N_b)). For a monoculture system all cells will express both, therefore N_a and N_b will be replaced with N . In this example, I assume that each population type can be tightly controlled and that the proportion of Cell A to Cell B can be robustly maintained at a 1:1 ratio. Under this assumption, without loss of generality, I consider $N_a = 1$, $N_b = 1$ and $N = 2$, so that the monoculture and consortium have equal total number of cells. The rate of starch input, s_{0in} , and the rate of loss of each substrate, d_{s_0} , d_{s_1} and d_s , can be adjusted for the desired bioreactor setup. These equations were combined with the ODEs for Cell A and Cell B as shown in Figure 2.3 and summarised in Materials and Methods 7.1.3.

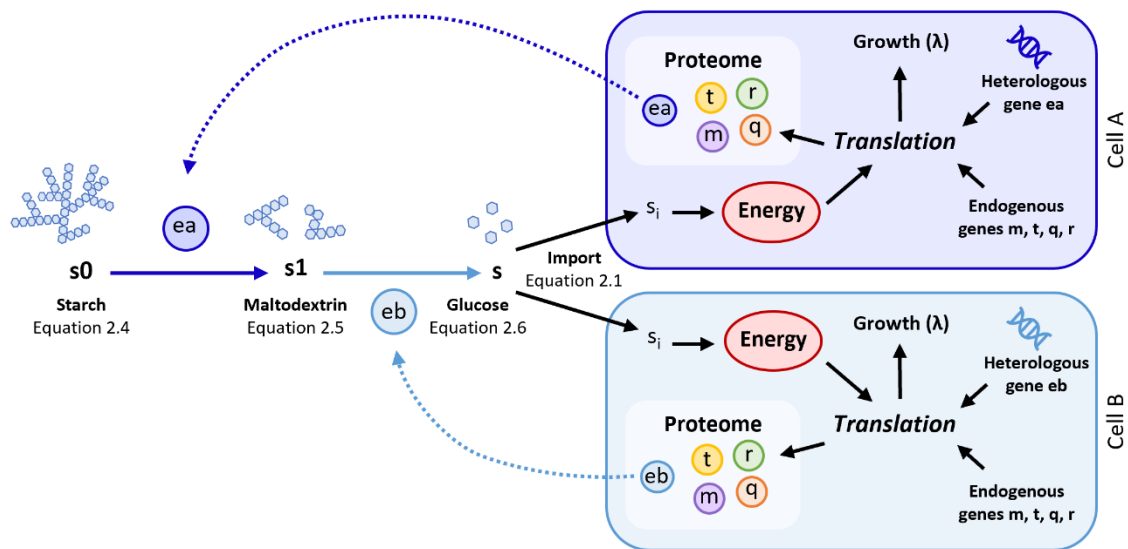


Figure 2.3 Model for starch utilisation with division of labour in a two-strain consortium.

Each cell is modelled using the Weiße *et al.* (2015) modelling framework, adapted to account for the additional expression of either α -amylase (**ea**) or glucoamylase (**eb**). These amylases each catalyse a step in the degradation of starch. The first step is an α -amylase-dependent reaction, which degrades starch (**s0**) and produces maltodextrin intermediates of various lengths (**s1**). These intermediates are then degraded to glucose (**s**) via a glucoamylase-dependent reaction. Glucose is imported into each cell and the intracellular molecules of the two cells are modelled with the whole-cell model based on Weiße *et al.* (2015).

2.5. DOL can achieve higher growth rate on starch than an equivalent monoculture system

To investigate the relationship between amylase expression, starch degradation and burden when starch is used as the primary carbon source, I used the model in Equations 2.1–6 to numerically simulate growth rate for a range of expression levels of amylases.

To estimate realistic levels of transcription for the two amylases I looked at the available Anderson promoters. Anderson Promoter J23116 (a medium-level promoter) had a total expression per cell of 1.3266 molecules per second per cell (or 79.6 moles/min/cell), although in this case they did have multiple copies of the plasmid per cell. They calculated the “transcription rate” to be 2.203×10^{-7} M/min. Anderson Promoter J23100, the strongest promoter, gave a total expression of 8.5326 molecules per second per cell (or 512 moles/min/cell) (iGEM Parts Registry, n.d.).

While the model is not a population-based model, and therefore the assumptions it is based on only work at a single-cell level, by changing the ratio of Cell A to Cell B the model can give some insight into the effect of unequal ratios of the two strains. A parameter sweep of N_a and N_b (the number of each type of cell) revealed that approximately equal ratios showed the best average

growth rate for the consortium (Figure 2.4). Hence, a 1:1 ratio was assumed for all simulations. When a higher number of cells are used, a higher growth rate is achieved when N_a is slightly higher than N_b , for example 9:8 is higher than 9:9. This indicates that if larger populations were considered, then having more Cell A than Cell B might be advantageous. However, for the purpose of this project, this model was kept at a single-cell level and did not consider population-level dynamics.

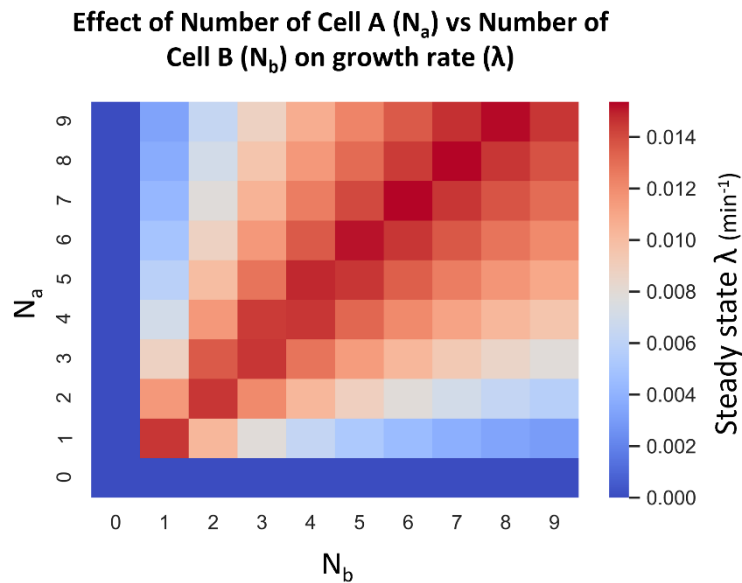


Figure 2.4 The effect on growth rate of changing the values of N_a (the number of Cell A) and N_b (the number of Cell B) in the consortium

By changing the number of Cell A (N_a) and number of Cell B (N_b) parameters the model shows that the best growth is achieved when N_a and N_b are roughly equal, especially at the lower values. At higher values the ratios where $N_a > N_b$ by 1 achieve a higher growth rate, which might indicate that, if the model considered populations, then a consortium with more Cell A might improve growth.

Figure 2.5A illustrates the growth rate of a monoculture single cell as a function of the maximum transcription rate (ω) of α -amylase (ω_{ea}) and glucoamylase (ω_{eb}). Figure 2.5B shows the average growth rate of a 2-cell consortium, where Cell A expresses only α -amylase, and Cell B expresses only glucoamylase. The monoculture reaches its maximum growth rate of 0.01784 min^{-1} , corresponding to a doubling time of 38.85 mins at $\omega_{ea} = 15$ and $\omega_{eb} = 12 \text{ mRNA min}^{-1}$. At this 1:1 ratio, the maximum average growth rate achieved by the consortium was 0.01793 min^{-1} , or a doubling time of 38.66 mins, which was achieved at $\omega_{ea} = 29$ and $\omega_{eb} = 24 \text{ mRNA min}^{-1}$. At this maximum, Cell A is growing at 0.01779 min^{-1} (doubling time 38.96 mins), while Cell B is growing at 0.01807 min^{-1} (doubling time 38.36 mins).

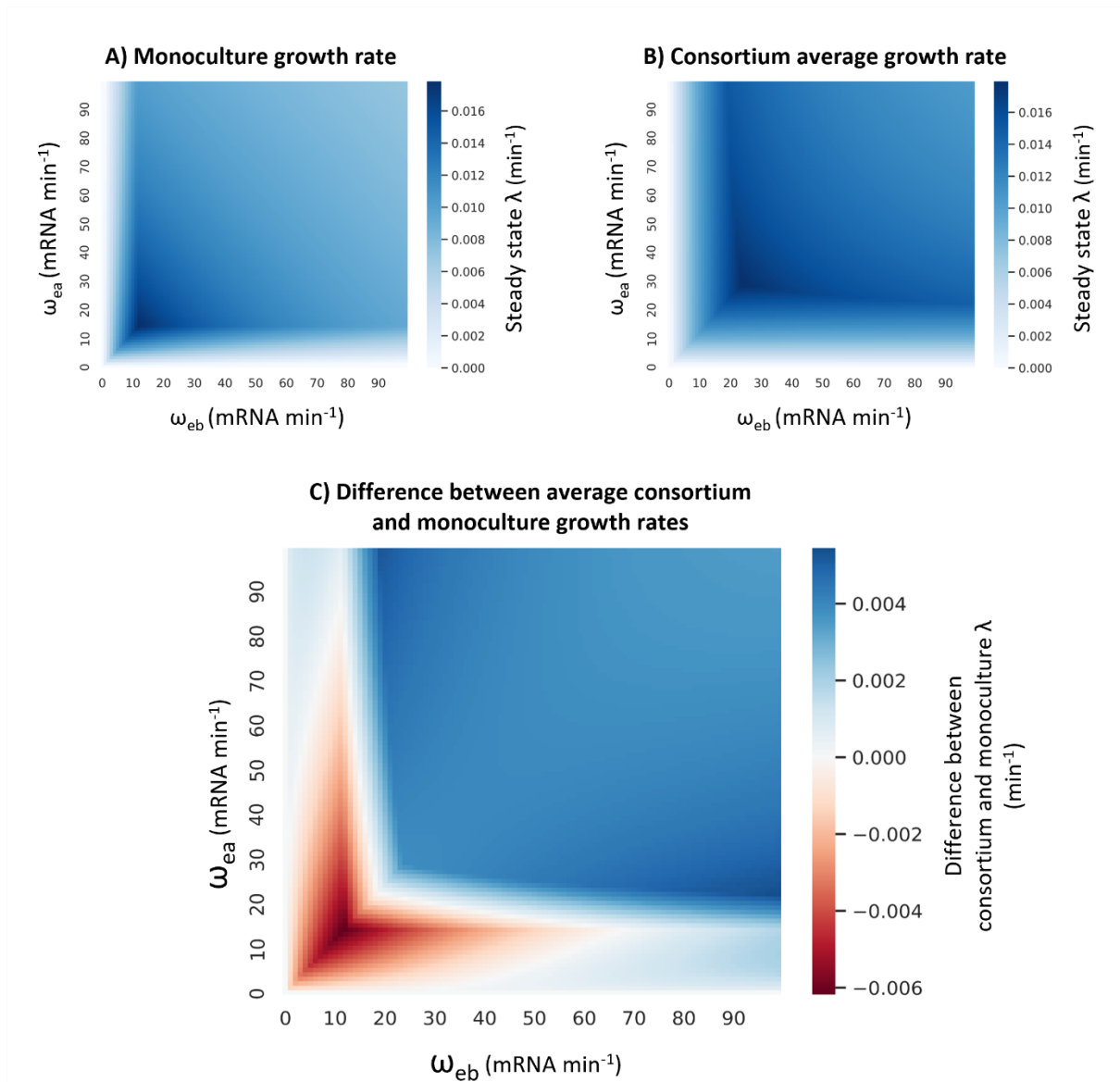


Figure 2.5 Changes in the steady state growth rate with varying transcription rates for α -amylase (ea) and glucoamylase (eb), in monoculture and division of labour (DOL) systems of expression.

Changes in growth rate (λ , min^{-1}) for an average cell in a monoculture or a two-strain consortium, as the maximum transcription rates of α -amylase and glucoamylase (ω_{ea} and ω_{eb} respectively) are varied. To obtain biologically realistic values for each parameter combination, a numerical simulation with glucose as the main carbon source was first run until steady-state values were reached. Using these steady-state values as initial conditions, I then performed a second run with starch as the main carbon source. **A)** Steady-state growth rate of an average cell co-expressing α -amylase and glucoamylase. **B)** Steady-state growth rate of an average cell in the consortium. Cell A expresses α -amylase, while Cell B expresses glucoamylase. The consortium growth rate is calculated as the average growth rate of the two cells. **C)** Comparison of monoculture and consortium growth rate (difference measured as the average growth rate of the consortium minus the monoculture growth rate). This Figure was previously published in Atkinson *et al.* 2022b.

When I considered the cell types in the consortium separately, the fastest growth rate for Cell A was 0.01915 min^{-1} (doubling time 36.20 mins), at $\omega_{ea} = 22$ and $\omega_{eb} = 99 \text{ mRNA min}^{-1}$. At these transcription rates, Cell B grows much more slowly, with a growth rate of 0.01004 min^{-1} (doubling time of 69.04 mins). Conversely, Cell B reaches its maximum growth rate of 0.01924 min^{-1} (doubling time 36.03 mins), at $\omega_{ea} = 99$ and $\omega_{eb} = 19 \text{ mRNA min}^{-1}$, where Cell A has a lower growth rate of 0.01098 min^{-1} or a doubling time of 63.13 mins. This suggests that each cell type in the consortium grows best when its consortium partner's transcription rate is high.

Figure 2.5C highlights the difference between the average growth rate of the consortium and the monoculture. At lower transcription rates, the monoculture has a faster growth rate than the consortium (red region in Figure 2.5C), while the consortium outperforms the monoculture at higher transcription rates (blue region in Figure 2.5C).

The monoculture performs better at lower transcription rates because, although it produces less per cell, it generates more total heterologous proteins, as discussed in Figure 2.2. This results in a higher concentration of enzymes in the environment compared to the consortium (Appendix 9.3), leading to faster starch degradation and thus, quicker growth. By comparing the glucose levels in the monoculture (Figure 2.6A) and the consortium (Figure 2.6B), the model reveals that the monoculture consistently reaches a higher steady-state glucose concentration than the consortium. Figure 2.6A and B show white regions where the steady-state glucose is close to zero, indicating that the cells are consuming all the glucose produced by amylase activity. The monoculture reaches a point of glucose surplus at lower transcription rates than the consortium. Referring back to Figure 2.5C, the red region corresponds to the parameter space where the consortium's steady-state glucose remains at zero, whereas the blue region corresponds to the consortium achieving a glucose surplus.

This suggests that when growth is restricted by glucose production rather than the burden of gene expression, DOL is not a good strategy for improving growth. At higher transcription rates, the monoculture is more affected by burden than the consortium since it co-expresses two heterologous proteins which divert resources away from essential endogenous genes. As transcription rates increase, this burden becomes more significant, allowing the consortium to surpass the monoculture in growth rate. Burden must be sufficiently limiting to growth for DOL to be an appropriate strategy to improve growth on starch.

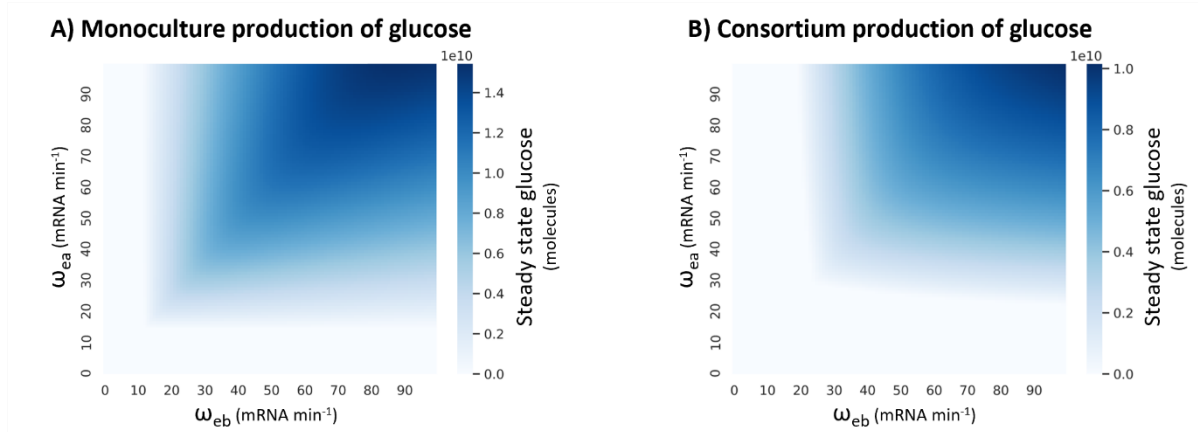


Figure 2.6 Changes in the steady state glucose with varying transcription rates for α -amylase (ea) and glucoamylase (eb), in monoculture and division of labour (DOL) systems of expression.

Steady-state level of glucose produced from starch **A)** by a 2-cell monoculture or **B)** by a 2-cell consortium (with 1 Cell A and 1 Cell B as the transcription rates of α -amylase and glucoamylase (ω_{ea} and ω_{eb} respectively) are varied. For the monoculture and the consortium there is a region of expression where the glucose level is 0. This is where the cell is consuming all the glucose that the amylases are creating. The monoculture reaches saturation of glucose (where there is >0 glucose at steady state, the blue region) at lower transcription rates than the consortium. Where glucose steady state is >0 , the monoculture produces higher excess glucose at all combinations of the maximum transcription values (ω_{ea} and ω_{eb}).

2.6. Adapting the model to yeast

The model has been run using the *E. coli* parameters from the original Weiße model. However, *in vivo* I planned to use yeast as the host organism. Yeast, as a eukaryote, may have different mechanisms for burden than a prokaryote like *E. coli*, however there is evidence that the fundamental assumptions of the whole-cell model apply to yeast as well. The growth rate laws that are represented in the Weiße model were first developed in Scott *et al.* (2010). In *E. coli*, the ribosomal protein fraction is a linear function of the growth rate λ when translational efficiency is varied (Scott *et al.*, 2010a). *S. cerevisiae* also has a linear correlation between growth rate and translation elongation rates, protein synthesis rates and amino acid incorporation rates (von der Haar, 2008; Boehlke & Friesen, 1975; Waldron & Lacroute, 1975). The Weiße model also adheres to the principle that translation accounts for the majority of energy usage in the cell. In *S. cerevisiae* there is a strong correlation between burden and the length of a heterologous protein, even when promoter strength is the same (Tomala & Korona, 2013). In addition, amino acid supplementation in the yeast media can alleviate the effects of burden (Van Rensburg *et al.*, 2012; Görgens *et al.*, 2005). Both these observations support translation as the primary process underpinning metabolic burden in yeast. (Lee *et al.*, 2011; Airoidi *et al.*, 2009; Lang, Murray &

Botstein, 2009; Levy & Barkai, 2009; von der Haar, 2008). Therefore, the mechanistic trade-offs implemented in the Weiße model for *E. coli* should apply to yeast as well.

To adapt the model for a yeast system, alternate parameters are required. *Y. lipolytica* is a non-model yeast which does not have extensive kinetic parameters defined in literature compared to a model organism such as *E. coli*. Instead, I used parameters for the model yeast *Saccharomyces cerevisiae* (Materials and Methods 7.1.4). Any unknown parameters were kept at the same level as for *E. coli*.

The doubling time for yeast predicted by the simulation, using the substitute parameters, was 97.46 mins, close to the expected doubling time of 90 mins for *S. cerevisiae*. But the values of the intracellular molecules were much higher than expected. The total protein:total RNA ratio predicted by the model was 35686, while in literature the total protein:total RNA ratio in yeast is 709 (Lahtee et al., 2017). The rRNA as a fraction of the total RNA was lower in the model, at 0.612, than the fraction in literature, at 0.830 (von der Haar, 2008). And the ribosomal proteins as a fraction of total proteins was much higher, at 0.567 in the model, compared to 0.004 in literature (Futcher et al., 1999; Warner, 1999).

In the original Weiße model some parameters were obtained by Bayesian approximations fitted to experimental data. The unknown parameters were the maximal transcription rates (ω_q , ω_r , ω_t , ω_m), the transcriptional energy-thresholds (θ_r , θ_x), the parameters for housekeeper inhibition (K_q , h_q), and the translational energy-threshold (K_v). These parameters could have been determined for *Y. lipolytica* using the same method described in Weiße et al. (2015). To do so *Y. lipolytica* would be grown on different media and with different concentrations of a translational inhibitor, both of which have linear relationships with growth (Scott et al., 2010a). However, I did not complete the experiments necessary to fully re-parametrise the model to yeast. Within the time constraints and bearing in mind the objectives of the project I did not think deriving these parameters precisely would be beneficial.

2.7. Limitations of this model

One key limitation of this model is the absence of experimental validation for its quantitative predictions. While the model highlights the reduced metabolic burden observed when DOL is implemented, this advantage has primarily been demonstrated through experimental data from DOL in biosynthesis pathways, where higher productivity or molecule production can be achieved compared to single-cell systems (Jones et al., 2016, 2017; Zhang et al., 2015). Additionally, various experimental studies support the use of consortia for degrading

lignocellulosic biomass (Li et al., 2019; Chen et al., 2018; Kogo et al., 2017; Tsai, Goyal & Chen, 2010). However, experimental comparisons between consortia expressing different degradation enzymes and equivalent co-expressing monocultures are relatively rare. As a result, while this model offers theoretical predictions of how such consortia might behave in comparison to monocultures, these predictions still require experimental verification.

Another limitation of the current model is that it assumes a stable ratio of cell types in the consortium, thereby overlooking the potential effects of asymmetric growth rates on population dynamics. In a monoculture, the primary substrate is shared equally, as all cells grow at the same rate. However, in a consortium, if one cell type grows faster than the other, it may consume more of the substrate, creating an imbalance with its consortium partner. The current 2-strain consortium model suggests that if transcription rates for one protein are set very high and the other very low, significant differences in growth rates between the two cell types could arise. In this case, the lower-expressing cell would have a survival advantage, potentially dominating the co-culture. Despite this, the model assumes a stable, fixed ratio between the two cell types, leaving no insight into how population dynamics might evolve over time. To capture both cellular and population-level dynamics more accurately, simplified host-aware coarse-grained models or agent-based modelling approaches may be more appropriate. Correctly balancing metabolic burden may play a key role in ensuring cooperative populations remain stable.

Finally, in terms of application of this system *in silico* versus *in vivo*, most industrial settings use batch or fed-batch reactors, but the model predicts the behaviour in a chemostat. While the intracellular molecules are dynamic, only the final steady state is output. Therefore, the results presented do not consider the time taken to reach that steady state. *In vivo*, the growth of cells is often measured as an increase in density of a population or an increase in biomass over time. These experimental measurements may not be reflected by the doubling time at exponential growth, since the latter does not consider the time-delay in starch degradation.

2.8. Conclusions for this chapter

In this chapter I have adapted a whole-cell model for metabolic burden to observe and predict the difference in growth when expressing heterologous genes by DOL versus in a single cell. Here, I have shown that a consortium of two cells each expressing a single heterologous protein achieves a higher growth rate than a monoculture co-expressing two heterologous proteins, because fewer ribosomes are redirected toward heterologous gene expression. Each cell in the consortium produces more of its respective enzyme than a single cell in the monoculture, but at the population level, when the monoculture and consortium have the same total number of cells,

the monoculture produces a greater overall number of heterologous proteins. Therefore, the choice between monoculture and a DOL system depends on the desired outcome.

The suitability of a DOL system for improving the growth of engineered *E. coli* on starch depends on balancing the productive expression of amylases for the production of glucose and the non-productive expression that creates an unnecessary burden on the cells. When starch degradation to glucose is the primary growth-limiting factor, a monoculture system is more advantageous because it produces a greater overall number of amylases. However, there is a threshold where amylase expression results in a surplus of glucose, and growth is no longer limited by substrate availability but by the metabolic capacity of individual cells to process the available sugars. Here, the reduced metabolic burden in a DOL consortium allow cells to process these sugars more efficiently toward biomass and other endogenous processes than cells in the monoculture that are burdened by expressing all enzymes. Hence, beyond this threshold, the consortium achieves a higher growth rate than the monoculture. Therefore, when the burden of gene expression becomes the main limitation to growth, the DOL system is the more effective strategy.

In this chapter I have looked at DOL for the degradation of starch *in silico*. In the next chapter I begin to apply the principles, learned from the model, *in vivo* in *Y. lipolytica*.

3. Chapter 3: 2-strain *Yarrowia lipolytica* consortia for division of labour in the degradation of a polysaccharide substrate.

3.1. Introduction to this chapter

Polysaccharides, such as starch, xylan and inulin, are long chains of monosaccharide units linked by glycosidic bonds which can be broken down by a combination of different hydrolysing enzymes. Where multiple hydrolysing enzymes are required for the most efficient degradation, this offers an opportunity for DOL. In a monoculture, cells face a higher metabolic burden, as resources are divided between producing multiple enzymes and growing. In contrast, in DOL, cells are specialised to produce fewer enzymes, reducing this burden and allowing for more efficient growth. In Chapter 2, modelling suggested that a DOL strategy could lead to higher growth rates compared to a monoculture when metabolic burden is limiting to growth. Building on this, I hypothesised that this effect will also be observed *in vivo*, where a 2-strain consortium of cells, each expressing a single hydrolysing enzyme, will grow faster than an equivalent monoculture where each cell produces both enzymes (Figure 3.1). While the hypothesis was supported by the model *in silico*, testing it *in vivo* introduces new challenges, such as ensuring efficient and equal resource sharing between cells, maintaining the stability of the consortium, and accounting for environmental variability.

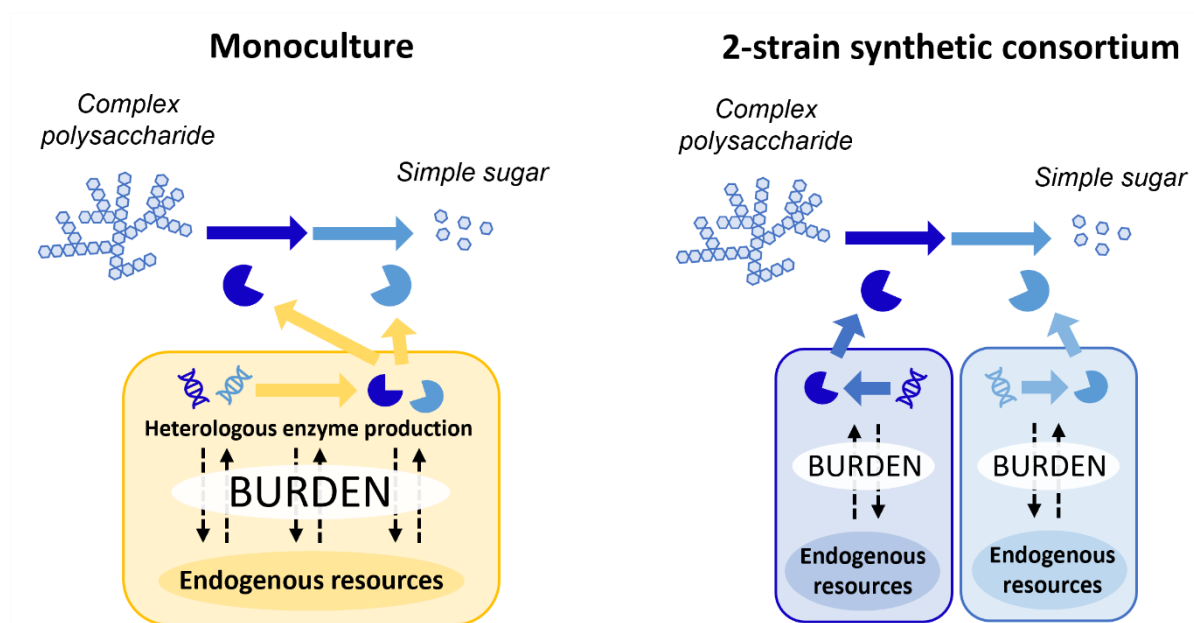


Figure 3.1 Monoculture and consortium systems for polysaccharide degradation

In a monoculture all heterologous genes required to degrade a complex substrate are expressed in one cell. This may cause significant burden by consuming cellular resources and interfering with essential host bioprocesses. In a consortium the labour is divided between cells, reducing the metabolic burden on each individual cell.

In this chapter, I mainly focus on the degradation of starch. The successful engineering of a *Y. lipolytica* strain capable of growing on raw starch as its sole carbon source was achieved by co-expressing an α -amylase from *Oryza sativa* and a glucoamylase from *Aspergillus niger* (Ledesma-Amaro, Dulermo & Nicaud, 2015). However, the engineered strain showed significantly reduced growth on glucose compared to the wild-type (WT), suggesting that metabolic burden might be a limiting factor. This finding aligns with previous observations that metabolic burden can lead to decreased biomass yields in *Y. lipolytica* (YaPing et al., 2017; Roth, Moodley & Van Zyl, 2009). By dividing labour between two cells, each expressing a single amylase, I wanted to see if burden is reduced, allowing the consortia to outperform the single-cell system.

3.2. Comparing the growth of strains expressing amylases with different promoter and signal peptide (SP) combinations

Since the benefit of DOL works on the premise that it reduces burden upon the cell, the first test was to see if I could observe this burden in amylase-expressing cells. I engineered *Y. lipolytica* strain Po1d to express either *Oryza sativa* α -amylase (OsAA) or *Thermomyces lanuginosus* glucoamylase (TlGAMY). Both these amylases have been previously shown to function well in *Y. lipolytica* (Celińska et al., 2018; Ledesma-Amaro, Dulermo & Nicaud, 2015).

In addition, I wanted to observe the effect of expression and secretion on burden, to see if the model created in Chapter 2 would be an accurate model for the *Y. lipolytica* starch-degrading consortia. I used combinatorial Golden Gate assembly (Materials and Methods 7.4.1) with three different promoters to control the expression level: the weak native promoter pPGM, the strong native promoter pTEF and the synthetic promoter 8UASpTEF, which is made of the native pTEF sequence preceded by eight upstream activating sequences (UAS), making a very strong promoter. I controlled the secretion level using a panel of five synthetic signal peptides (SPs) created by Celińska et al. (2018). The strains created are summarised in Table 3.1.

The strength of SPs is context-specific, and while Celińska et al. had already tested the SPs with TlGAMY, their strength with OsAA were unknown. To test the strength of the SPs with OsAA, I created the strains YTO1-5, each expressing OsAA under the control of pTEF and one of the five SPs. Supernatants of YTO1-5 were assessed for activity by micro-SIT assay (Materials and Methods 7.10.1), which uses iodine to stain starch granules. As the starch granules are degraded by OsAA the coloured stain decreases, and the less starch remaining the stronger the SP. SP1 and 5 were the strongest, SP3 and 4 were mid-level and SP2 was the weakest (Figure 3.2). This demonstrates the context-dependency of SPs, as for TlGAMY SP3 and 4 are strong, SP1 and 2 are mid-level and SP5 was the weakest (Celińska et al., 2018).

Table 3.1 Amylase strains engineered for this chapter

Strain	Insert/s	Strain	Insert/s
Single-amylase strains: express either α-amylase or glucoamylase			
YTO1	pTEF-SP1-OsAA	YTT1	pTEF-SP1-TIGAMY
YTO2	pTEF-SP2-OsAA	YTT2	pTEF-SP2-TIGAMY
YTO3	pTEF-SP3-OsAA	YTT3	pTEF-SP3-TIGAMY
YTO4	pTEF-SP4-OsAA	YTT4	pTEF-SP4-TIGAMY
YTO5	pTEF-SP5-OsAA	YTT5	pTEF-SP5-TIGAMY
YPO1	pPGM-SP1-OsAA	YPT1	pPGM-SP1-TIGAMY
YPO2	pPGM-SP2-OsAA	YPT2	pPGM-SP2-TIGAMY
YPO3	pPGM-SP3-OsAA	YPT3	pPGM-SP3-TIGAMY
YPO4	pPGM-SP4-OsAA	YPT4	pPGM-SP4-TIGAMY
YPO5	pPGM-SP5-OsAA	YPT5	pPGM-SP5-TIGAMY
Y8TO1	8UASpTEF-SP1-OsAA	Y8TT1	8UASpTEF-SP1-TIGAMY
Y8TO2	8UASpTEF-SP2-OsAA	Y8TT2	8UASpTEF-SP2-TIGAMY
Y8TO3	8UASpTEF-SP3-OsAA	Y8TT3	8UASpTEF-SP3-TIGAMY
Y8TO4	8UASpTEF-SP4-OsAA	Y8TT4	8UASpTEF-SP4-TIGAMY
Y8TO5	8UASpTEF-SP5-OsAA	Y8TT5	8UASpTEF-SP5-TIGAMY
YTO1-RFP	1) pTEF-SP1-OsAA 2) pTEF-hrRFP	YTT3-GFP	1) pTEF-SP3-TIGAMY 2) pTEF-GFP
Y8TO1-RFP	1) 8UASpTEF-SP1-OsAA 2) pTEF-hrRFP	Y8TT1-GFP	1) 8UASpTEF-SP1-TIGAMY 2) pTEF-GFP
Y2x8TO1	2 transformations with 8UASpTEF-SP1-OsAA	Y2x8TT1	2 transformations with 8UASpTEF-SP1-TIGAMY
YTG1	pTEF-SP1-AnGAMY	YTG2	pTEF-SP2-AnGAMY
YTG3	pTEF-SP3-AnGAMY		
Double-amylase strains: express both α-amylase and glucoamylase			
YTT3TO1	pTEF-SP3-TIGAMY-pTEF-SP1-OsAA	YTT5TO1	pTEF-SP5-TIGAMY-pTEF-SP1-OsAA
YTT3TO2	pTEF-SP3-TIGAMY-pTEF-SP2-OsAA	YTT5TO2	pTEF-SP5-TIGAMY-pTEF-SP2-OsAA
YTT3TO5	pTEF-SP3-TIGAMY-pTEF-SP5-OsAA	YTT5TO5	pTEF-SP5-TIGAMY-pTEF-SP5-OsAA
Y8TT18TO1	8UASpTEF-SP1-TIGAMY- 8UASpTEF-SP1-OsAA	Y8TT38TO3	8UASpTEF-SP3-TIGAMY- 8UASpTEF-SP3-OsAA
Y8TT18TO3	8UASpTEF-SP1-TIGAMY- 8UASpTEF-SP3-OsAA	Y8TT58TO3	8UASpTEF-SP5-TIGAMY- 8UASpTEF-SP3-OsAA
Y8TT38TO1	8UASpTEF-SP3-TIGAMY- 8UASpTEF-SP1-OsAA	Y8TT58TO5	8UASpTEF-SP5-TIGAMY- 8UASpTEF-SP5-OsAA
Y2x8TT18TO1	2 transformations with 8UASpTEF-SP1-TIGAMY- 8UASpTEF-SP1-OsAA	YTG18TO1	1. pTEF-SP1-AnGAMY 2. 8UASpTEF-SP1-OsAA

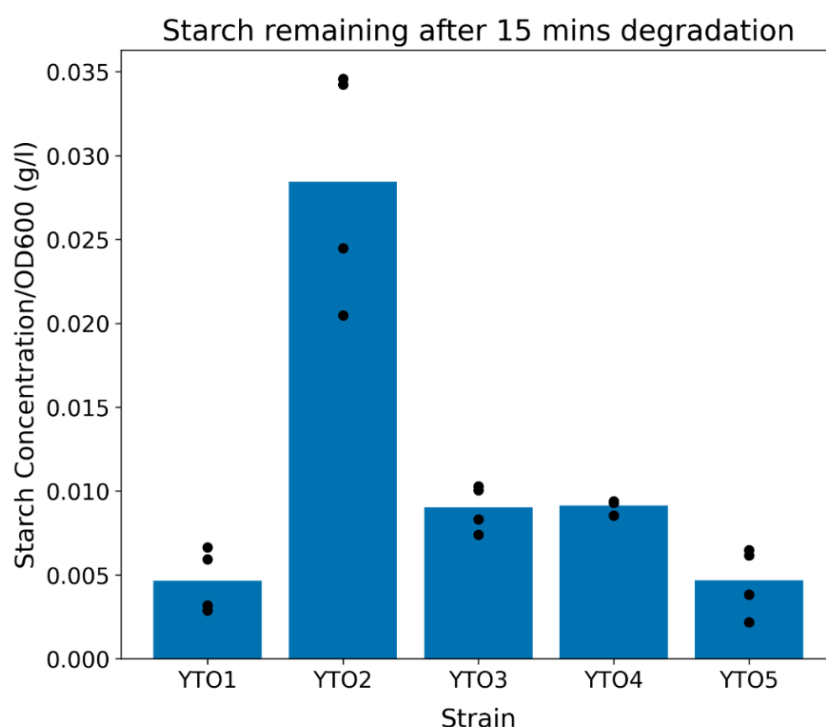


Figure 3.2 Micro-SIT for the supernatants of OsAA strains

Supernatants from cultures of single-OsAA-expressing strains were collected after 48 hours of growth and tested for activity by micro-SIT assay. Supernatants were incubated with 1% starch for 15 mins, and the remaining starch was stained with iodine and compared to a standard curve. Results were normalised by the final OD600 of the cultures. The YTO2 strain, using SP2, showed the highest starch remaining, making it the weakest signal peptide (SP) for OsAA. YTO1 and YTO5 had the least remaining starch, and were thus the strongest SPs. $n=4$.

Growth of these single-amylase strains, with glucose as the primary carbon source, was used to evaluate the impact of different promoters and SPs. Since amylases confer no survival advantage on glucose, any limitations reflect the burden of expressing the additional heterologous gene. Burden is typically indicated by a decrease in growth rate but can also manifest as a lower carrying capacity or an extended lag time, demonstrated by a later inflection point in the growth curve.

Strains were grown for 72 hours on YNB 2% glucose in 96-well plates with Absorbance at 600 nm (Abs600) measurements every 30 mins (Materials and Methods 7.11.1). Two separate transformants of each strain were tested, with three replicates of each. Growth parameters were obtained using curve-fitting (Materials and Methods 7.11.7) to a four-parameter Gompertz curve shown in Equation 3.1 (Tjørve & Tjørve, 2017).

$$Y(t) = Y_0 + (Y_s - Y_0) \cdot \exp(-\exp(-r(t - t_i)))$$

Equation 3.1 Gompertz model

Here $Y(t)$ is the Abs600 at time t , Y_0 is the initial Abs600, Y_s is the carrying capacity (Abs600 at stationary phase), r is the growth rate and t_i is time of the inflection point, where the growth rate reaches its peak.

Figure 3.3A, B and C show the growth parameters of each single-amylase strain. The most significant differences were seen for the strongest promoter, 8UASpTEF. Eight out of ten 8UASpTEF-SP variants had a decreased growth rate and later inflection point, with Y8TT1 and Y8TT4 being the exceptions; Y8TT4 had no significant difference and Y8TT1 had a significant increase in growth rate. Additionally, nine out of ten of the 8UASpTEF strains also had a slightly higher carrying capacity, with Y8TT1 being the exception. This correlation between reduced growth rate, later inflection point, and increase carrying capacity is an important consideration as it may indicate a higher burden might be better if you are aiming for a higher final Abs600, but worse if you want faster growth.

For the other promoter-SP-amylase combinations the change in the growth rate was more varied. While most show either a decrease or no significant difference from the WT, the pTEF-TIGAMY strains all seemed to have an increased growth rate compared to WT. It is unclear why this may be the case, but from the replicate points it seems like that at least for YTT1 and YTT2 one of the two transformants has a higher growth rate than the other, therefore it may be that the gene has integrated somewhere that increases the growth rate of the cell. Many of the other promoter-SP-amylase combinations also showed a significantly later inflection point, and a non-significant decrease in carrying capacity- all indicating a slower and lower overall growth when expressing these genes.

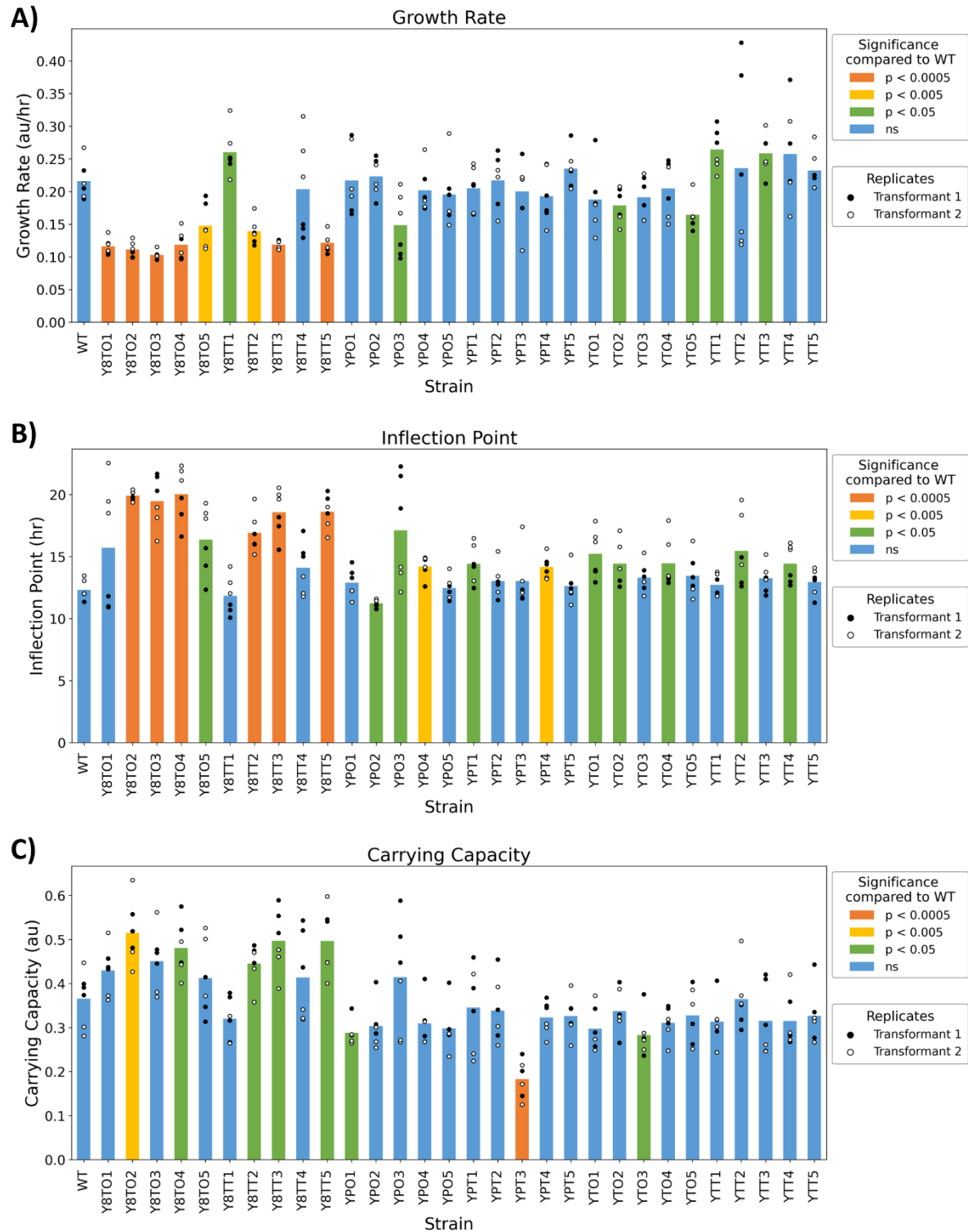


Figure 3.3 Comparison of growth parameters for single-amylase strains grown on glucose

Single-amylase strains were grown on 2% glucose YNB for 72 hours, and their growth was fitted to a Gompertz 4-parameter model to derive the parameters of **A)** growth rate **B)** inflection point (which reflects lagtime) and **C)** carrying capacity (the absorbance value at stationary phase). Growth rate was decreased in most of the 8UASpTEF controlled strains, while the lagtime is extended. This promoter also appeared to give a higher final carrying capacity. Data presented as the mean for n=6. Statistical analysis by Welch's T-test, pairwise analysis of each strain compared to the WT.

While the effect of the strongest promoter was quite clear, it was more difficult to parse the information for the other promoter-SP combinations for each gene. I used a standard least squares method of regression to further quantify the effects of the promoter and SP factors on the growth responses. The model incorporates both individual effects of the promoter and SP as well as their interaction, which assesses whether the impact of a particular promoter varies depending on the SP used. The model's fit to the data is evaluated using the adjusted R^2 statistic, which indicates how well the model explains the variability in each growth parameter, adjusted for the number of predictors. For OsAA, the model fit was poor for growth rate (adjusted $R^2 = 0.11$) but better for carrying capacity (0.67) and inflection point (0.64). In contrast, TlGAMY showed a somewhat improved fit for growth rate (adjusted $R^2 = 0.38$), with similar fits for carrying capacity and inflection point (0.63 and 0.64, respectively). This analysis revealed that the promoter had a significant effect on all growth parameters for both amylases, with p-values less than 0.01, indicating a strong influence. However, the SP alone did not show a significant impact. For TlGAMY, the interaction between promoter and SP (SP*Promoter) was significant for all three responses, while for OsAA, it was significant only for the inflection point (Appendix 9.4).

This model was used to predict the “desirability” of the different promoter and SP combinations (Materials and Methods 7.11.2), with the aim of maximising growth rate and carrying capacity and minimising inflection point – ie overall minimising any negative growth effects (Figure 3.4). For OsAA, the highest desirability scores were generally seen with SP2 and SP4 combinations, particularly with pPGM and pTEF promoters, indicating these combinations are possibly the least burdensome. In contrast, the lowest scores were observed with certain SP3 and SP4 combinations, especially with the 8UASpTEF promoter. For TlGAMY, the SP*Promoter effect was noticeable, with the different SPs showing different patterns based on the promoter used. The highest desirability scores were associated with SP1 for pTEF and 8UASpTEF, and SP5 for pPGM. Conversely, the lowest desirability scores were associated with SP3 for pPGM and 8UASpTEF, and SP2 for pTEF.

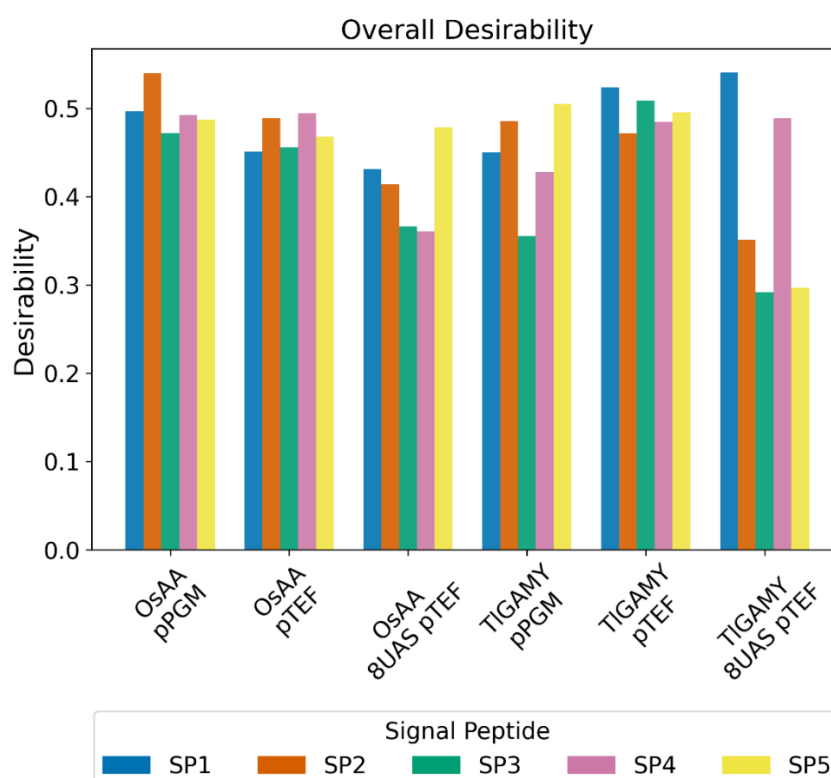


Figure 3.4 The desirability of each promoter-SP combination for OsAA and TIGAMY

The desirability of each promoter-SP combination was calculated with the aim to maximise growth rate and carrying capacity and minimise inflection point. SP3 was less desirable for both proteins, while SP2 and SP1 were more desirable for OsAA and TIGAMY respectively. The promoter 8UASpTEF was least desirable for both proteins.

3.3. Comparing growth of single- and double-amylase strains on glucose

To see if DOL had the potential to reduce burden, I compared single-amylase strains to strains which express both OsAA and TIGAMY. When designing the constructs to compare single-amylase and double-amylase expression, promoter and SP combinations were chosen to give a good range of expression outside the cell. Initially, pTEF was the chosen promoter as it is the most ubiquitous promoter in engineering for *Y. lipolytica*. For TIGAMY, SP3 (strongest) and SP5 (weakest) and for OsAA SP1 and SP5 (strongest) and SP2 (weakest) were used. The selected SPs were used in the assemblies to create double-amylase strains equivalent to consortia combining YTT3 or YTT5 (TIGAMY-expressing) with YTO1, YTO2 or YTO5 (OsAA-expressing).

Strains were grown for 48 hours on 1% glucose YNB media in 96-well plates (Materials and Methods 7.11.1). Growth curves were plotted over time, and the growth rate, inflection point and carrying capacity were calculated by fitting to a 4-parameter Gompertz curve (Materials and Methods 7.11.7). Two different transformants were run on separate days and combined for the total number of six replicates.

Existing knowledge about burden, and the model presented in Chapter 2, suggests that the double-amylase strains should have a slower growth rate than the single-amylase strains. The results for growth rate do seem to support this (Figure 3.5A). While the single-amylase expressing strains were not significantly different from the WT, the double-amylase YTT3TO1, YTT3TO5, YTT5TO2 and YTT5TO5 growth rates were all significantly lower than the WT. Double-amylase strains also generally had a lower growth rate than their single-amylase equivalents, but not all these decreases were statistically significant. Only YTT5TO2 showed a significantly lower growth rate than both its equivalent single-amylase strains, YTT5 and YTO2. YTT5TO1 did not have any statistically significant difference to either of its equivalent single-amylase strains. Every other double-amylase strain had a significantly decreased growth rate compared to their TlGAMY-expressing strains, YTT3 or YTT5, but not compared to their OsAA-expressing strains YTO1, YTO2 or YTO5.

There were no significant differences in carrying capacity for any of the pTEF-controlled strains (Appendix 9.5A). However, double-amylase-expression did result in an increase in the time of the inflection point, indicating a longer lag time (Figure 3.5B). While the single-amylase expressing strains were not significantly different, the double-amylase YTT3TO2, YTT5TO2 and YTT5TO5 showed a significant increase in inflection point compared to WT. Further, YTT5TO2 showed a significantly increased inflection point compared to both single-amylase equivalents YTT5 and YTO2, while YTT3TO2 and YTT5TO5 showed a significant increase compared to YTT3 and YTT5 respectively. The other double-amylase strains were not significantly different from their equivalents.

While the results aren't entirely clean-cut, taking both growth rate and inflection point into account, the double-amylase strains grow slower and have a longer lag time than their single-amylase expressing equivalents. The modelling in Chapter 2 suggests that these growth impacts will be further exacerbated by increasing the expression of the genes.

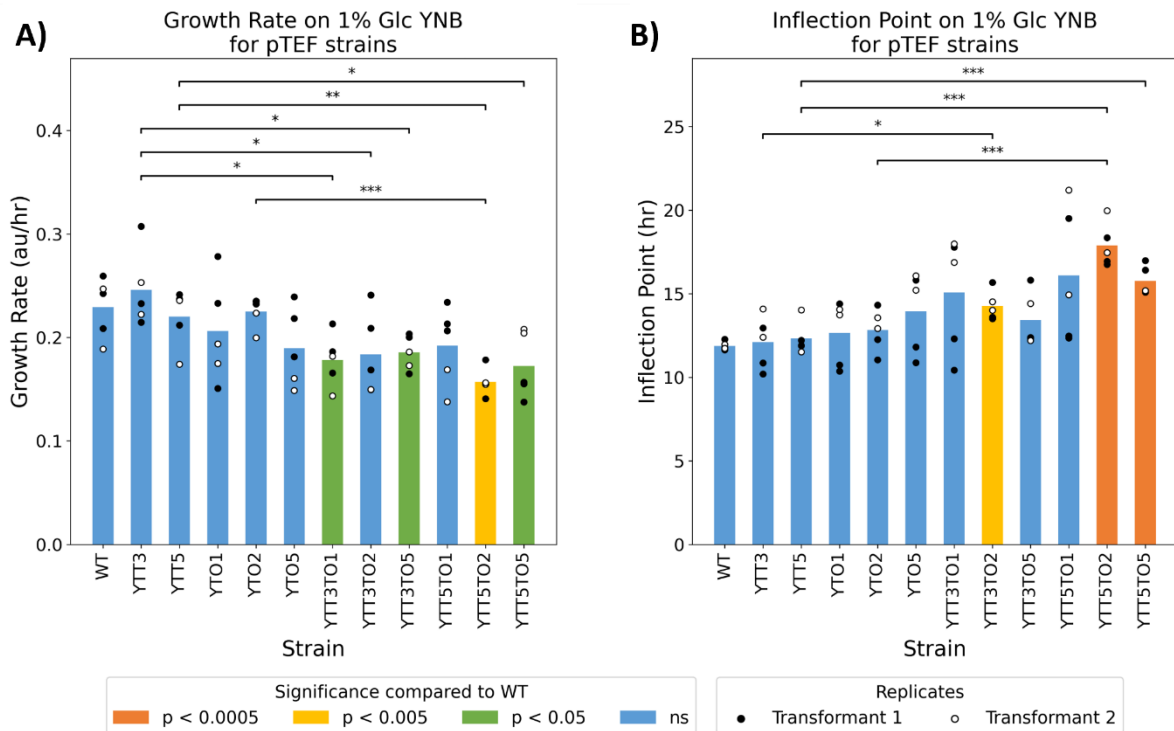


Figure 3.5 Growth rate and inflection point of pTEF-controlled single- and double-amylase strains on glucose

Strains were grown for 48 hours on 1% glucose YNB media and growth parameters were calculated by Gompertz 4-parameter curve fitting. **A)** Double-amylase strains had lower mean growth rate than their single-amylase equivalents. However, only YTT5TO2 showed a significantly decreased growth rate compared to both its equivalent single-amylase strains; the other double-amylases showed either non-significant differences, or only significant compared to the TIGAMY strain. **B)** Double-amylase strains had higher inflection points than their single-amylase equivalents. Again, only YTT5TO2 showed a significantly higher inflection point compared to both its equivalent single-amylase strains. Data presented as the mean for $n=5$. Statistical analysis by Welch's T-test; * represents a p -value < 0.05 , ** p -value < 0.005 and *** p -value < 0.0005 .

Repeating the investigation with the stronger 8UASpTEF promoter, which had previously shown more burden compared to WT on glucose, revealed fewer significant differences between single- and double-amylase strains. Again, there were no significant differences between any of the strains for carrying capacity (Appendix 9.5B). None of the double-amylase strains showed a significantly lower growth rate than both of their single-amylase equivalents, but three of them did show a significant decrease in growth rate for one of their pairs; Y8TT18TO1, Y8TT18TO3, Y8TT58TO5 were all lower than their equivalent TIGAMY-expressing strain Y8TT1 or Y8TT5 (Figure 3.6A). Like the pTEF-controlled strains, a decrease in growth rate was correlated with an increase in inflection point, with most of the strains showing a significant increase compared to WT (Figure 3.6B). Only Y8TT18TO3 compared to Y8TT1 showed a significant increase between double and single-amylase strains.

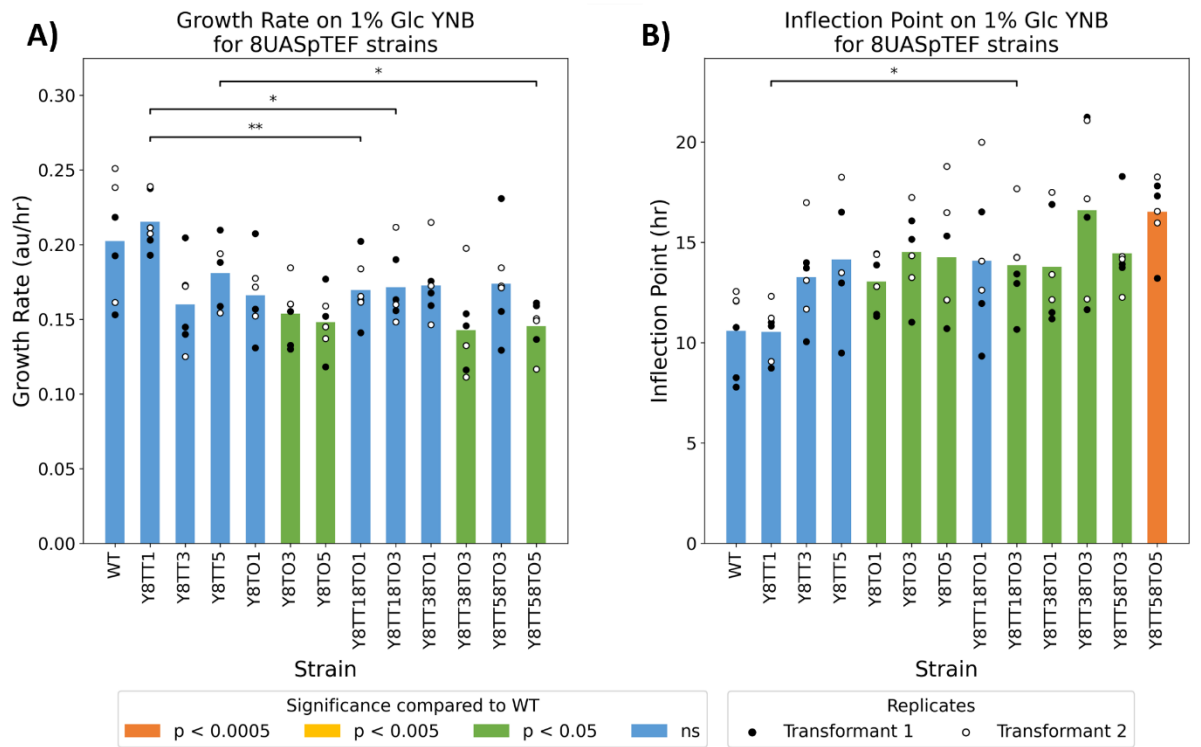


Figure 3.6 Growth rate and inflection point of 8UASpTEF-controlled single- and double-amylase strains on glucose

Strains were grown for 48 hours on 1% glucose YNB media and growth parameters were calculated by Gompertz 4-parameter curve fitting. **A)** All strains, except for Y8TT1, had a lower growth rate than the WT, although only 4 of these were significant. Y8TT18TO1, Y8TT18TO3 and Y8TT58TO5 showed significantly slower growth rate than their equivalent TIGAMY-expressing strain. **B)** 8 out of 12 8UASpTEF strains had a significantly higher inflection point than WT. But only one double-amylase strain was significantly higher than its TIGAMY-expressing equivalent. Data presented as the mean for $n=6$ (except Y8TT5 $n=5$). Statistical analysis by Welch's T-test; * represents a p -value < 0.05 , ** p -value < 0.005 and *** p -value < 0.0005 .

The 8UASpTEF-controlled strains showed an even less distinct relationship between single-amylase and double-amylase strains and burden. However, this may be because the 8UASpTEF-single-amylase strains are more burdensome than the pTEF-single-amylase strains, as established in Section 3.2. For pTEF strains, only double-amylase strains were significantly different from the WT for both growth rate and inflection point. But two of the 8UASpTEF-OsAA strains showed a significantly decreased growth rate compared to WT, and all three 8UASpTEF-OsAA strains were significantly different for inflection point. Possibly for the 8UASpTEF promoter the single-amylase strains have reached a limit of burden, over which the expression of a second gene does not further increase the burden. *Y. lipolytica* may have mechanisms for tolerance once a threshold of burden has been reached. This may mean that DOL will not confer a significant advantage for 8UASpTEF strains. However, thus far we have only looked at the burden side of the equation. In Chapter 2 the model showed that there is a balance

between decreasing burden and increasing starch-degradation. The 8UASpTEF promoter should contribute a higher amount to starch-degradation than the pTEF promoter, which may impact how degradation and burden balances out.

3.4. Comparing growth on starch of single-cell double-amylase strains and 2-strain consortia of single-amylase strains

With a better understanding of where the burden is being imposed on the cells in single- and double-amylase expressing cells, I moved onto the investigation to see if a consortia approach could help improve growth on starch. To explore this, double-amylase strains were grown on 1% starch YNB in 96-well plates (Materials and Methods 7.11.1 and 7.11.7) and compared to 2-strain consortia, each containing a strain expressing OsAA and a strain expressing TIGAMY. The two single-amylase expressing strains were inoculated in a 50:50 ratio. I chose to inoculate the monocultures at Abs600 0.01 and the consortia at 0.02, so that each strain was at 0.01. This meant that the consortia started with double the Abs600 of the monoculture, but it also meant the consortia had an equal number of cells producing each amylase compared to the monoculture.

I first started with the pTEF-controlled strains, as they had more significant differences between double-amylase and single-amylase strains. The first observation was that the consortia of YTT3+YTO2 and YTT5+YTO2 did not grow very well, possibly due to both having the weakest SP for the OsAA gene. The very low slope of the curves in these cases made the fit to the Gompertz curve less reliable. The poor growth of the YTT3+YTO2 and YTT5+YTO2 is reflected in their lower carrying capacities (Figure 3.7C) compared to all other consortia and monocultures, and some replicates had to be removed because their inflection point was coming out as almost-zero.

Figure 3.7 shows the growth rates (A), inflection point (B) and carrying capacity (C) of the pTEF-controlled consortia compared to their equivalent monoculture. The consortia of YTT3+YTO1 and YTT5+YTO1 exhibited increased growth rates compared to their equivalent double-amylase strains, although only the difference for the YTT3+YTO1 was statistically significant (Figure 3.7A). In all other strain combinations, the double-amylase strains showed higher growth rates than to the consortia, with the YTT5TO5 monoculture showing a significant increase compared to YTT5+YTO5. Four consortia showed a significantly decreased inflection point, indicating they had shorter lag times, compared with their equivalent monoculture (Figure 3.7B). YTT3+YTO1 and YTT5+YTO1, which had increased growth rate, had the most significant decrease in inflection point. These two consortia also exhibited a higher carrying capacity than their equivalent monocultures (Figure 3.7C). This is the inverse of what we observed previously with the

8UASpTEF single-amylase strains, where growth rate seemed to be inversely related to carrying capacity. Overall, the YTT3+YTO1 and YTT5+YTO1 have a higher growth rate, shorter lag time and higher carrying capacity than their equivalent YTT3TO1 and YTT5TO1 monocultures, which is a good reflection that this DOL strategy is improving growth on starch.

8UASpTEF-controlled strains were also tested, to see if their greater expression influenced the consortia's ability to outperform their monoculture. The consortia of Y8TT1+Y8TO1, Y8TT3+Y8TO1, and Y8TT5+Y8TO5 all had a significantly increased growth rate (Figure 3.8A) and a significantly decreased inflection point (Figure 3.8B) compared to their equivalent double-amylase strains. However, there was no significant change in carrying capacity (Figure 3.8C) for these consortia. In contrast Y8TT5+Y8TO3 had a significantly decreased growth rate compared to its monoculture, which was accompanied by a significant increase in carrying capacity. All other consortia had lower growth rate, although not significant, and no significant difference in their inflection points or carrying capacity, compared to their equivalent monocultures.

To compare the two promoters, there are two equivalent systems: YTT3+YTO1 vs Y8TT3+Y8TO1 and YTT5+YTO5 vs Y8TT5+Y8TO5. Both YTT3+YTO1 and Y8TT1+Y8TO3 had a faster growth rate than their equivalent monoculture, but the 8UASpTEF strain had a greater difference between its consortia and monoculture growth rate. The mean difference between consortia and monoculture growth rate for YTT3+YTO1 was 0.055 ± 0.019 (SEM) au. Whereas Y8TT3+Y8TO1 differs by 0.30 ± 0.11 for growth rate. For YTT5+YTO5 the consortia had a worse growth rate than its equivalent monoculture, but under the control of an 8UASpTEF promoter the consortia had a better growth rate than its equivalent monoculture. In the modelling in Chapter 2 there was a limit over which the benefit of expressing more amylase was outweighed by the benefit of having the extra burden. It could be that this limit exists between the YTT5+YTO5 and Y8TT5+Y8TO5 consortia. With pTEF, the starch-degradation is the limiting factor to growth, so DOL provides no benefit. With 8UASpTEF, the starch-degradation is sufficient, and burden becomes the limiting factor, so DOL becomes a valid strategy to improve growth

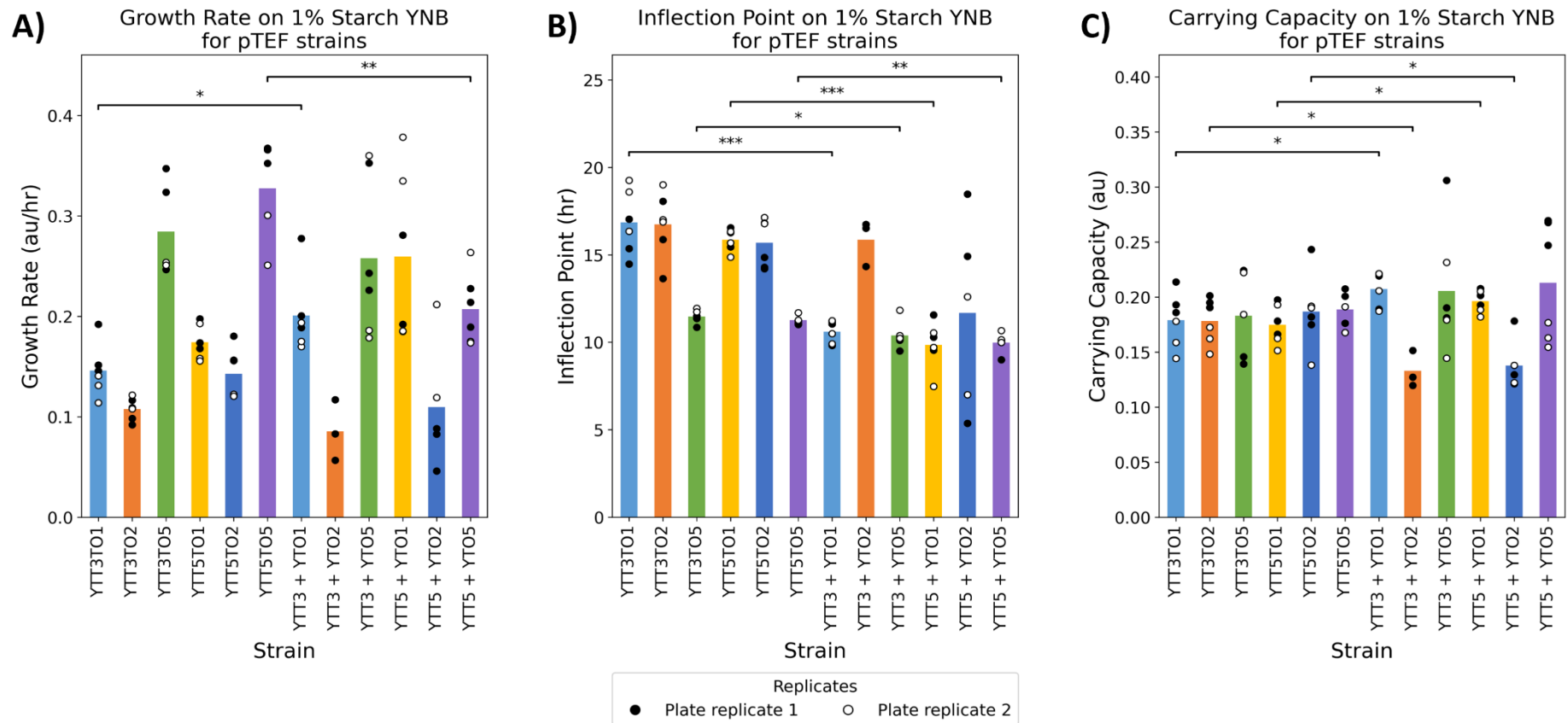


Figure 3.7 Growth parameters for pTEF-controlled double-amylase monoculture strains and 2-strain consortia on 1% soluble starch YNB

Double-amylase strains and 2-strain consortia, each containing a strain expressing OsAA and a strain expressing TIGAMY, were grown on 1% starch YNB and growth parameters were calculated by Gompertz 4 parameter curve fitting. **A)** YTT3+YTO1 and YTT5+YTO1 consortia had a higher growth rate than their double-amylase equivalents. All other consortia grew slower than their equivalent monoculture, with YTT5+YTO5 being significant. **B)** 4 out of 6 consortia had a significantly decreased inflection point, indicating a shorter lag time on starch, with YTT3+YTO1 and YTT5+YTO1 having the most significant difference. **C)** YTT3+YTO1 and YTT5+YTO1 had increased carrying capacity compared to their double-amylase equivalents, while YTT3+YTO2 and YTT5+YTO2 had a significant decrease. Data presented as the mean for $n=6$ ($n=3$ for YTT3+YTO2 and $n=5$ for YTT5+YTO2). Statistical analysis by Welch's T-test; * represents a p -value < 0.05 , ** p -value < 0.005 and *** p -value < 0.0005 .

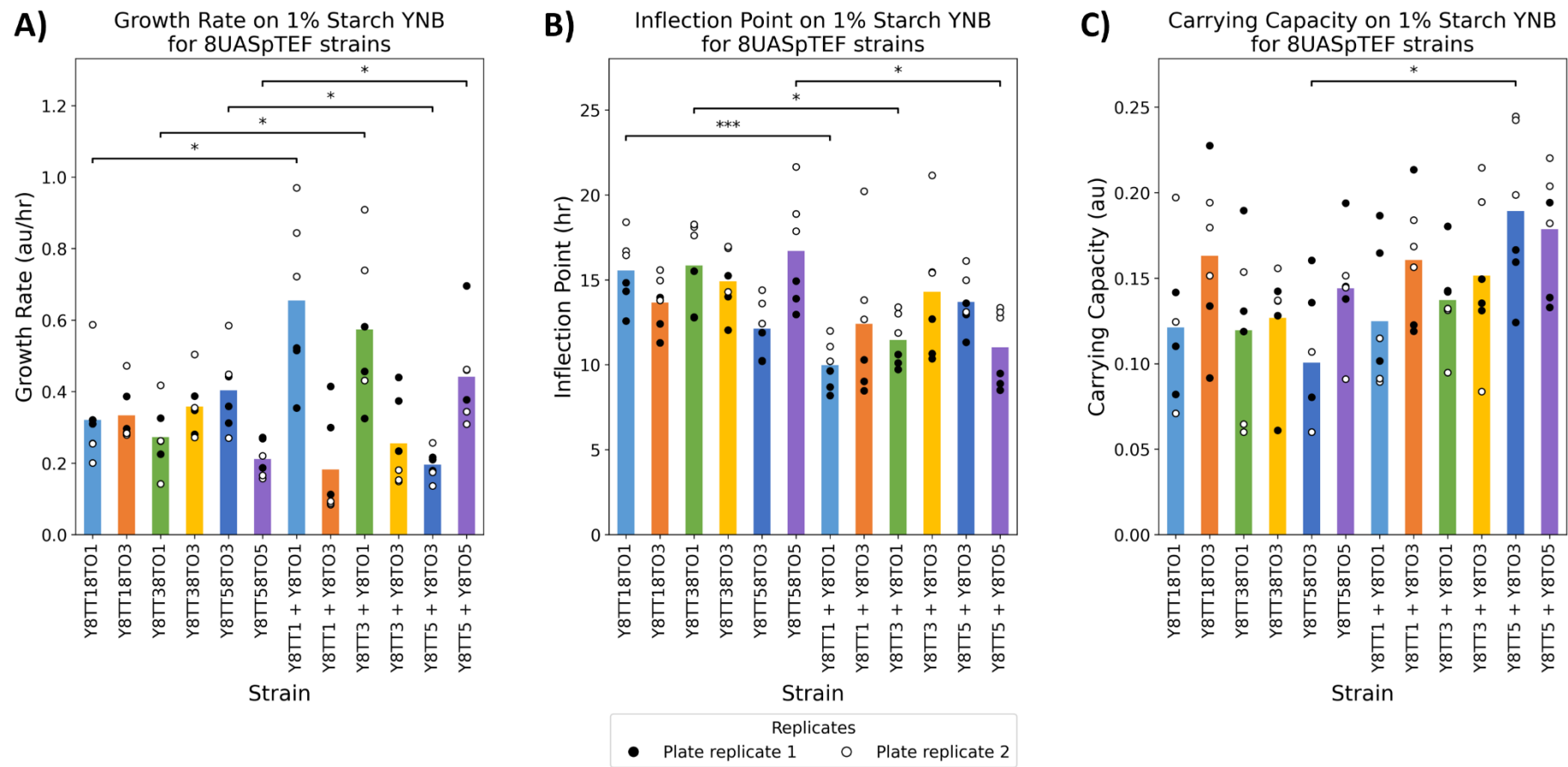


Figure 3.8 Growth parameters for 8UASpTEF-controlled double-amylase monoculture strains and 2-strain consortia on 1% soluble starch YNB

Double-amylase strains and 2-strain consortia, each containing a strain expressing OsAA and a strain expressing TIGAMY, were grown on 1% starch YNB and growth parameters were calculated by Gompertz 4 parameter curve fitting. **A)** Y8TT1+Y8TO1, Y8TT3+Y8TO1 and Y8TT5+Y8TO5 consortia had increased growth rate compared to their equivalent double-amylase strains. In contrast, Y8TT5+Y8TO3 had a significant decrease in growth rate. **B)** Y8TT1+Y8TO1, Y8TT3+Y8TO1 and Y8TT5+Y8TO5 consortia had significantly decreased inflection points compared to their equivalent monoculture. **C)** Only the Y8TT5+Y8TO3 had significantly increased carrying capacity compared to its monoculture equivalent. All other consortia were not significantly different. Data presented as the mean for n=6. Statistical analysis by Welch's T-test; * represents a p-value < 0.05, ** p-value < 0.005 and *** p-value < 0.0005.

The consortia which achieve higher growth rate than their monoculture equivalent do not entirely reflect the expression systems with the most burden. In Section 3.3, YTT5TO2 was the only monoculture which showed a significant difference in growth rate and inflection point compared to both its equivalent consortia strains. But unfortunately, the YTT5+ YTO2 consortia did not grow well on starch, as shown by their low carrying capacity. YTT3TO1, YTT3TO5, YTT5TO5, Y8TT18TO1, Y8TT18TO3 and Y8TT58TO5 were the monoculture strains which showed significantly reduced growth rates compared to their TIGAMY-expressing equivalents. Of their equivalent consortia, YTT3+YTO1, Y8TT1+Y8TO1, and Y8TT5+Y8TO5 had a significantly increased growth rate on starch compared to their monoculture. On the other hand, YTT3+YTO5, YTT5+YTO5 and Y8TT1+Y8TO3 had worse growth rate than their monoculture. So, there is no direct way to predict if DOL will be an effective strategy for improving growth on starch simply by observing burden on glucose.

Of note, pTEF-controlled double-amylase strains YTT3TO5 and YTT5TO5 have the highest growth rates and lowest inflection point of all the monocultures, while YTT3TO1 and YTT5TO1 have lower growth rate and higher inflection point. They only differ in the SP used for OsAA. These SPs had very similar activity in the iodine assays (Figure 3.2), and the double-amylase strains had similar burden (Figure 3.5), so their different growth rate on starch does not make much sense based on previous experiments. However, it does seem to make a difference for whether DOL is effective, as YTT3TO5 and YTT5TO5 have a higher growth rate than their equivalent consortia, while YTT3TO1 and YTT5TO1 had lower growth rate than their consortia. For the 8UASpTEF-controlled double-amylase strains a similar pattern is seen. Y8TT18TO1, Y8TT38TO1, and Y8TT58TO5 had the lowest growth rate and highest inflection point of the double-amylase monocultures, and they were also the strains which had a lower growth rate than their equivalent consortia. So, there is a clear connection between the worst performing monocultures and DOL achieving a higher growth rate. Instead of predicting the burden based on growth in glucose and assessing starch degradation by measuring supernatant activity, it may be necessary to grow directly on starch to simultaneously observe both the impact of starch degradation and the burden's effect on growth. Unfortunately, this makes designing a DOL system for starch degradation significantly more challenging, as it requires testing various promoters and SPs, as I have done, to identify the threshold at which DOL becomes effective.

I also tested the pTEF-controlled consortia on a different source of starch: starch from potato, which had a lower opacity than soluble starch and therefore was preferable for absorbance measurements. I experimented with using different methods of creating the starch media. I tested three methods, firstly dissolving the starch in boiling water and then autoclaving it, secondly microwaving the starch to solubilise it, and finally using entirely un-heated starch.

Un-heated starch was too opaque to read any increase in biomass in the plate reader. Microwaved and autoclaved starch gave similar growth curves, but microwaved starch was more prone to contamination, with many replicates being contaminated with bacteria and removed. In both cases 2% starch was the maximal concentration that could be fully dissolved. In both cases YTT3+YTO1 and YTT5+YTO1 were the only consortia to have a higher growth rate than their monoculture (Figure 3.9), consistent with the results from soluble starch.

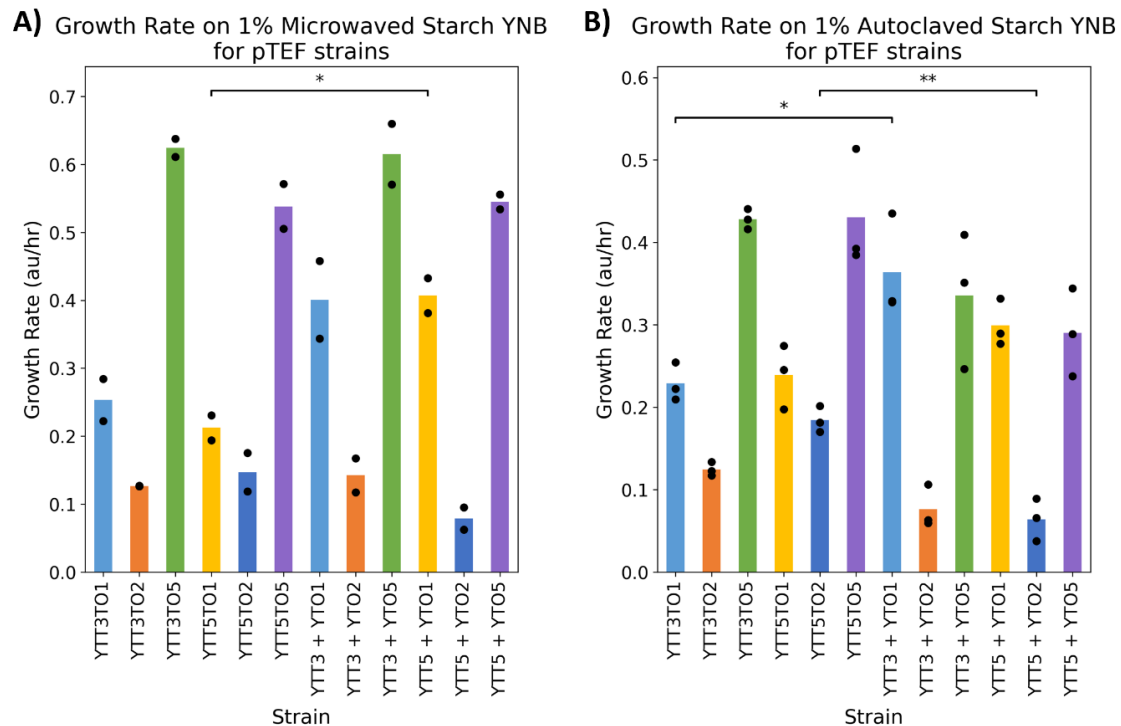


Figure 3.9 Growth rate for double-amylase monoculture strains and 2-strain consortia with different preparations of 1% potato starch YNB

1% potato starch was prepared by either **A)** suspending it in cold water and microwaving it until it dissolved or **B)** dissolving it in boiling water and then autoclaving it. Growth was measured in the plate reader and growth parameters were calculated by fitting to a 4-parameter Gompertz curve. The strains achieved a higher overall growth rate on microwaved starch, but the trends were very similar to autoclaved starch. In both cases only YTT3+YTO1 and YTT5+YTO1 showed a higher growth rate than their monoculture equivalent. Autoclaved starch had the benefit of higher sterility. For microwaved starch n=2, for autoclaved starch n=3.

The decrease in inflection point for many of the consortia is likely due to the higher initial Abs600 for the inoculation of the consortia. The growth rate and carrying capacity should be independent of the initial Abs600, but the observed growth dynamics may appear altered due to faster depletion of resources with the higher Abs600. With potato starch I compared inoculating the monocultures at both the original 0.01 and at an initial Abs600 of 0.02. At both initial Abs600s

there were significant decreases in inflection point for the consortia compared to the monoculture (Figure 3.10A & B). However, these experiments were not consistent with the previous experiments for the growth rate, as in neither case does the YTT3+YTO1 and YTT5+YTO1 show a higher growth rate than monoculture (Figure 3.10C & D). What caused this variation was unclear and attempts to improve the reproducibility in the plate reader failed. Therefore, I looked at different formats for culturing which were more consistent.

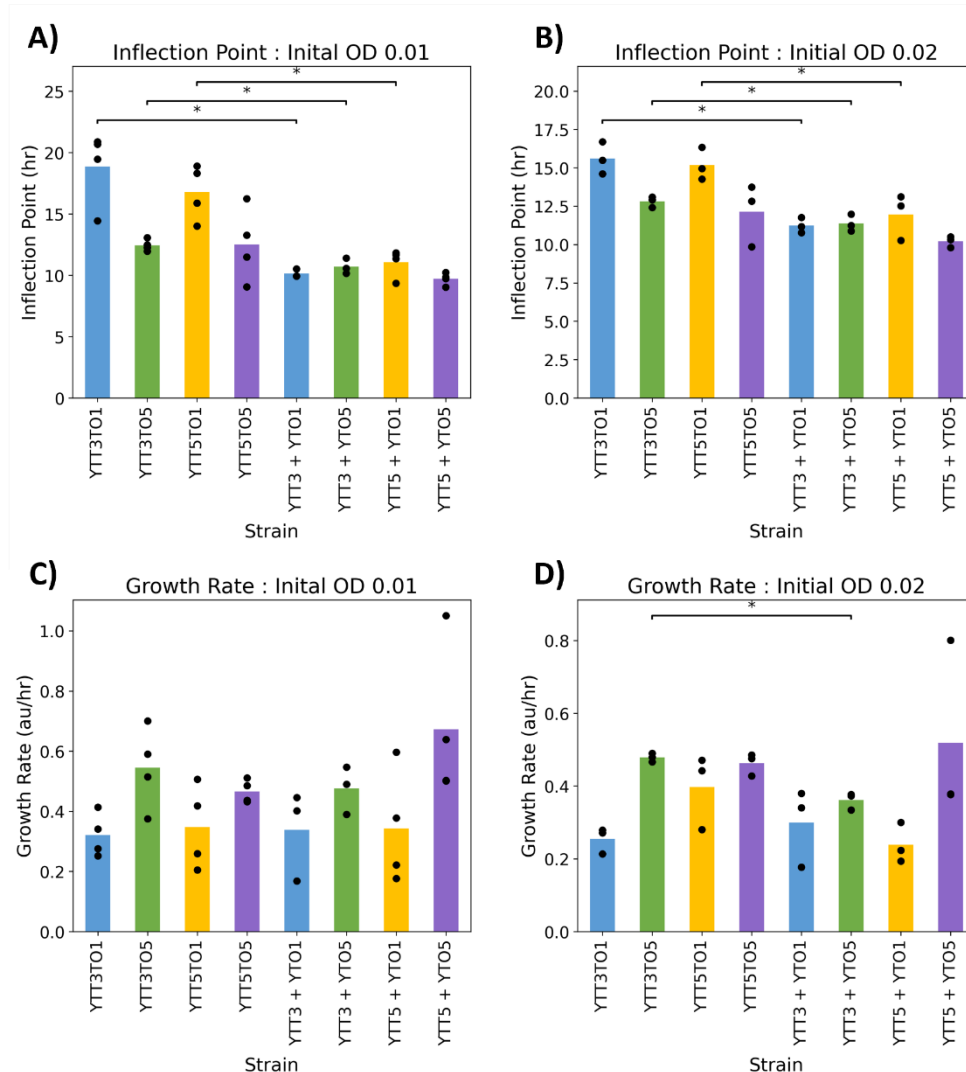


Figure 3.10 Inflection point and growth rate for double-amylase monoculture strains and 2-strain consortia on 1% soluble starch YNB with different inoculation Abs600s

Double-amylase strains were inoculated with either 0.01 Abs600 (A, C) or 0.02 Abs600 (C, D). The 2-strain consortium was inoculated with 0.01 Abs600 per strain, making a total of 0.02 Abs600, therefore either giving them an equal amount of cells making each amylase, or an equal total initial Abs600. **A & B)** At both initial Abs600s there were the same significant decreases in inflection point for the consortia compared to the monoculture. **C & D)** For growth rate there were no significant differences between consortia and monoculture for an initial Abs600 of 0.01, and for initial Abs600 0.02 only the decrease in growth rate for the YTT3+YTO5 consortia compared to monoculture was significant. This is different to the result obtained in all previous plate reader assays. n=3.

Because of the requirement for a high-throughput way of measuring all the different combinations of amylases, I was using a 96-well plate format. However, this is not the best growth format for *Y. lipolytica* for multiple reasons. *Y. lipolytica* is an obligate aerobe, and the Breathe-Easy (Sigma) films that are added to the plates, to be incubated in the plate-reader, do not allow efficient exchange of oxygen. *Y. lipolytica* grows to very high cell densities, which makes it difficult to measure the density using the limited range of the plate-reader absorbance. And *Y. lipolytica* tends to filament when it is stressed, which adds another level of variation to the absorbance results.

Therefore, I tested out some different formats for growth: a 96-well culture plate, a 24-well culture plate, both with Breathe-Easy films, 14 mL culture tubes with loose lids and 50 mL flasks with foam bungs (Materials and Methods 7.11.3). In all cases, the samples were grown in an incubator rather than in a plate reader, and at each timepoint, a sample was taken and diluted x10, placed into a fresh 96-well plate, and measured in the plate reader. I found that the flasks led to the least standard deviation between replicates completed on different days and led to the highest growth overall (Figure 3.11). Flasks with foam bungs allow higher aeration than can be achieved in the 96-well plate format and *Y. lipolytica* growth is improved as oxygenation increases. This was consistent with more thorough research done by (Celińska & Gorczyca, 2024), which found 96-well plates were very inconsistent, and that using wells with more corners, for better agitation, and metal lids, for better aeration, provides a better high-throughput way to grow *Y. lipolytica*. Due to the better aeration, the consortia and monoculture may behave differently in flasks compared to 96-well plates, therefore my next aim was to confirm that the consortia could achieve higher growth in flasks.

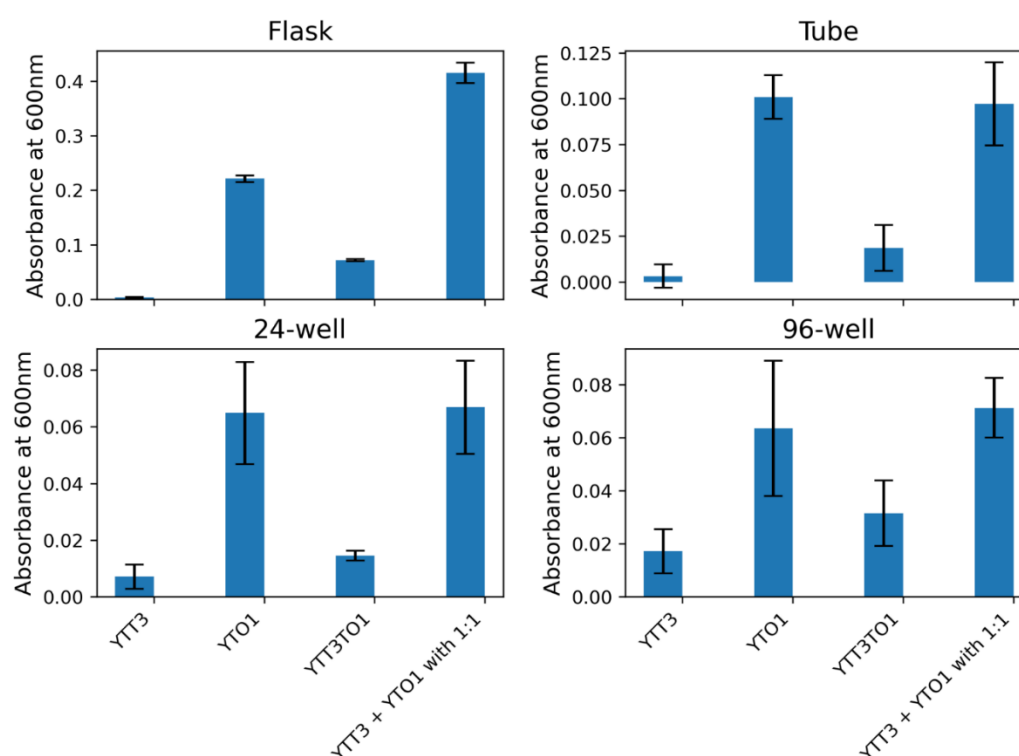


Figure 3.11 Testing different culture formats for growth of *Y. lipolytica* in 1% potato starch YNB

Cultures were grown on 1% potato starch YNB in 4 different formats 1. 50 mL Erlenmeyer flasks with 10 mL media with foam bungs 2. 14 mL culture tubes with 3 mL media with loose lids 3. 24 well plate with 600 μ L media and a Breathe-Easy film 4. 96-well plate with 200 μ L of media and a Breathe-Easy film. All formats were grown in a shaking incubator at 30°C for 72 hours and OD600 was taken every 24 hours. Here, only the 24 hour timepoint is shown to compare the standard deviations between different formats. Flasks have the smallest standard deviation compared to their means. n=3.

3.5. Flask cultures for the consortia which achieved higher growth rates than their equivalent monocultures

Using a flask format, I now tested whether the improvement seen for the YTT3+YTO1 consortium was consistent in this higher volume format. Cultures were grown on 1% potato starch YNB over 48 hours with measurements taken every 30-60 minutes (Materials and Methods 7.11.4). However, fitting the data to a growth curve was inaccurate, as I couldn't capture the growth overnight (Appendix 9.6). Instead, I chose to take measurements at select timepoints. Working with the pTEF-controlled strains first, the YTT3TO1 strain was inoculated at an initial optical density at 600 nm (OD600) of 0.1. For the YTT3+YTO1 consortium, I tested inoculating each strain at 0.05 OD600 (50:50 ratio), making the total initial OD600 the same as the monoculture, and each strain at 0.1 OD600 (100:100 ratio), making the total initial OD600 twice that of the monocultures. Both these versions of the consortium achieved a significantly higher OD600 than the monoculture after 48 and 72 hours (Figure 3.12A).

I wanted to compare how much starch remained at the end of the culture, to identify if it differed for the monoculture and consortium. I repeated the flask cultures, removed the cells, and used the remaining media in an iodine assay (Materials and Methods 7.10.1). The 1% starch YNB has 10 g/L of starch originally. By the end of 72 hours the YTO1 strain culture had 2.851 ± 0.0195 (SD) g/L of starch remaining, the YTT3TO1 strain had 2.835 ± 0.031 g/L starch remaining and the YTT3+YTO1 consortia had 2.871 ± 0.086 g/L remaining. There was no statistical difference between these three results. I also opted to observe starch degradation using the microscope, as starch granules are large enough to visualise with x200 magnification (Appendix 9.7). However, it was not possible to quantify how much the starch was broken down visually.

I now focussed on the 8UASpTEF-controlled strains, but for the Y8TT1+Y8TO1 consortium I only used the 50:50 ratio. Y8TT1+Y8TO1 also achieved a higher final OD600 than its monoculture, but the difference was significant only at 18 hours (Figure 3.12B). However, the overall growth on starch was better with the 8UASpTEF promoter. To try to see if there was glucose left in the media I sent filtered 8UASpTEF double-amylase and consortia cultures to HPLC, but it gave no detectable amount of glucose.

In both the pTEF and 8UASpTEF-controlled flask cultures, the growth before 24 hours was quite low. One issue that was raised was limiting nitrogen. Therefore, I increased the concentration of YNB in the media, and this did improve the growth at the earlier 18 hour timepoint (Figure 3.12C). On this new media, the Y8TT1+Y8TO1 consortium did achieve a statistically significant increase in OD600 over the equivalent monoculture, Y8TT18TO1, at both 18 and 24 hours.

I also adopted a new way of measuring growth curves, which requires setting up six replicates, with three of them on a 12-hour delay compared to the other three. OD600 was measured every 4 hours (Materials and Methods 7.11.6). This allowed me to capture the growth at timepoints I would otherwise miss and allowed me to fit this data to a Gompertz curve to get growth rate, carrying capacity and inflection point (Materials and Methods 7.11.7). The data for the flask growth curve was sparse for fitting the data to a curve, so the growth parameters predicted should be interpreted with caution due to limitations in the accuracy of the fit. The growth curves for Y8TT1+Y8TO1 and Y8TT18TO1 were quite similar (Figure 3.12D). For Y8TT18TO1 the carrying capacity was OD600 5.455, the growth rate was 0.230 hr^{-1} , and the inflection point was 12.091 hr. For Y8TT1+Y8TO1 the carrying capacity was OD600 6.075, the growth rate was 0.155 hr^{-1} and the inflection point was 13.064 hr. So, the curve fitting predicted that the monoculture grew faster, but the consortium grew to a higher final OD600.

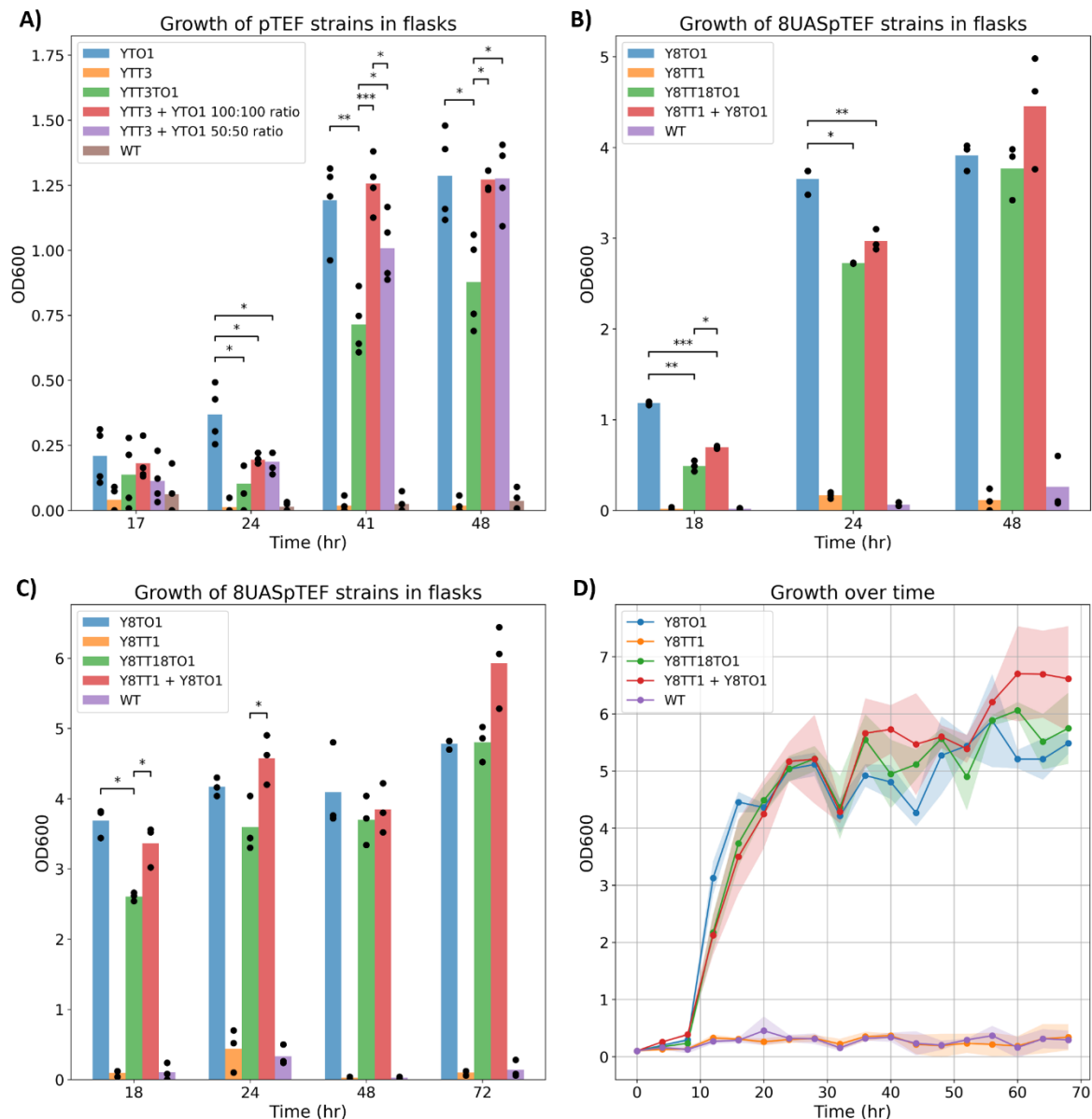


Figure 3.12 Growth of starch-degrading consortia and monocultures in flasks

A) The consortium YTT3+YTO1 was inoculated with either a 50:50 ratio, where each cell was inoculated at half the total initial OD600, or at a 100:100 ratio, where each cell was inoculated at the same initial OD600 as the monoculture YTT3TO1. In both cases the consortium achieved a statistically significant increase in OD600 compared to the monoculture after 41 and 48 hours. The single-amylase YTO1 strain achieved a significantly higher OD600 than both monoculture and consortium at 24 hours, and higher than the monoculture at 41 and 48 hours. **B)** The consortium Y8TT1+Y8TO1 was inoculated with a 50:50 ratio. It did have the highest mean OD600 after 48 hours, but only the 18 hour timepoint gave a significant difference from the monoculture. The single-amylase strain Y8TO1 achieved a significantly higher OD600 than both the monoculture and consortium at 18 and 24 hours. **C)** The amount of nitrogen (as YNB) in the media was increased, which improved growth after 18 hours. The consortium achieved a significantly higher OD600 than the monoculture at both 18 and 24 hours. **D)** Full growth curves for the 8UASpTEF-controlled consortium vs monoculture. Data presented as the mean for n=4 (A) or n=3 (B, C, D). Statistical analysis by Welch's T-test; * represents a p-value < 0.05, ** p-value < 0.005 and *** p-value < 0.0005. Coloured region on the line graph represents the standard deviation about the mean at each timepoint.

Unexpectedly, the YTO1 and Y8TO1 strains grew on starch. Previous literature has shown that *Y. lipolytica* will only grow sufficiently on starch if OsAA is co-expressed with a glucoamylase (Ledesma-Amaro, Dulermo & Nicaud, 2015). For my strains, not only did the OsAA-expressing strains grow on starch without the help of a TIGAMY-expressing strain, but YTO1 and Y8TO1 had a higher growth than in either consortia or monoculture at multiple timepoints (Figure 3.12A, B and C). Gompertz fitting for Y8TO1 predicted a carrying capacity of OD600 5.107, a growth rate of 0.381 hr⁻¹ and an inflection point of 10.505 hr. Not only that, but it is also noticeable that all the consortia which did achieve higher growth rate in the plate reader experiments were using either SP1-OsAA or SP5-OsAA: the strong SPs for OsAA. The weaker SP2-OsAA consortia grew very poorly overall, and worse than their equivalent monoculture. It may be that in the consortia the increased growth rate, compared to the double-amylase strains, is due to the strength of the OsAA-expressing strain.

3.6. Observing the changes in population of the two amylase strains

To investigate the role of the OsAA-expressing strains in the consortia in more depth I looked at what was happening to the two different cell types over time, by expressing RFP in the OsAA and GFP in the TIGAMY strain. Figure 3.13 shows the growth of the pTEF-controlled YTT3-GFP+YTO1-RFP consortium over time in the plate reader. I inoculated the culture with different ratios of the two strains (ratios are always given as OsAA strain : TIGAMY strain) (Materials and Methods 7.11.5). The growth of the YTO1-RFP correlates with the initial ratio- the higher its initial ratio, the higher its growth. On the other hand the YTT3-GFP strain does not grow the best when it is in the highest ratio (0:4), instead it requires the presence of YTO1-RFP to grow, with the 1:3 ratio being the best. This data was encouraging as it suggested that both cell types remain in the consortium, and the growth overall is not only due to the OsAA strain growing by itself.

Of note, by adding the extra marker genes I am adding additional burden to the two strains. In comparison to the previous plate-reader data with YTT3 and YTO1, YTT3-GFP and YTO1-RFP had a higher carrying capacity, a lower growth rate, and a higher inflection point (Table 3.2). To compare the burden of different markers, a WT strain was compared to WT-GFP and WT-RFP grown on glucose in 96-well plates (Materials and Methods 7.11.1). The marker strains had a lower carrying capacity, lower growth rate and higher inflection point, indicating there is some extra burden introduced when expressing the marker genes. These differences are mainly insignificant, except inflection point with GFP. However, this burden should be considered in the following fluorescence-based results.

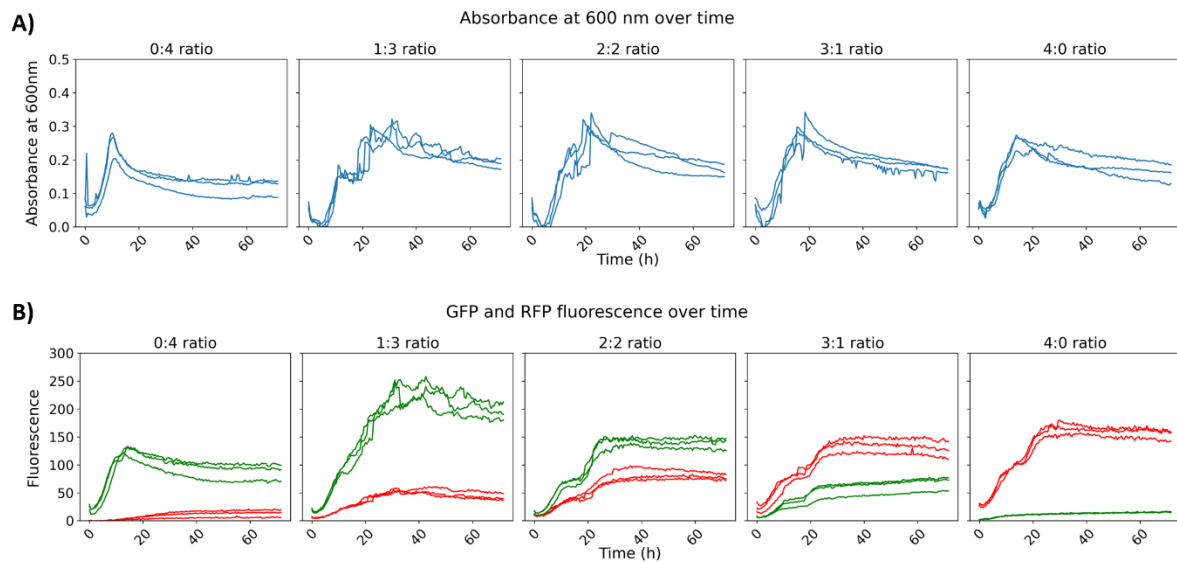


Figure 3.13 Growth of the YTT3-GFP+YTO1-RFP consortium

The consortium was inoculated at different ratios of the two strains (ratio's given as YTO1-RFP : YTT3-GFP). The YTO1-RFP strain showed growth proportional to its inoculation ratio, whereas the YTT3-GFP strain grew the most at a 1:3 ratio. n=3.

Table 3.2 Growth parameters for strains grown on glucose with and without RFP and GFP markers

YTT3 and YTO1 results were taken from Figure 3.5 and compared to YTT3-GFP and YTO1-RFP results taken from Figure 3.13. WT, WT-GFP and WT-RFP were grown in 1% glucose YNB in 96-well plates and fitted to a Gompertz 4-parameter curve. WT-GFP and WT-RFP parameters were compared to WT in a Welch's T-test.

Strain	Mean carrying capacity (Abs600 au)	Mean growth rate (au/hr)	Mean inflection point (hr)
YTT3	0.328768514	0.246037845	12.09909542
YTT3-GFP	0.548176806	0.098896973	15.03537149
YTO1	0.293088939	0.206154278	12.66369779
YTO1-RFP	0.329297583	0.176172472	14.53509893
WT	0.423947058	0.306752237	11.83988009
WT-GFP	0.375085947	0.196654685	18.04474187
<i>p-value</i>	0.3298	0.0808	0.0400
WT-RFP	0.353145242	0.285334062	14.38791572
<i>p-value</i>	0.2045	0.6454	0.0601

To get a more accurate measure of the ratio of the two strains, I used the flow cytometer to count the number of RFP+ and GFP+ cells at different timepoints. Figure 3.14 shows the growth and the percentage of each cell type for the 8UASpTEF-controlled Y8TT1-GFP+Y8TO1-RFP consortium, at 4 different timepoints. Noticeably, the ratios do not remain stable at their inoculation ratio. Even after 18 hours every consortium has changed to increase the Y8TO1-RFP population and decrease the Y8TT1-GFP population. Although, the 1:10 ratio does seem to recover some Y8TT1-GFP population after 24 hours. This might indicate that the population adjusts to have Y8TO1-RFP at earlier timepoints, and Y8TT1-GFP at later timepoints, which I might have expected since glucoamylase carries out the final step of starch degradation down to glucose. However, the 1:10 ratio also grows the slowest and to the lowest final OD600, so adjusting its population in this way doesn't improve growth. In all previous experiments I was using a 1:1 (or 50:50) ratio, however Figure 3.14 demonstrates that the 1:1 ratio stabilises at around 65% Y8TO1-RFP and 35% Y8TT1-GFP, or a 13:7 ratio, which might explain why the consortium previously seemed more dependent on their OsAA strain than their TIGAMY strain. Often, when one population dominates a consortium, it is because it has a higher growth rate than its fellow consortium members, and thus grows and consumes the shared resources faster. However, from Figure 3.5, the OsAA strains did not have a faster growth rate on glucose, in fact it is the opposite: Y8TT1 has a faster growth rate and a shorter lag time (from inflection point). However, as noted earlier, WT-GFP has a slower growth rate than WT-RFP, so this shift in population may reflect the different burden of the marker genes, more than the influence of the amylases themselves.

There is a clear correlation between inoculation ratio and OD600, most obvious at 18 and 24 hours, where the consortia inoculated at higher amounts of Y8TO1-RFP had a faster growth to their final OD600. The addition of the TIGAMY strain appears to hinder growth more than help. This may be because the competition for glucose is greater than the synergism of the two enzymes. When the OsAA strain is alone it can consume all the glucose and therefore grow at its maximum rate. However, when a TIGAMY strain is present it consumes half the produced glucose, but it does not double the amount of starch degradation, so the amount it contributes is unequal to the amount it consumes.

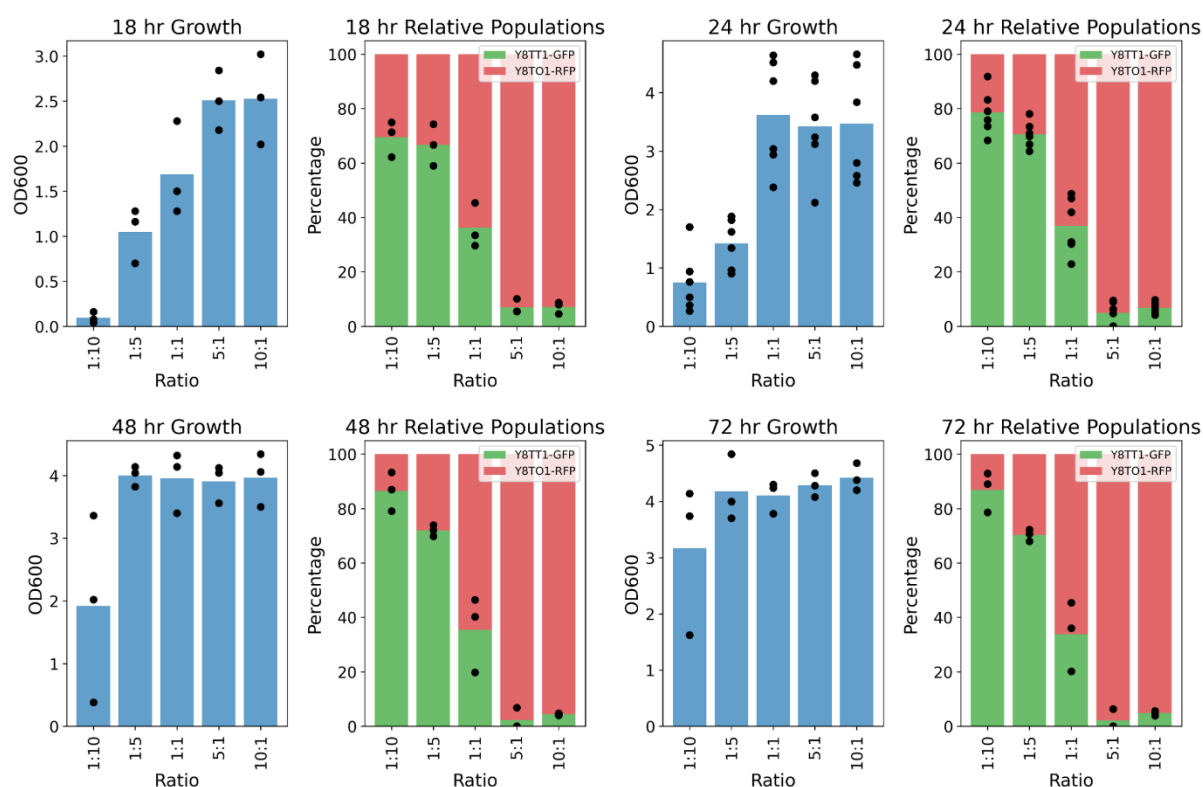


Figure 3.14 Growth and relative populations of the cell types in Y8TT1-GFP+Y8TO1-RFP consortium

The Y8TT1-GFP+Y8TO1-RFP consortium was inoculated at different ratios of the two strains. The ratios are given as Y8TO1-RFP : Y8TT1-GFP- so from left to right it is increasing in Y8TO1-RFP and decreasing in Y8TT1-GFP. The OD600 and flow cytometry measurements were taken at 18, 24, 48 and 72 hours. The 10:1 and 5:1 ratios grew the fastest, at 18hr, followed by the 1:1 ratio, at 24 hr, and the 1:5 ratio at 48 hr. By 72 hr the 1:10 ratio had not reached the same OD600 as the other consortium ratios. At all 4 timepoints, the flow cytometry data is very similar. Compared to their initial ratio, the Y8TO1-RFP population has increased. The 1:10 ratio does show that the Y8TT1-GFP population does begin to return towards its original ratio from 18 hr to 72 hr, settling at around 1:9. Data is presented as the mean of n=3 or n=6 (for 24 hr).

3.7. Activity of glucoamylase

Having observed that the OsAA strain grows best on its own, I needed to understand why the glucoamylase strain was acting to reduce growth rather than increase it, as would be suggested by the synergism of the two enzymes. The TlGAMY activity was well-published in literature. However, due to my results I decided to test the activity of all single-amylase strains. In a similar way to measuring the OsAA activity, I grew up each TlGAMY strain and then extracted the supernatant after 48 hours. Because TlGAMY breaks down starch into single glucose units, I used a micro-GOPOD (glucose oxidase peroxidase) assay instead of the micro-SIT that was used for OsAA. GOPOD provides a colorimetric output for glucose concentration after incubation of TlGAMY supernatant and starch (Materials and Methods 7.10.2). Initially I grew the strains on YNB

2% glucose media, but the remaining glucose left in the culture media caused issues with the assay, and therefore I switched to YNB 4% glycerol instead.

Figure 3.15 shows the glucose concentration, both unnormalized (A) and normalised to OD600 (B). Y8TT1 produces the most glucose, followed by Y8TT5, and Y8TT2 produces the least glucose. This contrasts with previously published results which found SP3 to be the strongest and SP5 to be the weakest for TIGAMY (Celińska et al., 2018). In addition, very little glucose is produced overall: from starch at 20mg/mL, Y8TT1 supernatant only produced 0.12 mg/mL of glucose after 48 hours, including around 0.04 mg/mL present in the WT.

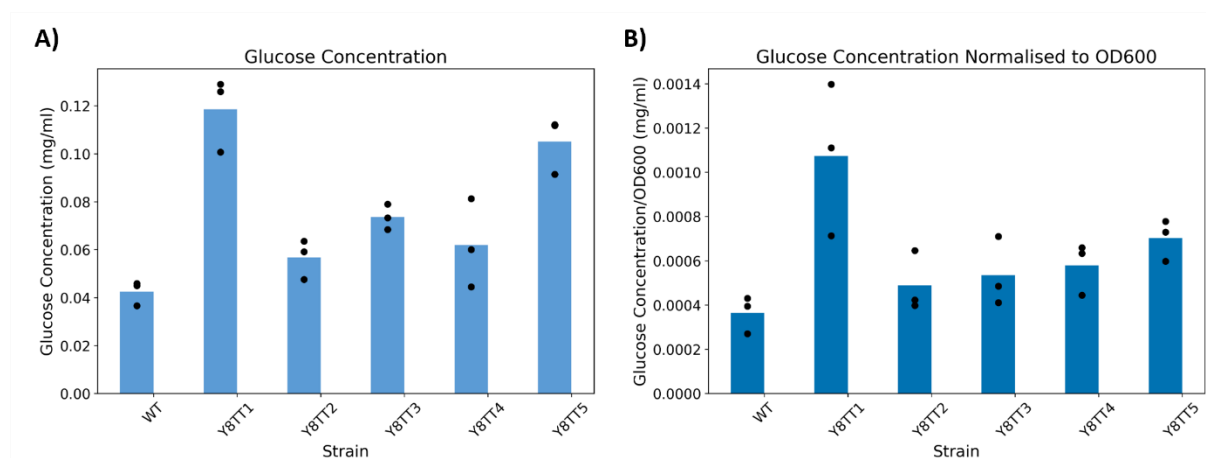


Figure 3.15 Micro-GOPOD assay of 8UASpTEF-TIGAMY strains

Supernatant and 2% starch were incubated for 48 hours. **A)** Glucose concentration prior to normalising to OD600. The glucose concentration prior to normalisation gives a more accurate reflection of how much glucose would be produced in a culture, where the OD600 will not be equal. **B)** Glucose concentration normalised to OD600 can be used to see the differences between the different signal peptides (SPs). The SPs can be ranked from strongest to weakest as SP1-SP5-SP4-SP3-SP2. n=3.

3.8. Multi-copy amylase strains do not increase the advantage of DOL

Since the glucoamylase seemed to have very weak activity, I also made strains with multiple copies of the amylase genes, by repeating the transformation with each amylase cassette. Figure 3.16 shows the growth in flask of strains (Materials and Methods 7.11.4) containing multi-copies of OsAA: Y2x8TO1, TIGAMY: Y2x8TT1, or both amylases: Y2x8TT18TO1, as well as the consortium of Y2x8TT1+Y2x8TO1. Compared to Y8TO1, Y2x8TO1 has a significant increase in OD600 after 72 hours, but Y2x8TT18TO1 does not provide any significant increase in growth compared to the single-copy strain. There is no significant difference between the consortium of Y2x8TT1+Y2x8TO1 and the monoculture Y2x8TT18TO1.

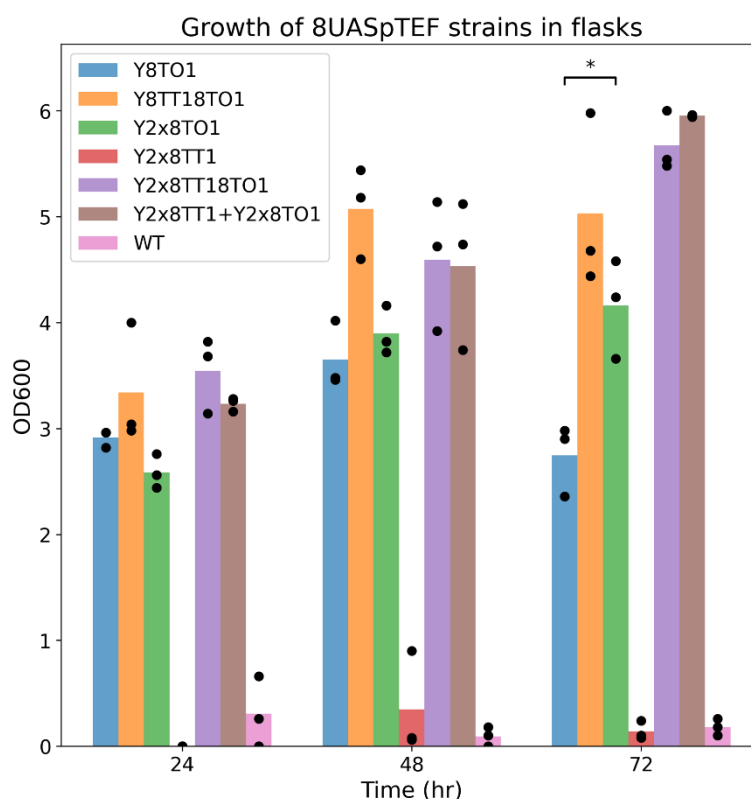


Figure 3.16 Growth of strains with two copies of the OsAA and TIGAMY genes

Strains were grown on 1% starch YNB for 72 hours. Y2x8TO1 has a significant increase in OD600 after 72 hours compared to the single-copy Y8TO1. No significant difference was seen for Y2x8TT18TO1 compared to the single-copy Y8TT18TO1. No significant difference was seen between the consortium of Y2x8TT1+Y2x8TO1 and the monoculture Y2x8TT18TO1. Data presented as mean of n=3. Statistical analysis by Welch's T-test; * represents a p-value < 0.05, ** p-value < 0.005 and *** p-value < 0.0005.

3.9. Using a stronger glucoamylase

If TIGAMY is not producing very much glucose, that would explain why in the consortia it is not providing any benefit to growth above OsAA alone. So, I looked for a glucoamylase with a higher activity, that might be able to contribute more toward starch degradation. Ledesma-Amaro, Dulermo & Nicaud, 2015, used glucoamylase from *Aspergillus niger*, henceforth referred to as AnGAMY. I engineered a strain YTG1 (pTEF-SP1-AnGAMY) and compared it to the YTT1 strain in a micro-GOPOD assay (Materials and Methods 7.10.2) and found it did have an increase in glucose released (Figure 3.17A). Unfortunately, I was not able to get all of the transformants for every promoter-SP pair. The SPs I did test were SP1 (YTG1), SP2 (YTG2) and SP3 (YTG3), of which YTG1 showed the highest glucose concentration produced from starch after 15 minutes of incubation (Figure 3.17B).

I then engineered a new monoculture strain, YTG18TO1, with both AnGAMY and OsAA. As with the previous TlGAMY+OsAA consortia, I grew the AnGAMY+OsAA consortia and monoculture on 1% potato starch YNB over 72 hours, measuring OD600 each day (Materials and Methods 7.11.4). Figure 3.17C shows the growth of the monoculture single-amylase strains Y8TO1 and YTG1, the double-amylase YTG18TO1 and a 2-strain consortium of YTG1+Y8TO1. In contrast to the TlGAMY+OsAA combination, the glucoamylase AnGAMY appears to perform better than the α -amylase OsAA. After 48 and 72 hours, the Y8TO1 had significantly lower growth than all other cultures, so the AnGAMY is successfully improving the starch degradation. However, now I observed the opposite problem where the Y8TO1 does not seem to improve the growth compared to YTG1 alone. The consortium of YTG1+Y8TO1 showed significantly lower growth than both YTG1 and Y8TO1+TG1. Figure 3.17D shows the full growth curves for the AnGAMY strains (Materials and Methods 7.11.6). YTG1 had an average carrying capacity of 41.626 OD600, a growth rate of 0.298 hr^{-1} , and an inflection point of 21.724 hr, making it the fastest grower. YTG18TO1 had a carrying capacity of 45.467 OD600, a growth rate of 0.226 hr^{-1} , and an inflection point of 25.218 hr. In comparison, YTG1 + Y8TO1 had a carrying capacity of 21.434 OD600, a growth rate of 0.095 hr^{-1} , and an inflection point of 19.660 hr.

Overall, using a stronger glucoamylase did not improve the consortium's performance compared to its monoculture. In both the TlGAMY+OsAA and AnGAMY+OsAA consortia the strain that can degrade starch the best, OsAA or AnGAMY respectively, will have faster growth in its own monoculture than in a consortium with a less-productive partner, or the benefit of adding the less-productive partner will be non-significant at best. While the synergism of α -amylase and glucoamylase is well documented, it may not be enough to support the competition for glucose.

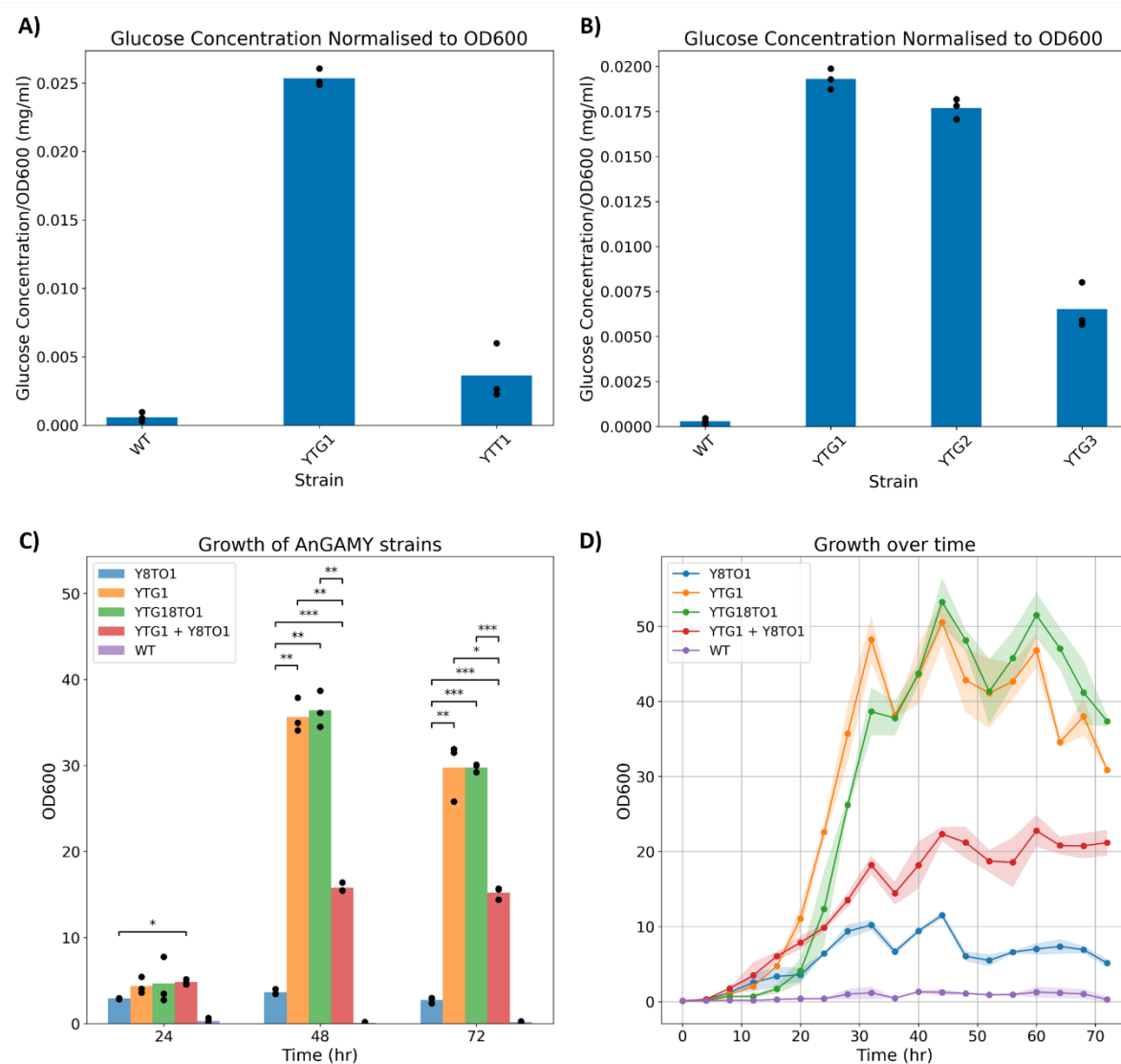


Figure 3.17 Starch degradation and growth of AnGAMY-expressing strains

A) Micro-GOPOD assay for supernatants of AnGAMY (YTG1) and TIGAMY (YTT1) strains. The YTG1 strain showed higher glucose concentration than YTT1, indicating AnGAMY has higher activity. **B)** Micro-GOPOD assay for supernatants of YTG1, YTG2, YTG3, three AnGAMY strains with different SPs. YTG1, containing SP1, had the highest glucose released, followed by YTG2 and then YTG3. **C)** AnGAMY and OsAA monocultures and a 2-strain consortium were grown on 1% potato starch YNB over 72 hours. After 24 hours, the consortium showed a significant improvement over Y8TO1 alone, but no significant difference from the AnGAMY-containing monocultures. After 48 and 72 hours, the Y8TO1 had significantly lower growth than all other cultures. The consortium of YTG1+Y8TO1 showed significantly lower growth than both YTG1 and Y8TO1TG1. **D)** The full growth curves for the AnGAMY strains show that YTG1 and YTG18TO1 have similar growth rates, with YTG1 having a slightly lower inflection point. The consortium YTG1+Y8TO1 has a much lower growth rate. A, B, C) Data presented as the mean for n=3. Statistical analysis by Welch's T-test; * represents a p-value < 0.05, ** p-value < 0.005 and *** p-value < 0.0005. D) Coloured region on the line graph represents the standard deviation about the mean at each timepoint.

3.10. Xylan degradation

I wanted to explore another polysaccharide component of plant waste, specifically one that had been previously shown to have improved degradation due to synergistic enzymes. Xylan can also be degraded by the action of two hydrolysis enzymes. I engineered *Y. lipolytica* to express an exoxylanase from *Aspergillus niger*, XlnD (AnXlnD), and/or an endoxylanase from *Hypocrea lixii*, Xyn2 (HlXyn2), based on the research of Wang *et al.*, 2014 and Duquesne *et al.*, 2014. Metabolism of xylose was achieved by overexpression of endogenous xylose reductase (XR), xylose dehydrogenase (XDH) and xylulokinase (XK) (Niehus *et al.*, 2018). Xylanase strains are shown in Table 3.3.

Table 3.3 Xylanase strains engineered for this chapter

Strain	Inserts
Single-xylanase strains: express either exoxylanase or endoxylanase, plus a second insert containing <i>Y. lipolytica</i> xylose dehydrogenase, xylose reductase, and xylulokinase	
YTA1XC	pTEF-SP1-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTA2XC	pTEF-SP2-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTA3XC	pTEF-SP3-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTA4XC	pTEF-SP4-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTA5XC	pTEF-SP5-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTH1XC	pTEF-SP1-HlXyn2 pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTH2XC	pTEF-SP2-HlXyn2 pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTH3XC	pTEF-SP3-HlXyn2 pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTH4XC	pTEF-SP4-HlXyn2 pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
YTH5XC	pTEF-SP5-HlXyn2 pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
Double-xylanase strains: express both exoxylanase and endoxylanase, plus <i>Y. lipolytica</i> xylose dehydrogenase, xylose reductase, and xylulokinase	
YTA1TH1XC	pTEF-SP1-AnXlnD-pTEF-SP1-HlXyn2 pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK
Control strain: expresses just the xylose cassette	
JMY5814 from (Niehus <i>et al.</i> , 2018).	pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK

These strains were grown on 2% glucose YNB in 96-well plates (Materials and Methods 7.11.1 and 7.11.7). Like the amylase strains, growth on glucose was used as an indicator of the burden of expressing the genes. Figure 3.18 shows that on glucose there was no significant difference in growth rate compared to WT (Figure 3.18A), but there was a slight increase in inflection point, with three of the xylanase strains being significantly higher (Figure 3.18B), and all of the strains had a decreased carrying capacity, with seven out ten being significantly lower (Figure 3.18C). However, most of this difference can be accounted for with the xylose cassette alone. Compared to JMY5814, there were no significant differences in growth rate and inflection point, and only YTH4XC had a significant difference (increase) in carrying capacity.

The xylanase strains were then grown on 1% xylan YNB. Each parameter was compared between the different SPs for each xylanase to see which provided the best growth on xylan (Figure 3.19). For AnXlnD, YTA5XC had the fastest growth rate (Figure 3.19A) and shortest inflection point (Figure 3.19B), but also the lowest carrying capacity, whereas YTA1XC had the highest carrying capacity (Figure 3.19C). For HlXyn2, YTH5XC had the highest growth rate, but a similar inflection points and carrying capacity to most of the other HlXyn2 strains. YTH2XC had the highest carrying capacity.

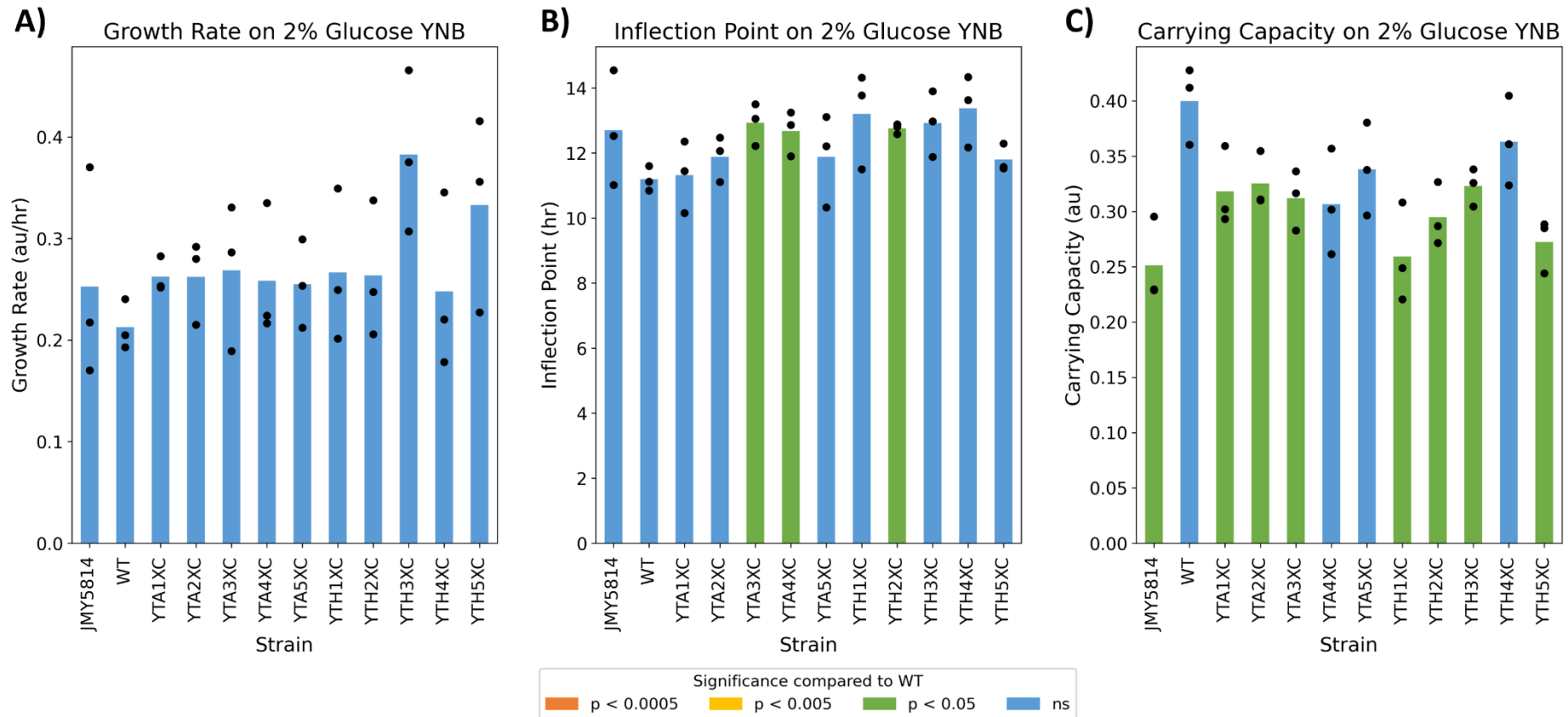


Figure 3.18 Growth parameters of xylanase strains on 2% glucose YNB

A) For growth rate there were no significant differences between the WT and any of the strains. **B)** For inflection point three strains had a significantly increased inflection point compared to WT. **C)** Eight strains showed a significant decrease in carrying capacity, however this also includes the control strain JMY5814. JMY5814 contains just the xylose-consumption cassette. Compared to this strain, the further addition of a xylanase gene did not have a significant effect. Data presented as mean of n=3. Statistical analysis by Welch's T-test.

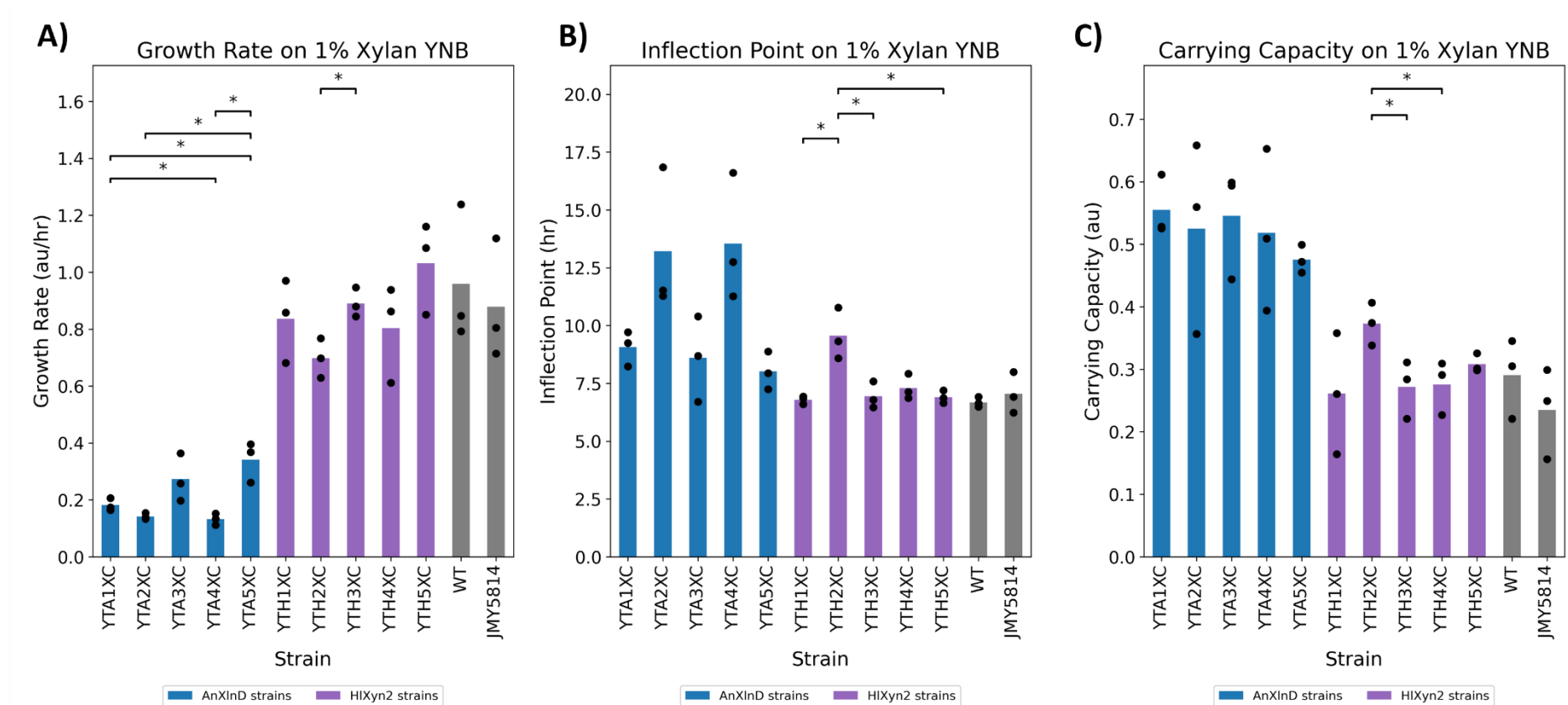


Figure 3.19 Growth parameters of xylanase strains on 1% xylan YNB

A) YTA5XC and YTH5XC had the highest growth rate among the different xylanase strains. **B)** YTA5XC has the lowest inflection point for AnXlnD, while all HIXyn2 strains had a similar inflection point except YTH2XC, which is higher. **C)** YTA1XC and YTH2XC had the highest carrying capacity. Data presented as mean of n=3. Statistical analysis by Welch's T-test compared different SPs for each xylanase; * represents a p-value < 0.05, ** p-value < 0.005 and *** p-value < 0.0005.

Ideally, I would have used the best and worst performing SPs to make double-xylanase monocultures. However, integration of both xylanases plus the xylose cassette proved challenging, only YTA1TH1XC was successfully integrated with all five genes. Still, I used this double-xylanase strain to compare to a 2-strain consortium with YTA1XC+YTH1XC. Figure 3.20 shows the growth curve (Materials and Methods 7.11.6), where YTA1TH1XC and YTA1XC+YTH1XC gave almost identical curves, and YTA1XC alone was also very similar. When fitted to a Gompertz curve (Materials and Methods 7.11.7), YTA1XC and YTA1TH1XC had more similar growth rates to each other, with 0.275 and 0.249 hr^{-1} respectively, while YTA1XC+YTH1XC had a lower growth rate of 0.198 hr^{-1} . Although, this may be due to the limitations of the fitting, since the exponential phases of YTA1XC+YTH1XC and YTA1TH1XC look visually more similar. There were no significant differences between the three for carrying capacity and inflection point.

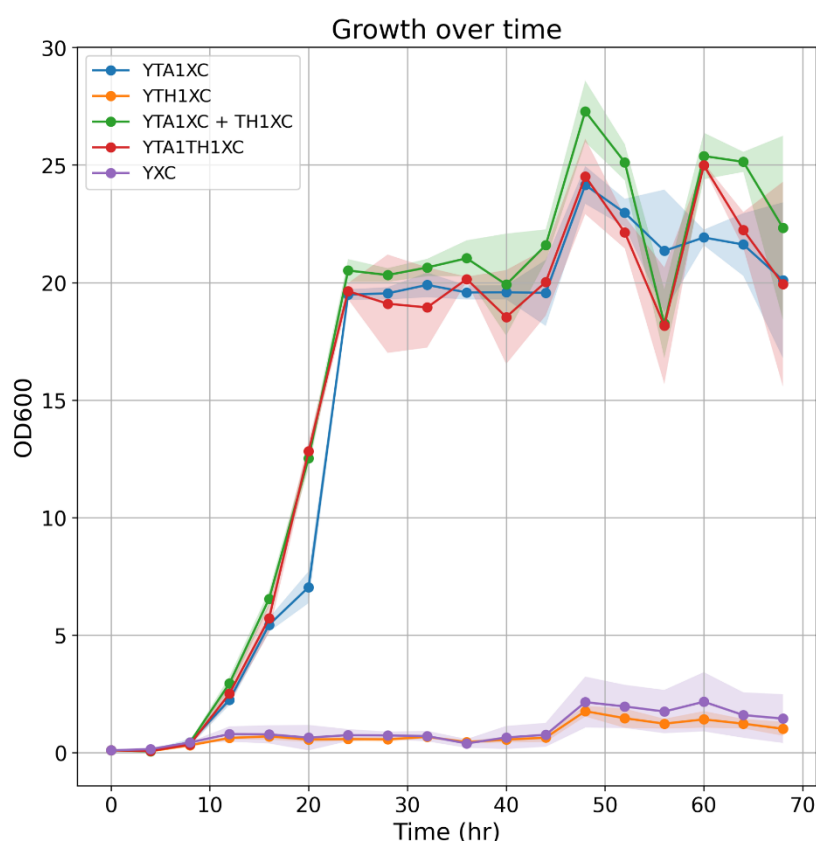


Figure 3.20 Growth of a xylanase consortium and monocultures on 1% xylan YNB

Strains were grown on 1% xylan YNB for 68 hours. YTA1XC, YTA1XC+YTH1XC and YTA1TH1XC had very similar exponential phases, but YTA1XC+YTH1XC reaches a slightly higher carrying capacity. Data presented as the mean of $n=3$, where the coloured region is the standard deviation about the mean.

3.11. Conclusions for this chapter

In this chapter I applied DOL to degradation of a single polysaccharide in *Y. lipolytica*. Firstly, I characterised the burden caused by expressing either one or two amylases under the control of different promoters and SPs. Using a stronger promoter resulted in higher burden, as did co-expressing two amylases, although for the stronger promoter there was a limit to the burden that could be observed. I then compared the growth on starch of a 2-strain consortia, where each strain expresses one amylase, and an equivalent double-amylase-expressing monoculture. This showed that several consortia were able to achieve a higher growth rate than their monoculture equivalent. However, the burden observed in the preliminary experiments was not an accurate predictor of which consortia would achieve a higher growth rate, indicating there were further factors involved. The measurement of burden on glucose does not account for the beneficial effect of stronger expression which results in the release of more glucose from starch. In addition, burden may not be the same when growth is on either starch or glucose, as the available substrate concentration will be different and may result in varied metabolism.

In both the TIGAMY+OsAA and AnGAMY+OsAA systems, one of the single-amylase strains, OsAA or AnGAMY respectively, grew faster than either the consortium or the co-expressing monoculture. In the TIGAMY+OsAA system, the OsAA strain grew the fastest, while the consortium grew faster than the monoculture. This can be explained by the less efficient glucoamylase imposing a metabolic burden without significantly contributing to faster growth. In the consortia where growth rate was improved, the results suggest that the OsAA strain generated enough glucose to support the growth of both cell types, and the reduced burden allowed them to outgrow their equivalent monocultures. However, while the OsAA strain alone may have shown a high growth rate, the TIGAMY+OsAA consortium achieved a higher final OD600. This suggests the contribution of TIGAMY was small, not enough to offset the burden when co-expressed, but it was still present. A similar effect was seen for the xylanase-expressing 2-strain consortium. The consortium of AnXlnD+HlXyn2 strains achieved a slightly higher carrying capacity than the equivalent AnXlnD and double-xylanase monocultures. However, in this xylanase consortium, the effect of reducing burden was barely noticeable, as the consortium and monocultures had very similar growth curves overall.

In contrast, in the AnGAMY+OsAA system, the AnGAMY strain grew the fastest, but the double-amylase monoculture, despite potentially bearing a higher burden, outperformed the consortium both in growth rate and final OD600. Here, AnGAMY efficiently produced glucose on its own, leading to far greater growth compared to OsAA. In the co-expressing monoculture, every

cell produced AnGAMY, whereas in the consortium only half the cells did. Competition for glucose then placed the consortium at a disadvantage compared to both the AnGAMY strain and the co-expressing strain.

Based on these results, I determined that competition between the strains for the resultant degraded substrate would likely be a limiting factor to improving a starch-degrading or xylan-degrading consortium. Therefore, in the next chapter, I focus on DOL combining starch and xylan degradation, which allowed me to reduce the competition between the cells based on their preference for two different substrates.

4. Chapter 4: DOL between two different substrate degradation pathways

4.1. Introduction to Chapter 4

In Chapter 3 I focussed on DOL for the degradation of one substrate at a time. However, most sustainable feedstocks are a mixture of different macromolecules, and therefore, for complete valorisation, consortia must be able to degrade a wide range of polysaccharides simultaneously. In the context of synthetic consortia designed *de novo*, DOL can be applied to the simultaneous utilisation of simple sugars such as hexoses and pentoses. For example, the glucose-xylose-arabinose consortia created by Xie, Eiteman & Altman or Verhoeven *et al.*, where three strains were engineered to consume only one sugar, demonstrated that making cells specialists to one or the other sugar is beneficial over having a more generalist consumer that eats everything (Verhoeven *et al.*, 2018; Xia, Eiteman & Altman, 2012). Orthogonal substrate utilisation allows consortia to efficiently utilise all the substrates within the environment and in nature this increases robustness in changing conditions.

Alternatively, cross-feeding between strains in nature is ubiquitous, and allows cells to lose certain functions which are provided for by others in their community. As discussed in Chapter 1 (Section 1.5.2), rationally designed cross-feeding can make strains within a consortium mutually-dependent on each other, for example amino-acid auxotrophic strains can be designed to exchange essential amino acids (Mee *et al.*, 2014). In consortia of auxotrophic *E. coli* cells cross-feeding each other two reciprocal amino acids, every pair showed an increased growth rate compared to prototrophic wild-type *E. coli* cells (Pande *et al.*, 2014). This cross-feeding population was stable because the frequency of each auxotroph in the population acts as a selection pressure on its complementary partner, called frequency-dependent selection (Losoi, Santala & Santala, 2019; Pande *et al.*, 2014).

In this chapter I aim to see if the rational design strategies for DOL, already in use for simple sugars and metabolites, can also be used for complex substrates. Starch and xylan are two different polysaccharides which require individually specific enzymes to degrade them to glucose and xylose respectively. To achieve degradation of mixed starch and xylan substrates, you can choose to make a generalist strain monoculture capable of digesting both substrates, or you can apply DOL to separate the two digestion pathways into different cells in a 2-strain consortium. Further, I aim to see if orthogonality and mutual dependence can be engineered by knocking out the sugar consumption, creating strains that can consume only glucose or xylose, and whether these strategies provide the same benefits as seen for simple sugars or amino acids.

4.2. Combining a starch-degrader and a xylan-degrader

In Chapter 3, I found that in the 2-strain amylase consortia I created, my OsAA-expressing strain of *Y. lipolytica* was, in effect supporting both partners growth, while the TIGAMY-expressing strain reduced growth rather than supported it (Figure 3.14). I needed to ensure a similar imbalance would not exist for a 2-strain consortium of a starch and a xylan degrader. I hypothesised that in this instance, having two different substrates would prevent either degrader strain alone from singlehandedly consuming all the substrate available to them. Initially, I combined starch-degrading YTO1 (OsAA-expressing strain) and xylan-degrading YTA1XC (AnXlnD-expressing strain), *Y. lipolytica* strains that were already created as part of Chapter 3, to see if there was any benefit to growing these starch and xylan degraders together. Note that in this 2-strain consortium YTO1 can only consume glucose, while YTA1XC can eat both glucose and xylose. In the plate reader, the consortia all gave a biphasic growth curve, due to their ability to consume the two different sugar feedstocks. Therefore, I used a biphasic logistic growth model (Equation 4.1) to fit the data (Zwietering et al., 1990) (Materials and Methods 7.11.7). For YTO1, since it cannot grow on xylose, I continued to use Gompertz, as in Chapter 3 (Equation 3.1). For YTA1XC it was difficult to determine which curve fitting gave the best fit to the data, since it can eat both sugars, so could have biphasic growth, but it cannot degrade starch, so there should be no glucose available to it. I chose to use the biphasic model in the end, as it seemed to give a slightly better fit to the data.

$$A(t) = \frac{A_1}{1 + e - (k_1(t - t_1))} + \frac{A_2}{1 + e - (k_2(t - t_2))} \quad \text{Equation 4.1 Biphasic growth model}$$

Where $A(t)$ is the Abs600 at time t , is A_1 and A_2 are the carrying capacity for the first and second phase of growth respectively, k_1 and k_2 are the growth rates for the first and second phase, and t_1 and t_2 are the inflection points (midpoints) of the two phases.

Consortia of YTO1+YTA1XC were grown in the plate reader on 0.5% starch + 0.5% xylan YNB, with different inoculation ratios (given as YTO1:YTA1XC) (Materials and Methods 7.11.1 and 7.11.5). The consortia with a 3:1 ratio grew the best, achieving a higher final Abs600 than either the 0:1 or 1:0 ratios with either strain alone (Figure 4.1A). While YTO1 and YTA1XC do reach a carrying capacity by 48 hours, the growth curves for the consortia do not plateau, indicating they may continue to grow after this point. In the first phase of growth, the growth rate appears to be dependent on the YTO1 amylase strain (Figure 4.1B), which may indicate that mainly glucose is consumed. In the second phase the 1:1 ratio has the highest growth rate, but it is a much slower

growth rate than the first phase. While YTA1XC xylanase strain alone (0:1 ratio) achieves quite a high final OD600, it has the slowest growth rate.

I repeated this experiment in flasks (Figure 4.1C) (Materials and Methods 7.11.4). This experiment only presented a small increase in final OD600 for the YTO1+YTA1XC consortium compared to the YTA1XC strain alone. However, when combined with the growth rate results in the plate reader, the results demonstrate that the consortium of a starch degrader and a xylan degrader was able to achieve higher growth, either in the form of a higher final OD600 or in the form of higher growth rate. Since Chapter 3 showed that an 8UASpTEF promoter increased growth on starch, I chose to use the 8UASpTEF-controlled amylase strains going forward. And I hypothesised that by adapting the sugar uptake of the two strains I could further improve the consortium's growth compared to the component strains.

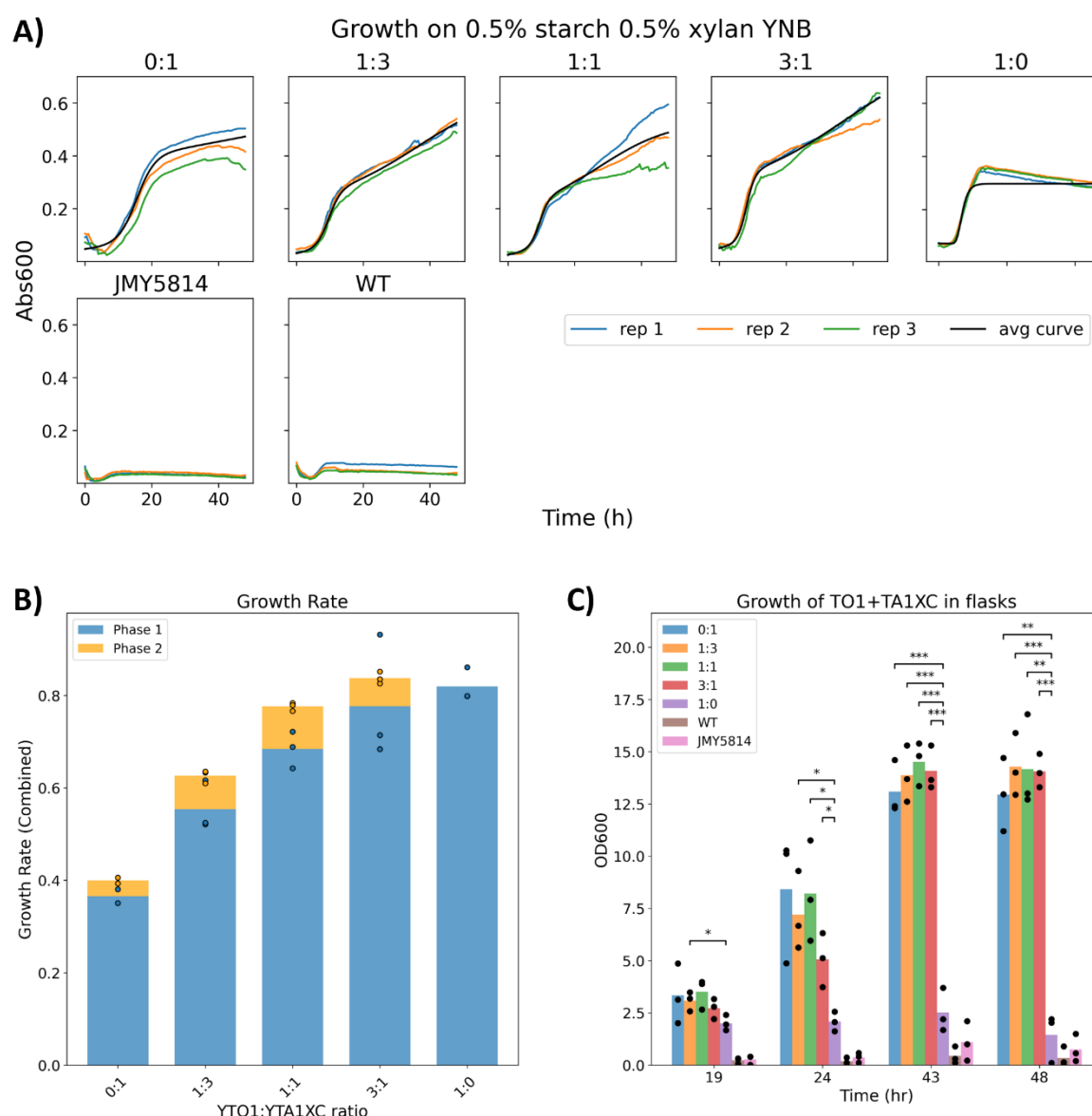


Figure 4.1 Growth of YTO1+TA1XC consortia on a mixture of starch and xylan

Cultures were grown in 96-well plates, on 0.5% starch + 0.5% xylan YNB, for 48 hours. **A)** Growth curves were fitted to a biphasic logistic curve for those which could consume both glucose and xylose. A Gompertz curve was used for Y8TO1 which could only consume glucose. The 3:1 ratio for the consortium achieved the highest growth overall. **B)** Growth rate for both phases of growth. YTO1 on its own has the fastest growth rate in Phase 1, however it did not show biphasic growth. The 1:1 ratio has the fastest growth rate in Phase 2. **C)** In flasks, all the consortia did better than the Y8TO1 alone, but only achieved a slightly higher OD600 (non-significant) than the YTA1XC strain alone. Data is presented as the mean of $n=3$ ($n=2$ for 0:1 ratio). Statistical analysis by Welch's T-test; * represents a p -value < 0.05 , ** p -value < 0.005 and *** p -value < 0.0005 . Coloured region on the line graph represents the standard deviation about the mean at each timepoint.

4.3. Designing different DOL strategies

When designing a consortium that can degrade and consume two distinct substrates, different design strategies can be employed which are inspired by previous consortia work with simple sugars. Firstly, I imagined the monoculture strategy for degrading starch and xylan. The strain is a generalist for degradation, by which I mean it expresses both amylase and xylanase, degrades both starch and xylan, and a generalist for consumption, meaning it can metabolise both glucose and xylose (Figure 4.2A).

Then I designed three 2-strain strategies for the DOL between starch and xylan degradation. In the first design, which I named the general-consumer design (Figure 4.2B), each strain expresses either an amylase or a xylanase, making each cell a specialist degrader, but both strains can consume both glucose and xylose. All the cells in this consortium are fed by both degradation pathways and therefore should be able to grow as well as a monoculture equivalent. However, as discussed in Chapter 3, this generalist consumer strategy also means the strains will be competing with one another for sugar, and therefore this consortium might not remain stable over time. Potentially one strain could outgrow the other and dominate the consortium to such an extent that one of the degradation pathways is lost.

In the second design strategy, which I call the orthogonal specialist consortia (Figure 4.2C), each strain is entirely specialist and separate from the other for both degradation and consumption. One strain makes amylase and eats the resulting glucose, and the second makes xylanase and eats the resulting xylose. There should be no competition between these two strains for sugar, however both cells will only be able to consume part of the given feedstock. Despite this, there is still a likelihood that one cell will be able to out-grow the other and dominate the consortium because of differences in their growth rate on the different substrates.

The third and final design, named the cross-feeding specialist consortia (Figure 4.2D), also uses specialist cell strains for both degradation and consumption, but there is a disconnect between degradation and consumption. The first cell makes amylase to degrade starch, but has its glucose metabolism knocked out, so instead it consumes only xylose. In contrast, the other cell expresses xylanase to degrade xylan but consumes only glucose. This creates a mutual dependence on one another for survival. This strategy should help reduce the amount that one cell can dominate the consortium and may allow for a more robust consortium over long periods of culture.

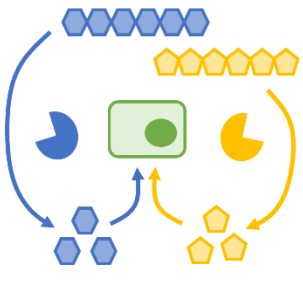
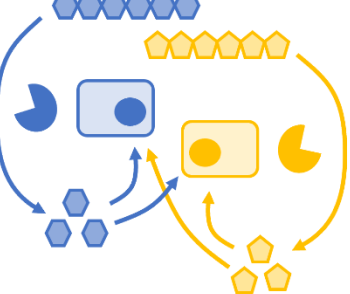
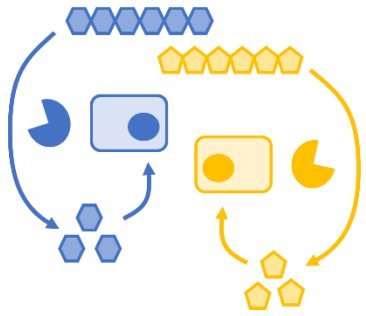
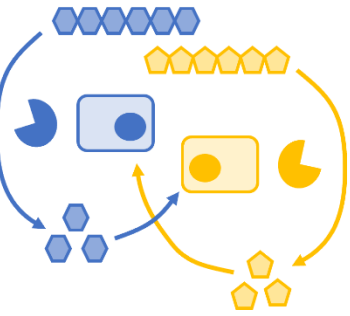
A) MONOCULTURE		B) GENERALIST CONSUMER	
STRAINS		STRAINS	
Y8TO1TA1XC		Y8TO1XC	YTA1XC
DEGRADES		DEGRADES	
Starch AND Xylan		Starch	Xylan
CONSUMES		CONSUMES	
Glucose AND Xylose		Glucose AND Xylose	Glucose AND Xylose
			
C) ORTHOGONAL SPECIALIST		D) CROSS-FEEDING SPECIALIST	
STRAINS		STRAINS	
Y8TO1	YG-TA1XC	YG-8TO1XC	YTA1
DEGRADES		DEGRADES	
Starch	Xylan	Starch	Xylan
CONSUMES		CONSUMES	
Glucose	Xylose	Xylose	Glucose
			

Figure 4.2 Design of division of labour (DOL) between a starch-degrader and a xylan-degrader

DOL between starch and xylan degrading can take advantage of several different strategies to optimise the relationship between the strains in the consortium. **A)** A monoculture that can degrade both starch and xylan, consuming both glucose and xylose. This monoculture may experience considerable metabolic burden and may not be as robust to environmental changes as a consortium. **B)** A consortium where one cell degrades starch and the other cell degrades xylan may have reduced metabolic burden, but the cells will compete for sugars. **C)** A consortium where one cell degrades starch, the other degrades xylan and they don't compete for sugar may have reduced burden and no competition for sugar, but the cells may have different growth dynamics on the two sugars, leading one cell to dominate the consortium. **D)** A consortium where one cell degrades the substrate that the other consumes will have a mutual dependency, which may improve the stability of the consortium.

I engineered *Y. lipolytica* to express an amylase, OsAA, and/or a xylanase, AnXlnD, in the combinations required to achieve the rational design strategies described above (Table 4.1). To create the strains that don't consume glucose, I utilised a strain, RLA1074, from Hapeta *et al.*, 2021, where glucose metabolism has been eliminated by the deletion of hexose kinases.

Table 4.1 Strains engineered for this chapter

Strain	Parental strain	Insert/s
Y8TO1TA1XC	Po1d	8UASpTEF-SP1-OsAA pTEF-SP1-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK
Y8TO1	Po1d	8UASpTEF-SP1-OsAA
Y8TO1XC	Po1d	8UASpTEF-SP1-OsAA pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK
YG-8TO1XC	Po1d Δ HXT	8UASpTEF-SP1-OsAA pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK
YTA1	Po1d	pTEF-SP1-AnXlnD
YTA1XC	Po1d	pTEF-SP1-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK
YG-TA1XC	Po1d Δ HXT	pTEF-SP1-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK
YG-XC	Po1d Δ HXT	pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK
Y8TO1TA1XC-GFP	Po1d	8UASpTEF-SP1-OsAA pTEF-SP1-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK pTEF-GFP
Y8TO1-RFP	Po1d	8UASpTEF-SP1-OsAA pTEF-RFP
Y8TO1XC-RFP	Po1d	8UASpTEF-SP1-OsAA pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK pTEF-RFP
YG-8TO1XC-RFP	Po1d Δ HXT	8UASpTEF-SP1-OsAA pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK pTEF-RFP
YTA1-GFP	Po1d	pTEF-SP1-AnXlnD pTEF-GFP
YTA1XC-GFP	Po1d	pTEF-SP1-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK pTEF-GFP
YG-TA1XC-GFP	Po1d Δ HXT	pTEF-SP1-AnXlnD pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK pTEF-GFP
YTG1-RFP	Po1d	pTEF-SP1-AnGAMY pTEF-RFP
YTG1XC-RFP	Po1d	pTEF-SP1-AnGAMY pTEF-yIXDH-pTEF-yIXR-pTEF-yLXK pTEF-RFP

4.4. Consortia of a starch-degrading strain and a xylan-degrading strain can achieve a higher growth than either a starch-degrader or xylan-degrader alone

Again, my first aim with the three new consortia designs described in Section 4.3 was to explore whether they could achieve a higher growth rate or final OD600 as a 2-strain consortium than each of their component strains could when cultured alone.

Figure 4.3 shows the growth of the three different DOL strategies on 0.5% starch + 0.5% xylan YNB media in flask cultures over 72 hours, with different inoculation ratios of the two strains (Materials and Methods 7.11.4 and 7.11.5). The three consortia were grown with 1:3, 1:1 and 3:1 inoculation ratios of amylase (OsAA) expressing strain : xylanase (AnXlnD) expressing strain. Each single-enzyme-expressing component strain was also grown alone (1:0 or 0:1 inoculation ratio). The OD600 measurements were taken twice a day.

The generalist consumer consortium Y8TO1XC+YTA1XC (Figure 4.3A) achieved a higher final OD600 than either of its component strains, Y8TO1XC or YTA1XC cultured alone, although the xylanase-expressing YTA1XC alone did grow reasonably well. The 1:3 ratio of Y8TO1XC:YTA1XC (favouring the xylanase strain) grew the fastest, growing to 15.59 after 24 hours, compared to 13.55 and 13.72 for the 3:1 and 1:1 ratio respectively. The generalist consumer consortium DOL strategy grew to the highest OD600 overall, compared to the other DOL strategies. The generalist consumer consortium can eat all the available substrate, whereas in the two specialist consortia each cell only has access to half the available substrate for growth.

The orthogonal specialist consortium Y8TO1+YG-TA1XC (Figure 4.3B) also achieved a higher final OD600 than either of its component strains Y8TO1 or YG-TA1XC cultured alone. The 1:3 ratio of Y8TO1:YG-TA1XC (favouring the xylanase strain) grew marginally faster than the 1:1 ratio, which in turn grew faster than the 3:1 ratio. The xylanase-expressing YG-TA1XC strain alone had some unusual results. Two of the replicates grew to an OD600 of 10.28 and 11.08 respectively, while the third only grew to 1.58. Originally, I considered this third replicate to be anomalous and removed it from the results. I have now chosen to reinstate it and further experiments described in this chapter will demonstrate why this decision has been made.

The cross-feeding specialist consortium YG-8TO1XC+YTA1 (Figure 4.3C) also achieved a higher final OD600 than either of its component strains, YG-8TO1XC or YTA1, which indeed did not grow when cultured alone. This lack of growth when these two strains were in isolation from one another demonstrates the desired mutual dependence on each other. The 1:1 ratio of YG-8TO1XC:YTA1 and the 1:3 ratio grew the fastest. The orthogonal specialist and cross-feeding specialist consortia both grew to similar final ODs, regardless of the initial inoculation ratio.

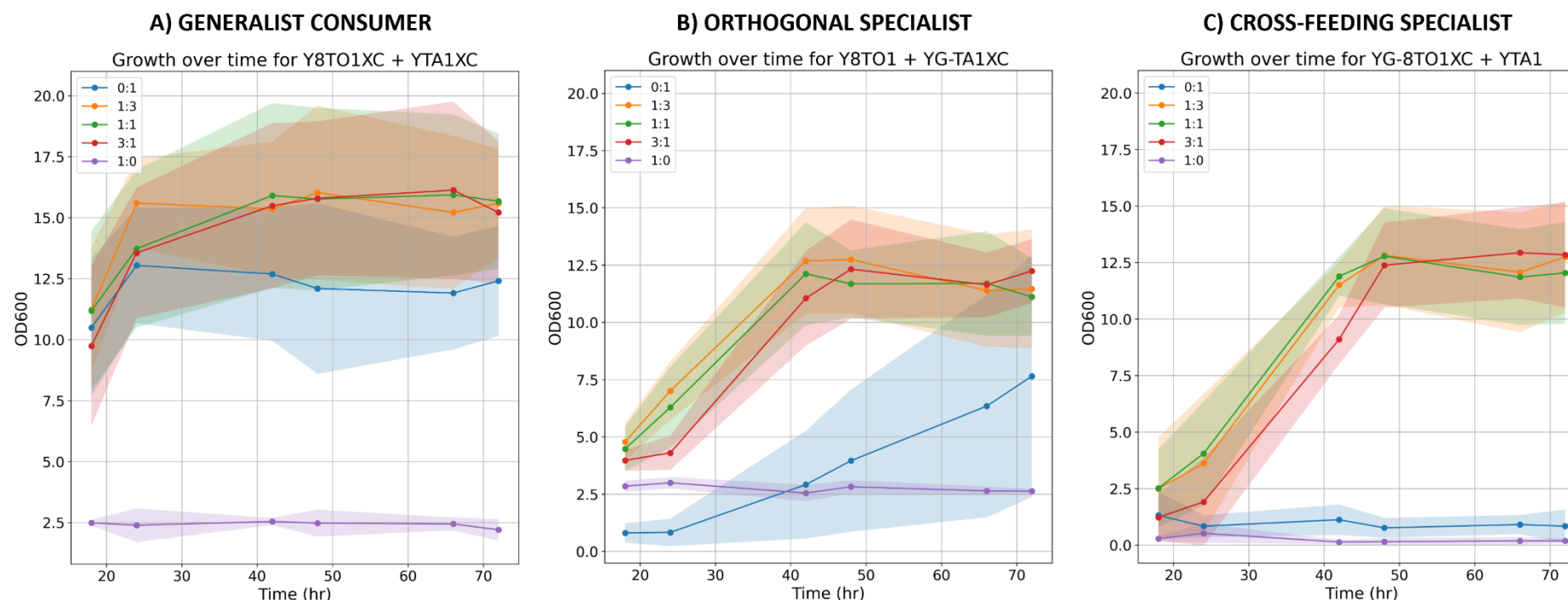


Figure 4.3 Growth of different division of labour (DOL) strategies on a mixture of starch and xylan, with different inoculation ratios

The consortia were inoculated at different ratios (given as amylase strain:xylanase strain) and grown for 72 hours on 0.5% starch 0.5% xylan YNB. **A)** The generalist consumer consortium: both strains can consume both glucose and xylose, Y8TO1XC expresses amylase, and YTA1XC expresses xylanase. In this case the YTA1XC strain alone grows quite well, but all three consortia achieve a higher final OD600, and the 1:3 ratio has the fastest growth. **B)** The orthogonal specialist consortium: Y8TO1 expresses amylase and can only consume glucose, YG-TA1XC expresses xylanase and can only consume xylose. All three consortia achieve a similar final OD600, with the 1:3 ratio growing the fastest. YG-TA1XC grows slowly to a similar final OD600 to the consortia. **C)** The cross-feeding specialist consortium: YG-8TO1XC expresses amylase but can only consume xylose, YTA1 expresses xylanase but can only consume glucose. In this case only the consortia grow, showing that the strains are mutually dependent on each other, with the 1:1 ratio growing the fastest. Data is shown as the mean of $n=3$, the coloured region represents the standard deviation about the mean.

4.5. How growth and population dynamics change over time for each DOL strategy

Having assessed the general growth of the three DOL strategies, I now wanted to observe how each strain behaves within each consortium. This would allow me to see if the expected dynamics would be achieved. Based on the rational design I expected that in the generalist consumer both strains would grow at a similar rate, since they both have access to the same substrates, but that the growth of the consortium would be dependent on the “stronger” enzyme, so the population might shift slightly towards one or the other strain over time, as was seen with the amylase-expressing consortia in Chapter 3. For the orthogonal specialist consortium, I expected to see one of two things. Either, the substrates are consumed simultaneously, in which case the two strains would grow at different rates, leading to a population shift toward one strain. Or the substrates are consumed sequentially, then I would expect to see one strain dominating at the beginning of the culture, and the other dominating at the end of the culture. For the cross-feeding specialist consortium I expected to see a more stable population than the orthogonal specialist due to the mutual dependence of the strains; if one strain diminishes then the other strain loses its substrate. Tracking the population over time allowed me to see if these hypotheses were correct.

To do this I added an RFP marker gene to the amylase strains, and a GFP marker gene to the xylanase strains. Strains were grown on 4% glycerol YP media overnight, as opposed to the previously used 2% glucose YPD media, since some of the strains cannot consume glucose, and to ensure all the strains start from the same overnight conditions. Consortia were grown in 0.5% starch + 0.5% xylan YNB media in flasks for 72 hours (Materials and Methods 7.11.6). Cultures with inoculation ratios of 3:1, 1:1 and 1:3 amylase-expressing strain : xylanase-expressing strain were tested first. However, these produced very similar results to one another (Appendix 9.8), possibly suggesting that the difference between the inoculation ratios was insufficient. I therefore decided to increase the degree of difference in the ratios tested.

Instead, the three consortia strategies were grown with 1:10, 1:1 and 10:1 initial inoculation ratios of amylase-expressing strain : xylanase-expressing strain. Each component single-enzyme-expressing strain was also cultured alone (1:0 or 0:1 inoculation ratio). The OD600 and flow cytometry (Guava flow cytometer) measurements were taken every 24 hours (Materials and Methods 7.12.1). Figure 4.4 shows the results for their growth and the changes in population composition.

For the generalist consumer consortium, Y8TO1XC+YTA1XC, the fastest growth within 24 hours was seen for the 1:1 inoculation ratio, with a mean OD600 of 16.45 (Figure 4.4A). The amylase-

expressing Y8TO1XC strain alone grew to an OD600 of 3.05, while the xylanase-expressing YTA1XC strain alone grew to an OD600 of 13.07 after 24 hours. This meant that the 1:1 consortia growth was approximately the same as the combined growth of its component xylanase and amylase strains when grown alone. After 72 hours the 1:10, 1:1 and 10:1 inoculation ratios had OD600s of 14.71, 14.93 and 15.98 respectively, while the component strains, YTA1XC and Y8TO1, grown alone had OD600s of 10.74 and 2.97 respectively (Figure 4.4C). In this case by the end of the culture the generalist consumer consortium attained a higher growth than the sum of the growth of the two component strains cultured on their own.

Additionally, for the generalist consumer consortium I found that the final population composition of the two strains remains dependent on and consistent with the initial ratios. The 1:1 ratio was maintained by the end of the culture at 72 hours. The 1:10 ratio was roughly stable at 2:8, however there was a slight shift towards the amylase-expressing Y8TO1XC strain, from 16:83 at 24 hours to 22:77 at 72 hours. The 10:1 ratio was also roughly stable at 8:2 with a very small shift towards the xylanase-expressing YTA1XC strain over the 24-72 hour period, of only 3 percent. This overall stability was somewhat unexpected for the generalist consumer consortia. In this DOL strategy both cell types can consume both sugars, so the two strains are directly competing for feedstock. Therefore, it might be expected that the better competitor would come to dominate the consortium. In this case, since the YTA1XC xylanase strain alone grew to a higher final OD600 than the Y8TO1XC amylase strain, it might be expected that it would also dominate in a consortium. However, the final OD600 is not always reflective of the growth rate, as demonstrated in Section 4.2 where the xylanase-strain alone had a high final OD600 but a low growth rate. In this experiment I did not measure the growth rates, only three timepoints for OD600 so I cannot draw a conclusion about the growth rate of the Y8TO1XC and YTA1XC strains here. However, since they have similar background and can both eat both sugars, they may well have a very similar growth rate, which helps to maintain their population ratios.

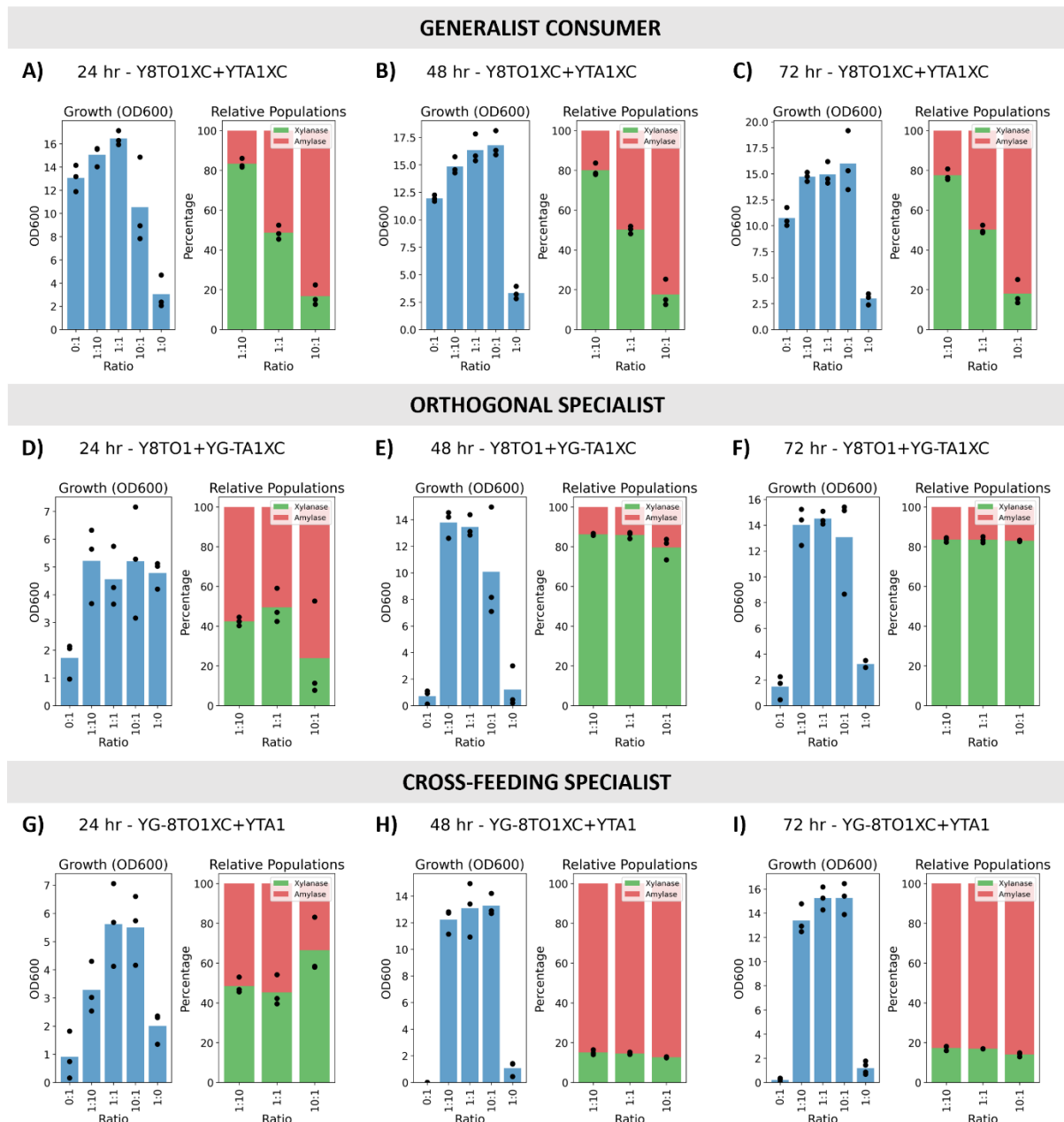


Figure 4.4 Growth and population dynamics for different division of labour (DOL) strategies for a consortium with a starch-degrading strain and a xylan-degrading strain

The generalist consumer consortium (**A, B, C**) roughly maintains a ratio proportional to the inoculation ratio. The 1:1 ratio is maintained over 72 hours, while the 1:10 and 10:1 ratios become closer to 2:8 and 8:2 respectively, with the 1:10 having the least stability as it shifts towards the amylase strain over time. The 1:1 inoculation ratio achieves the highest OD600 after 24 hours, while the 10:1 has the highest OD600 after 48 and 72 hours. For the orthogonal specialist consortium (**D, E, F**) after 24 hours the 1:1 ratio remains at approximately 1:1, while the 1:10 ratio becomes 6:4, the percentage of the amylase strain Y8TO1 increasing. After 48 and 72 hours however, regardless of inoculation ratio, the population composition has shifted to favour the xylanase strain YG-TA1XC. After 24 hours the consortia have a similar OD600 to the YG-TA1XC strain alone, but by 48 hours the consortia have overtaken the xylanase strain, with the 1:10 and 1:1 ratios growing to the highest final OD600. The cross-feeding specialist consortium (**G, H, I**) shifts towards the xylanase-expressing strain after 24 hours, but then at 48 and 72 hours has shifted towards a dominant amylase-expressing YG-8TO1XC strain. Data is presented as the mean of n=3.

For the orthogonal specialist consortium, Y8TO1+YG-TA1XC, the consortia and the amylase-expressing Y8TO1 strain grew to a similar OD600 after 24 hours (Figure 4.4D), but the consortia continued growing so that by 72 hours (Figure 4.4F) they reached an OD600 of 14.03 (1:10), 14.51 (1:1) and 13.07 (10:1), while the single-enzyme-expressing strain cultures were only OD600 3.23 (Y8TO1) and 1.48 (YG-TA1XC). This was an unexpectedly significant difference, since the degradation and consumption pathways should be entirely orthogonal from each other. Each strain should only be able to consume its portion, in this case half, of the available substrate. Therefore, each individual strain on its own should act as a benchmark for how much growth is achievable with one substrate. The consortium of the two strains together might be expected to achieve a final OD600 equal to the sum of the two component strains cultured alone over the same time. However, even when you consider the highest growth of the two component strains, which was seen at 24 hours and is 4.78 for Y8TO1, and 1.72 for the YG-TA1XC strain, this only adds to an OD600 of 6.5. Therefore, some additional synergistic effect was occurring when the two strains are grown together in the consortium that produces a final OD600 of more than double that accounted for by the two component strains grown individually.

Of note here, the xylanase-expressing YG-TA1XC strain did not grow above an OD600 of 2. This explains my decision to reinstate the third replicate in the growth curves in the previous section (Figure 4.3)- since it would seem the low growth was the more consistent result. The low growth of YG-TA1XC alone was a sharp contrast to the OD600 achieved by the xylanase-expressing YTA1XC strain alone. When grown without the amylase-expressing strain, I would expect the YG-TA1XC and YTA1XC strains to behave the same, with only xylan being degraded and only xylose being consumed. The only difference between the two strains is their ability (YTA1XC) or inability (YG-TA1XC) to consume glucose. The fact that glucose and xylose consuming YTA1XC alone had a growth advantage over xylose consuming YG-TA1XC alone may indicate the presence of glucose even in the absence of the amylase-expressing strain. The negative controls, the WT (eats only glucose), JMY5814 (eats glucose and xylose) and YG-XC (eats only xylose), were grown alongside the consortia on the same media, and did have a very small amount of growth, but it was under 2 OD600 (Appendix 9.9) for all three. To me this suggests there is insufficient glucose, or other carbon source, to account for the difference in YTA1XC and YG-TA1XC growth. Possibly there is a small amount of glucose, which gives YTA1XC sufficient growth to begin producing enough xylanase to efficiently degrade xylan. The YG-TA1XC doesn't receive this early boost from glucose and therefore can't degrade enough xylan to grow to a high OD600. I considered that the origin of this glucose could either be from starch, or it could be available in the xylan media. The fact that strain YTA1XC did grow to an OD600 of over 20 in 1% xylan YNB

(Figure 3.13 in Chapter 3) may indicate that a carbon source boost was available in this culture as well. While xylan is primarily made of xylose chains, its branching structures can contain a variety of hexoses, including glucose. The potential presence of pre-existing glucose also presents an additional possibility for the difference between xylose-only consumer YG-TA1XC and glucose- and xylose-consumer YTA1XC, which is catabolite repression. If glucose represses the consumption of xylose, then the YTA1XC strain can remove this repression by consuming the glucose, while the YG-TA1XC strain cannot remove it, hence it cannot grow. Catabolite repression will be discussed further throughout the chapter.

The amylase-expressing Y8TO1 strain on its own grew slightly more within 24 hours than the Y8TO1XC strain alone, but less after 48 and 72 hours. This is only a small difference, but it may be caused by the additional burden of the xylose cassette in the Y8TO1XC strain.

For the orthogonal specialist consortia, in terms of the population composition, after 24 hours (Figure 4.4G) the 1:1 inoculation ratio remained at 1:1, while the 1:10 ratio became 6:4; switching from a majority xylanase strain to a majority amylase strain. This could also be due to the presence of pre-existing glucose giving Y8TO1, the amylase-expressing glucose-consumer, a boost in growth early, before YG-TA1XC, the xylanase-expressing xylose-consumer, can begin to degrade xylan and consume the resultant xylose. The 10:1 inoculation ratio had two replicates which remained at around 10:1, while the third replicate became 1:1. This third replicate may be anomalous, or it may just be slightly ahead of the other two. Because after 48 and 72 hours (Figure 4.4H and Figure 4.4I) all three inoculation ratios of the orthogonal cross-feeding consortium have the exact same population composition, of around 2:8, favouring the xylanase-expressing xylose-consuming YG-TA1XC strain.

For the cross-feeding specialist consortium, YG-8TO1XC+YTA1, at all timepoints the consortia grew better than its component strains grown alone; the latter never grew above an OD600 of 2. For the consortia, the 1:1 and 10:1 inoculation ratios grew the best, reaching a mean OD600 of 15.24 and 15.26 respectively after 24 hours. This DOL strategy had a higher final OD600 than the orthogonal specialist strategy, indicating that the mutual dependence of the two strains might provide a benefit for growth. In terms of population composition, at 24 hours the 1:1 ratio shifted slightly to 55:45, while the 1:10 ratio shifted closer to 1:1, both favouring the amylase-expressing strain, and similar to the orthogonal specialist consortium at this timepoint. However this DOL strategy did differ for the 10:1 inoculation ratio; at 24 hours the orthogonal specialist consortia 10:1 ratio still favoured the amylase strain, but in the cross-feeding specialist consortium it was closer to a 3:6 ratio, switching from an amylase-expressing majority to a xylanase-expressing

majority. In complete contrast to the orthogonal specialist consortium, after 48 and 72 hours, the cross-feeding consortium, regardless of inoculation ratio, has a 8:2 ratio, favouring the YG-8TO1XC amylase strain.

Notably both the orthogonal and cross-feeding specialist consortia ended with a higher population of the strain that consumes xylose, YG-TA1XC and YG-8TO1XC respectively. This occurred regardless of the initial inoculation ratio, indicating that the xylose-consuming strain has an advantage over the glucose-consumer. This is supported by the fact that the xylanase-expressing strains cultured on xylan alone grew to a higher OD600 than the amylase strains on starch alone (Chapter 3). I suggest three possibilities for these findings. Firstly, it could be due to an imbalance in the xylose metabolism vs the glucose metabolism. Glucose is recognised as a highly favourable substrate for *Y. lipolytica*, and the glycolysis pathway is very efficient, but perhaps the use of the pentose-phosphate pathway in xylose metabolism is providing an added benefit to the xylose-consumer. Secondly, there are potentially more units of xylose in 0.5% xylan (2.00×10^{21} assuming that xylan is 100% xylose, which is not the case) than there are units of glucose in 0.5% starch (1.67×10^{21} glucose), although they are still within the same order of magnitude. Thirdly, this imbalance may be due to a difference in activity of the xylanase vs the amylase. If the xylanase is more active there will be more xylose available than glucose, and the strain that can consume xylose therefore prospers.

While both the orthogonal and cross-feeding specialist consortia end the culture at 72 hours with a higher amount of the xylose-consumer, this is not the case at 24 hours. The change in population composition between 24 and 48 hours for both these DOL strategies demonstrates the different timing of consumption of the two substrates. The orthogonal specialist consortium 1:10 ratio shifts towards the amylase-expressing Y8TO1 at 24 hours, and the cross-feeding specialist consortium 10:1 ratio shifts towards the xylanase-expressing YTA1 strain at 24 hours. Both these strains are glucose-consumers. Therefore, for these consortia, at 24 hours the glucose-consuming strains increase in population first, indicating glucose is being consumed first, while after 48 hours the xylose-consuming strain population increases, after the glucose has predominantly been consumed. This supports the idea that these substrates are consumed sequentially not simultaneously, and that there may be some catabolite repression which causes the xylose to be consumed later. Since the starch and xylan are in equal ratios, you might expect that even if the substrates are consumed sequentially, that they would end the culture with an approximately equal ratio, or that the glucose-consumer would remain higher than the xylose-consumer, because it has the advantage of growing first. This indicates that the xylan or xylose is providing higher growth than the starch or glucose.

To verify the flow cytometry data, the same experiment was repeated, inoculating different ratios of the two strains in the consortia and measuring them at 24-hour intervals. The samples were analysed by imaging under the microscope and counting the cells with each fluorescence (Materials and Methods 7.12.2). The results under the microscope were consistent with the data from flow cytometry. The generalist consumer remained the most stable (Figure 4.5A), while the two specialist consortia ended with a majority of xylose-consuming cells (Figure 4.5B & Figure 4.5C). These microscopy images increased the confidence in the flow cytometry measurements and gave a visual representation of the populations, as demonstrated by the exemplar images for the 1:1 ratios of each DOL strategy at 72 hours (Figure 4.5D, E & F).

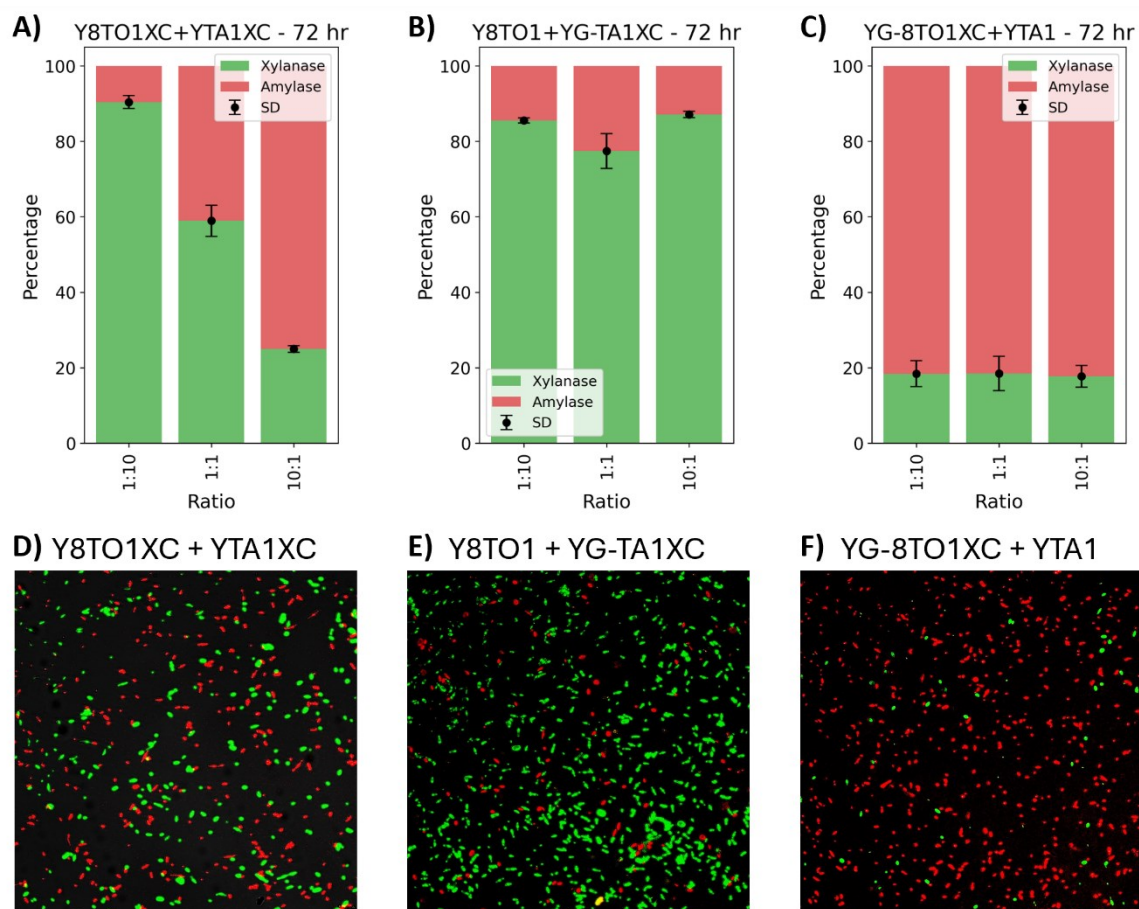


Figure 4.5 Population dynamics of division of labour (DOL) strategies observed under the microscope

DOL strategies were inoculated at different ratios of the amylase strain to the xylanase strain, and microscopy images were taken at 72 hours and analysed to count the number of GFP+ and RFP+ cells. This figure shows the results of the analysis as well as an example of the microscopy images at 72 hours. The generalist consumer consortium (**A & D**) roughly maintains a ratio proportional to the inoculation ratio, with the 10:1 ratio showing the least stability as it shifts towards the xylanase strain after 72 hours. For the orthogonal specialist consortium (**B & E**), the population composition has shifted to favour the xylanase strain YG-TA1XC. In contrast, the cross-feeding specialist consortium (**C & F**) has shifted towards the amylase strain YG-8TO1XC. Data in the bar graphs is presented as the mean of $n=5$, with error bars representing the standard deviation.

To investigate whether the observed shift towards the xylose-consumer strains over time was due to the xylanase activity, or due to the xylose metabolic pathway, I looked at a simplified system of a glucose-consumer strain (WT) and a xylose-consumer strain (YG-XC) without any degradation enzymes. The consortium of WT+YG-XC was inoculated with a 1:1 ratio. As expected, when grown on 2% glucose YNB alone, after 72 hours 99% of the culture was WT strain, and when grown on 2% xylose YNB alone, 99% of the culture was YG-XC strain. However, on a 1:1 mixture of 1% glucose + 1% xylose YNB, the WT still accounted for more than 95% of the culture at 72 hours (Figure 4.6). This shows that the glucose-consumer, with no degradation pathway, will dominate a culture. Therefore, it is likely that the xylose-consumer dominates on starch + xylan media due to the degradation pathway rather than the sugar consumption pathway.

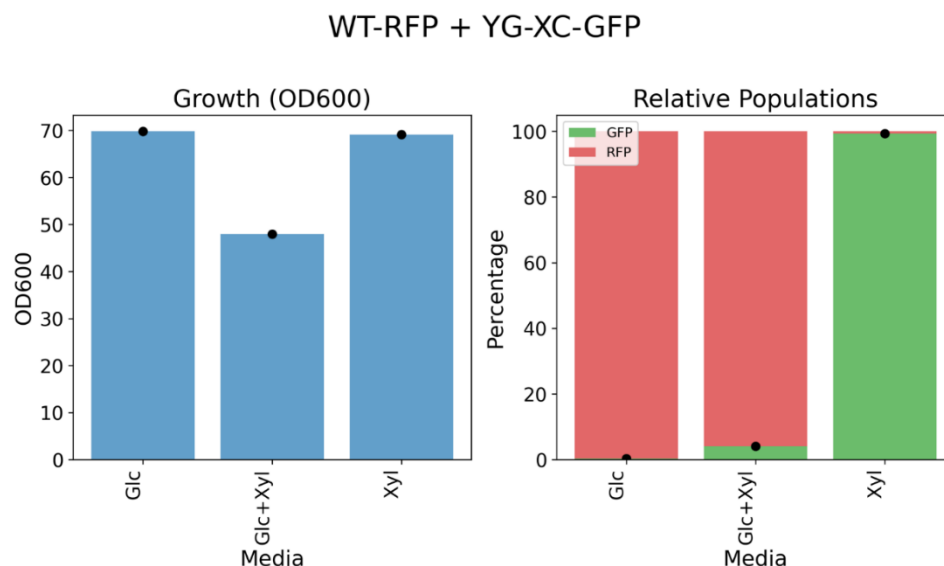


Figure 4.6 Growth and population dynamics for a 2-strain consortium of a glucose-consumer and a xylose-consumer

The WT-RFP (glucose-consumer) and the YG-XC (xylose-consumer) strains were inoculated at a 1:1 ratio, and the OD600 and flow cytometry data was taken after 72 hours. Media was prepared with 2% glucose (Glc), 2% xylose (Xyl) or a mixture of 1% glucose + 1% xylose YNB (Glc + Xyl). Here, the consortium achieves a similar final OD600 on both Glc and Xyl YNB media but has a lower final OD600 when grown on a mixture of the two substrates. WT and YG-XC strains both dominate the consortium in their respective sugar. In the mixture of the two sugars, the WT also dominates the consortium after 72 hours. N=1.

4.6. How different DOL strategies grow on different concentrations of starch + xylan media

Having looked at the growth and population dynamics of the DOL strategies, I then investigated how these DOL strategies might provide a benefit over a monoculture strain which degrades and

consumes both starch and xylan. The Y8TO1TA1XC monoculture strain expresses both amylase, OsAA, and xylanase, AnXlnD, and can consume both glucose and xylose.

In a monoculture designed to degrade both starch and xylan, every cell produces all the enzymes necessary to fully consume the two polysaccharide substrates. This could cause enough burden to make DOL worth it. But DOL comes with a cost of only producing one degradation enzyme per cell, compared to the two that the monoculture produces, which may give the monoculture an advantage just based on the balance between degradation and burden. However, another factor to consider is that the monoculture will produce both amylase and xylanase in fixed ratios, dependent on the promoter and SP combinations. On the other hand, rationally designed consortia, as demonstrated in the last section, can change their population composition, meaning they can adapt the ratio of the two degradation enzymes. In this section I investigate whether the three DOL strategies can achieve higher final OD600, or more stable and consistent growth, across a wide range of substrate mixtures than the monoculture strategy.

I prepared media with different concentrations of the two polysaccharides, with starch ranging from 0-2% and xylan ranging from 0-10%. In all consortia the strains were inoculated at a 1:1 ratio, and they were cultured in flasks for 72 hours. The OD600 was measured every 4 hours and for the consortia the populations of the two component strains was measured using the Attune flow cytometer. Figure 4.7 shows the growth of the monoculture (A), the generalist consumer consortium (B), the orthogonal specialist consortium (C) and the cross-feeding specialist consortium (D).

In 2% starch (no xylan) the monoculture grew better than any of the three the DOL strategies, to an average OD600 of 24.49 (A), while the three DOL strategies were 14.27 for the generalist-consumer (B) 15.10 for the orthogonal specialist (C) and 16.33 for the cross-feeding specialist (D). In 10% xylan (no starch) the monoculture and generalist consumer consortium reached OD600 166.60 and 170.47 respectively (a 2.32% increase with DOL); the orthogonal specialist consortium reached OD600 123.00 and the cross-feeding specialist consortium reached 43.63. The strains capable of consuming both glucose and xylose, as found in the monoculture and the generalist consumer consortium, had a growth advantage on xylan media alone. I surmise that this could be due to existing glucose in the media before any starch degradation occurs, as previously discussed. This theory is further supported by the fact that the cross-feeding specialist consortium had any growth on full-xylan media alone; as this consortium should only grow if there is both xylose and glucose feedstock available to support growth of the mutually-dependent strains.

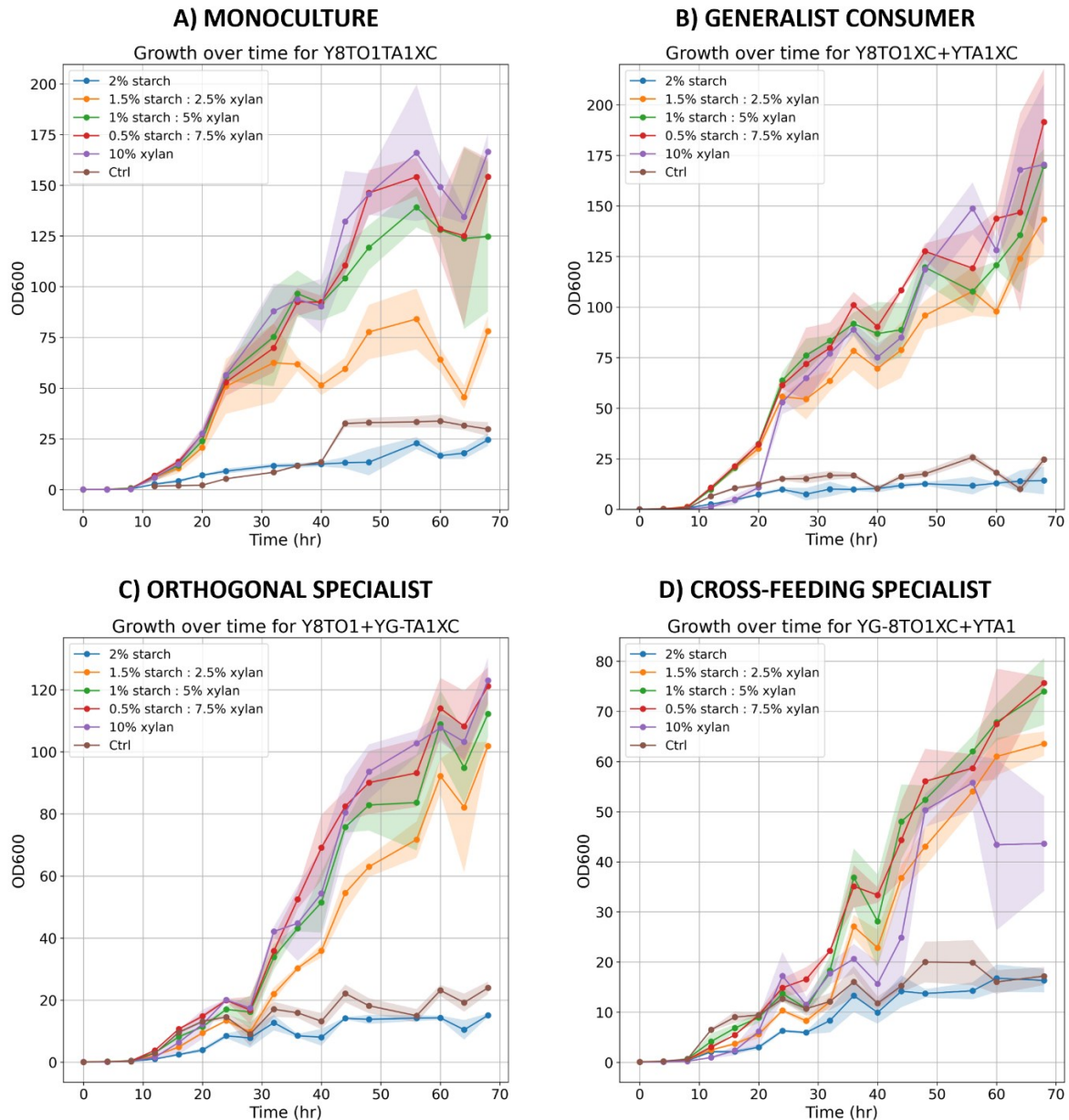


Figure 4.7 Growth curves for division of labour (DOL) strategies on different media compositions

Consortia with a 1:1 ratio of amylase strain:xylanase strain were grown on starch + xylan YNB media in flasks over 68 hours. **A)** The monoculture Y8TO1TA1XC growth was proportional to the amount of xylan in the media; it achieved the highest final OD600 in 10% xylan media and the lowest in 2% starch media. **B)** The generalist consumer consortium had the highest OD600 out of all the DOL strategies and higher than the monoculture. The 0.5% starch+7.5% xylan media gave the highest final OD600. The 10% xylan was the second highest but had a lower growth until 24 hours into the culture. The 1.5% starch+2.5% starch growth was improved compared to the monoculture strain. **C)** The orthogonal specialist consortium had the second highest growth of the different DOL strategies but was lower than the monoculture strain. The growth was proportional to the amount of xylan in the media, with the 10% xylan reaching the highest OD600 after 68 hours. Again, 1.5% starch+2.5% starch growth was improved compared to the monoculture strain. **D)** The cross-feeding specialist consortium had the worst growth of all the DOL strategies. However, unlike the other strategies the 10% xylan culture had the second lowest growth after 72 hours, although there is a period between 48 and 56 hours where it overtakes the 1.5% starch+2.5% starch media. Data presented as n=3 ; coloured region represents the standard deviation about the mean. Note: a different spectrophotometer was used in this experiment, making the OD600 non-comparable to other Figures.

In 1.5% starch + 2.5% xylan mixed media both the generalist consumer and the orthogonal specialist consortia had a higher final OD600, with 143.34 and 101.83 respectively, compared to the monoculture at 78.13. In 1% starch + 5% xylan and 0.5% starch + 7.5% xylan, only the generalist consumer consortium achieved a higher final OD600, with 169.80 and 191.53 respectively, than the monoculture, with 124.73 and 154.20 respectively. The generalist consumer consortium in these media concentrations demonstrated the highest growth of any of the DOL strategies on any of the mixed media.

Notably, the monoculture and the orthogonal consortium grew better the more xylan the media contained. In contrast, the generalist consumer and the cross-feeding consortium grew better when there was a mixture of starch and xylan, both growing best in the 0.5% starch + 7.5% xylan YNB media.

Figure 4.8 shows the change in population composition from the initial 1:1 inoculation ratios of amylase-expressing strain : xylanase-expressing strain, cultured over time for the generalist consumer consortium (A,B,C) the orthogonal specialist consortium (D,E,F) and the cross-feeding specialist consortium (G,H,I).

On 2% starch (no xylan), all three consortia behaved as I expected (A,D,G). For the generalist consumer, the strains stayed at roughly the same ratio throughout the culture. The slight oscillations were an artefact of the 3 replicates with and without a 12 hour delay; most likely they had some slight variation from the beginning. For both the orthogonal and cross-feeding specialist consortia, the first 12 hours showed a slight shift toward the strain that consumes xylose (YG-TA1XC or YG-8TO1XC). These strains should not be able to grow on starch, however before 12 hours the consortia were still in their lag phase, so this may be reflective of a lack of growth of the glucose-consumers rather than due to the tangible xylose-consumers growing. Indeed, from 12 hours onwards both these consortia had a strong shift in the opposite direction and plateaued at around 95% glucose-consumer (Y8TO1 or YTA1). This makes sense as only starch and therefore glucose is available.

On the mixture of 1% starch and 5% xylan, the generalist consumer consortium (B) showed some variation from the 1:1 ratio, shifting first towards xylanase-expressing YTA1XC for a short time, and then towards amylase-expressing Y8TO1XC. From 24 hours onwards it slowly recovered towards a more equal ratio, so that after 68 hours it was 6:4 in favour of Y8TO1XC. On this mixed media for both the orthogonal (E) and cross-feeding (H) specialist consortia the population dynamics were quite dramatic. Within 16 hours the population composition had shifted to be almost 95% glucose-consumer (Y8TO1 or YTA1). Then after 16 hours the population shifted again

in the opposite direction, plateauing at around 40 hours to a 8:2 or 2:8 ratio, favouring the xylose-consuming strain (YG-TA1XC or YG-8TO1XC).

The results for the 0.5% starch + 7.5% xylan and the 1.5% starch + 2.5% xylan mixtures is not shown here, since all the consortia showed similar dynamics to those already presented in Figure 4.8 (Appendix 9.10). The generalist consumer consortium on 1.5% starch + 2.5% xylan had similar dynamics to those on the 2% starch media, showing a relatively stable ratio of 1:1. On 0.5% starch + 7.5% xylan the generalist consumer consortium showed similar dynamics to those on 1% starch + 5% xylan. For both the 0.5% starch + 7.5% xylan and the 1.5% starch + 2.5% xylan, the orthogonal and cross-feeding specialist consortia showed the same dynamics as those for 1% starch + 5% xylan media.

For all three DOL strategies, the population dynamics on 10% xylan (no starch) looked very similar to the dynamics on the mixture of the two substrates (C,F,I). For the two specialist consortia, whose component strains cannot individually consume both sugars, there is a noticeable bump in the beginning, at around 20 hours, to the strain which consumes glucose (Y8TO1 or YTA1). Growing the WT strain on the 10% xylan mixture for 72 hours led to an OD₆₀₀ of 6.00. This confirms that there is glucose, or some other metabolizable carbon source, present in the xylan YNB.

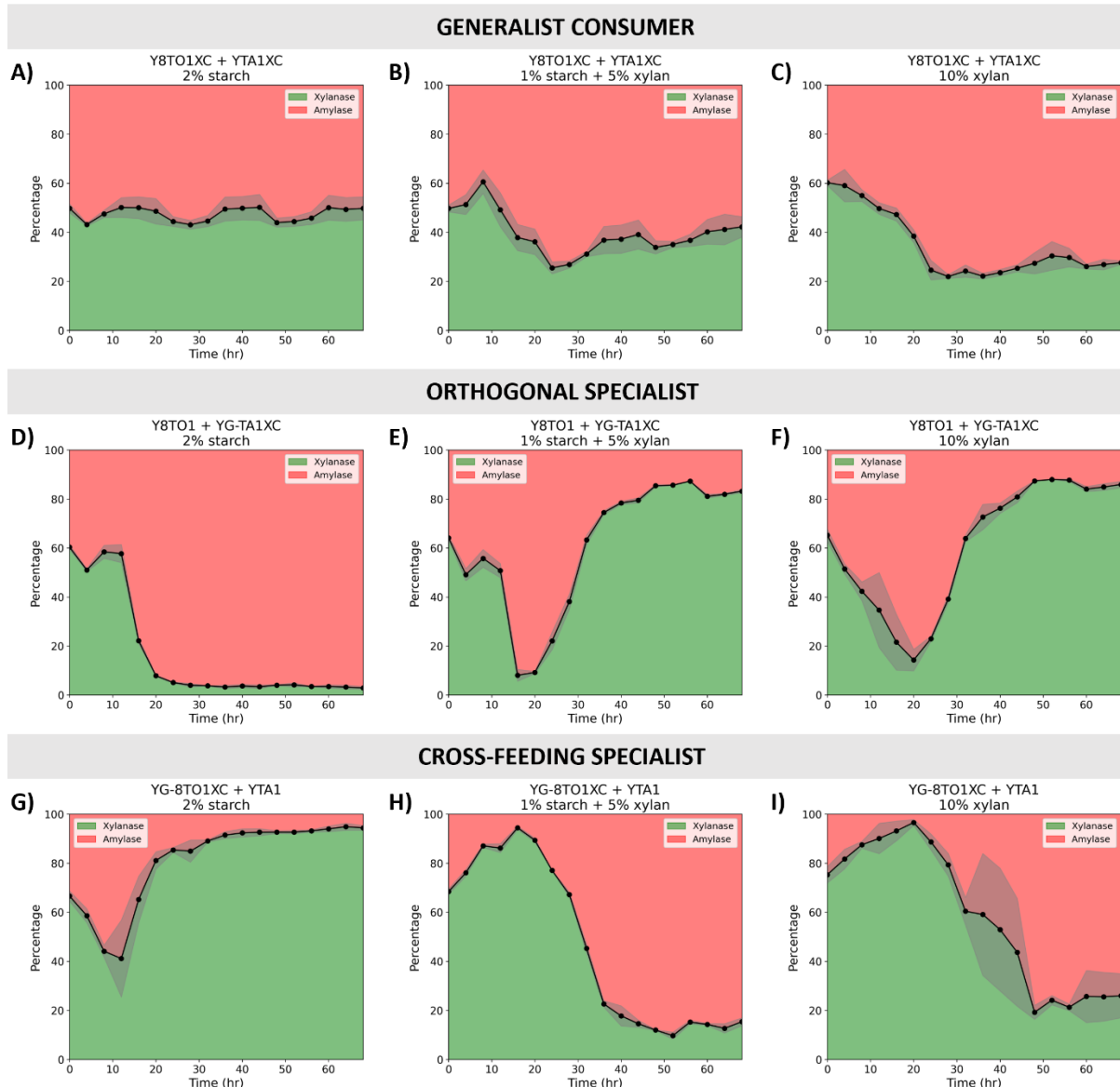


Figure 4.8 Population dynamics of different division of labour (DOL) strategies on different media compositions

Consortia with a 1:1 ratio of amylase strain:xylanase strain were grown on starch + xylan YNB media in flasks over 68 hours. Amylase strains have an RFP marker gene and xylanase strains have a GFP marker gene. The percentage of each fluorescence was calculated out of the total fluorescence events. The generalist consumer consortium has a stable 1:1 ratio in 2% starch (A), and favours the amylase strain, Y8TO1XC, in both a mixture of the two polysaccharides (B) and in 10% xylan (C). The orthogonal specialist consortium has 95% amylase strain Y8TO1 (glucose-consuming) by the end of the culture in 2% starch (D), and in both a mixture (E) and in 10% xylan (F) it shows first a shift towards the amylase strain Y8TO1 (glucose-consuming), and then a shift to the xylanase strain YG-TA1XC (xylose-consumer). The cross-feeding specialist consortium shows the opposite pattern to the orthogonal specialist in terms of the degradation strain (amylase/xylanase), but the same pattern in terms of the consumer (glucose/xylose). In 2% starch (G), the xylanase strain YTA1 (glucose-consuming) is 95% of the population by the end of the culture. In both a mixture (H) and in 10% xylan (I) it shows first a shift towards the xylanase strain YTA1 (glucose-consuming), and then a shift to the amylase strain YG-8TO1XC (xylose-consumer). Data is presented as the mean of $n=3$, where the grey region is the standard deviation about the mean.

4.7. The population dynamics observed for 10% xylan can be changed by first culturing with the WT strain

Following the repeatedly unexpected growth of glucose consuming strains on xylan substrate in the absence of starch I needed to try and eliminate any carbon sources other than xylan. To try and remove the carbon source that is providing feedstock for the glucose-consuming strains, I first prepared a batch of “pre-treated” xylan. 10% xylan YNB was prepared and inoculated with the WT strain. This culture was incubated for 5 days, to ensure any glucose, or other carbon source, present in the media is removed by the WT cells. The cells were spun down, and the media filtered, to create a xylan stock without any sugar source that can be metabolised by a WT strain. It is important to note that theoretically this stock may no longer be 10%, but my main aim was to obtain a “pure” batch of xylan. Additionally, it may now contain metabolites that have been secreted by the WT strain but probably in insufficient quantity to be problematic.

Using this new pre-treated xylan, I grew the monoculture and three consortia in flasks with 1% starch, 1% xylan and a mixture of 0.5% starch + 0.5% xylan YNB for 72 hours, measuring OD600 and relative populations (Guava flow cytometer). Figure 4.9 shows the growth curves and the growth parameters for the curves fitted to a Gompertz model are shown in Table 4.2. On 1% starch alone, the monoculture and all three consortia grew to an OD600 of around 5, the worst growth of the three media conditions (Figure 4.9A). In contrast, all strategies had the best final OD600 on 1% xylan alone (Figure 4.9C), with the mixed media (Figure 4.9B) falling somewhere in between the full-starch and full-xylan media. Again, the generalist consumer consortium, Y8TO1XC+YTA1XC, grew to the highest final OD600 overall, and it was the only DOL strategy to achieve a higher final OD600 than the monoculture in all three media conditions. Gompertz fitting predicted that in the mixed polysaccharide media, the generalist consumer consortium achieved a 12.55% increase in growth rate compared to the monoculture, although this difference was statistically insignificant (Table 4.2). The difference in predicted carrying capacity between monoculture and generalist consumer was statistically significant in all three media conditions.

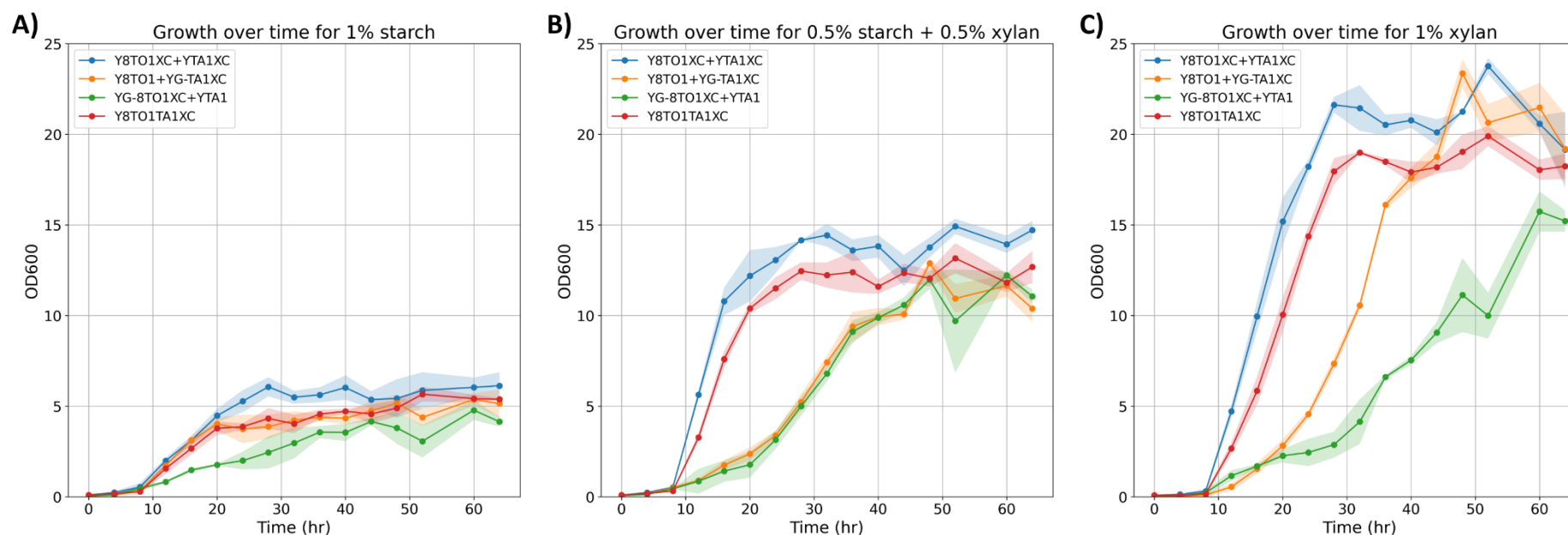


Figure 4.9 Growth of different division of labour (DOL) strategies on starch and pre-treated xylan media

Xylan was pre-treated by first culturing with a WT strain to remove any free glucose. Consortia with a 1:1 ratio of amylase strain:xylanase strain were grown on **A)** 1% starch **B)** 0.5% starch + 0.5% xylan or **C)** 1% xylan YNB media in flasks over 68 hours. The generalist consumer consortium (Y8TO1XC+YTA1XC) had faster and higher growth than the monoculture (Y8TO1TA1XC) on all three media compositions. The specialist consortia grew worse than the generalists in all conditions. The orthogonal specialist consortium (Y8TO1+YG-TA1XC) grew better than the cross-feeding specialist consortium (YG-8TO1XC+YTA1) in pure-sugar, 1% starch or 1% xylan, but the two specialists grew similarly on the mixture of two polysaccharides. The cross-feeding consortium was the only one where the mixture of polysaccharides had a faster growth than the 1% xylan media, although it did not achieve a higher final OD600 after 68 hours. Data is presented as the mean of $n=3$, where the coloured region is the standard deviation about the mean.

Table 4.2 Growth parameters for different division of labour (DOL) strategies grown on different media compositions

Media	DOL strategy	Carrying capacity	Growth rate	Inflection point
0.5% starch + 0.5% xylan	Y8TO1TA1XC	12.334	0.263	13.229
	Y8TO1XC+YTA1XC	13.934	0.296	11.760
	Y8TO1+YG-TA1XC	11.660	0.125	25.837
	YG-8TO1XC+YTA1	11.669	0.125	26.569
1% starch	Y8TO1TA1XC	5.086	0.130	13.478
	Y8TO1XC+YTA1XC	5.874	0.201	13.188
	Y8TO1+YG-TA1XC	4.823	0.157	12.532
	YG-8TO1XC+YTA1	4.514	0.084	19.450
1% xylan	Y8TO1TA1XC	18.875	0.193	16.957
	Y8TO1XC+YTA1XC	21.187	0.210	14.434
	Y8TO1+YG-TA1XC	21.653	0.136	28.433
	YG-8TO1XC+YTA1	29.216	0.038	49.442

In terms of growth rate, the orthogonal specialist consortium was the only strategy that had a lower growth rate on the mixture of polysaccharides than on either 1% starch or 1% xylan alone. All three other strategies had the highest growth rate on mixed polysaccharides, with the generalist consumer having the highest growth rate on all media.

The cross-feeding specialist consortium is the only DOL strategy that achieved a higher OD600 for the mixture of polysaccharides over the 1% xylan media at any point in its growth curve, which it did from 24 to 48 hours (Appendix 9.11). This is due to the mutual dependence of the two component strains, meaning they grow best when both starch and xylan are being degraded. Further, this DOL strategy did not reach stationary phase within 72 hours in the 1% xylan media. The Gompertz fitting predicted that it will plateau at a higher OD600 than any other DOL strategy, although without the data this is only an extrapolation. This is once again surprising because this DOL strategy should not survive when cultured in 1% xylan. In xylan media alone, the glucose-consuming YTA1 strain should not grow, and therefore should not be able to provide xylanase to create substrate for the xylose-consuming YG-8TO1XC. It is possible that there is still enough glucose in my pre-treated xylan media to allow the YTA1 strain to function. However, I think it is more likely due to the early secretion of the enzymes into the media. The YTA1 strain is present at the beginning of the culture, so it can secrete an amount of xylanase into the environment. This xylanase may then be capable of degrading xylan to feed YG-8TO1XC beyond the point at which YTA1 dies out for lack of glucose.

Figure 4.10 shows the change in the population dynamics of the three DOL strategies from the initial 1:1 inoculation ratios of amylase-expressing strain : xylanase-expressing strain, when using the pre-treated xylan media. The generalist consumer consortium (A, B, C) Y8TO1XC+YTA1XC maintained a relatively steady ratio of both strains in 1% starch, 1% xylan and the mixed polysaccharide media. Here both strains can eat both substrates, and in the full-starch and full-xylan media one strain can provide the degradation needed to support the other. Interestingly, I did not see any negative effect of the competition between these two strains; this generalist consumer consortium achieved the highest growth and neither strain dominated the consortium over time.

The orthogonal specialist consortium (D, E, F) and the cross-feeding specialist consortium (G, H, I) now had the expected population dynamics on full-xylan media, where the xylose-consuming strain (YG-TA1XC or YG-8TO1XC) dominated the consortium by the end of the culture. After around 20 hours both had 90%+ xylose-consumer. However, for the orthogonal specialist consortium, the glucose-consumer Y8TO1 did seem to recover slightly over time, with a final ratio at 72 hours of 2:8 in favour of YG-TA1XC. For the mixed polysaccharide media, the orthogonal and cross-feeding specialist consortia displayed the same pattern, with an adjustment towards the glucose-consumer at 12-16 hours, which was early in the exponential phase of growth. At 40-44 hours another adjustment occurred, which coincided with the time growth starts to plateau. Both specialist consortia plateaued with a population composition around 1:3 or 3:1 respectively, both in favour of the xylose-consumer. This again, supports my premise of starch/glucose being consumed first, and xylan/xylose consumed second.

Overall, the generalist consumer DOL strategy has the least changeable population dynamics, it will behave consistently regardless of the level of each substrate you give it. Meanwhile, the orthogonal and cross-feeding consortia both show adaptable populations dependent on the level of substrate available. Their population changes also suggested that the starch/glucose consumption occurs first, followed by the xylan/xylose consumption. Glucose metabolism is a natural feedstock for *Y. lipolytica*, and a highly favourable one, therefore it is not surprising that it is favoured over xylose. The sequential consumption of substrate also supports the idea that there is some catabolite repression, which explains the difference in growth of YTA1XC vs YG-TA1XC discussed in Section 4.5.

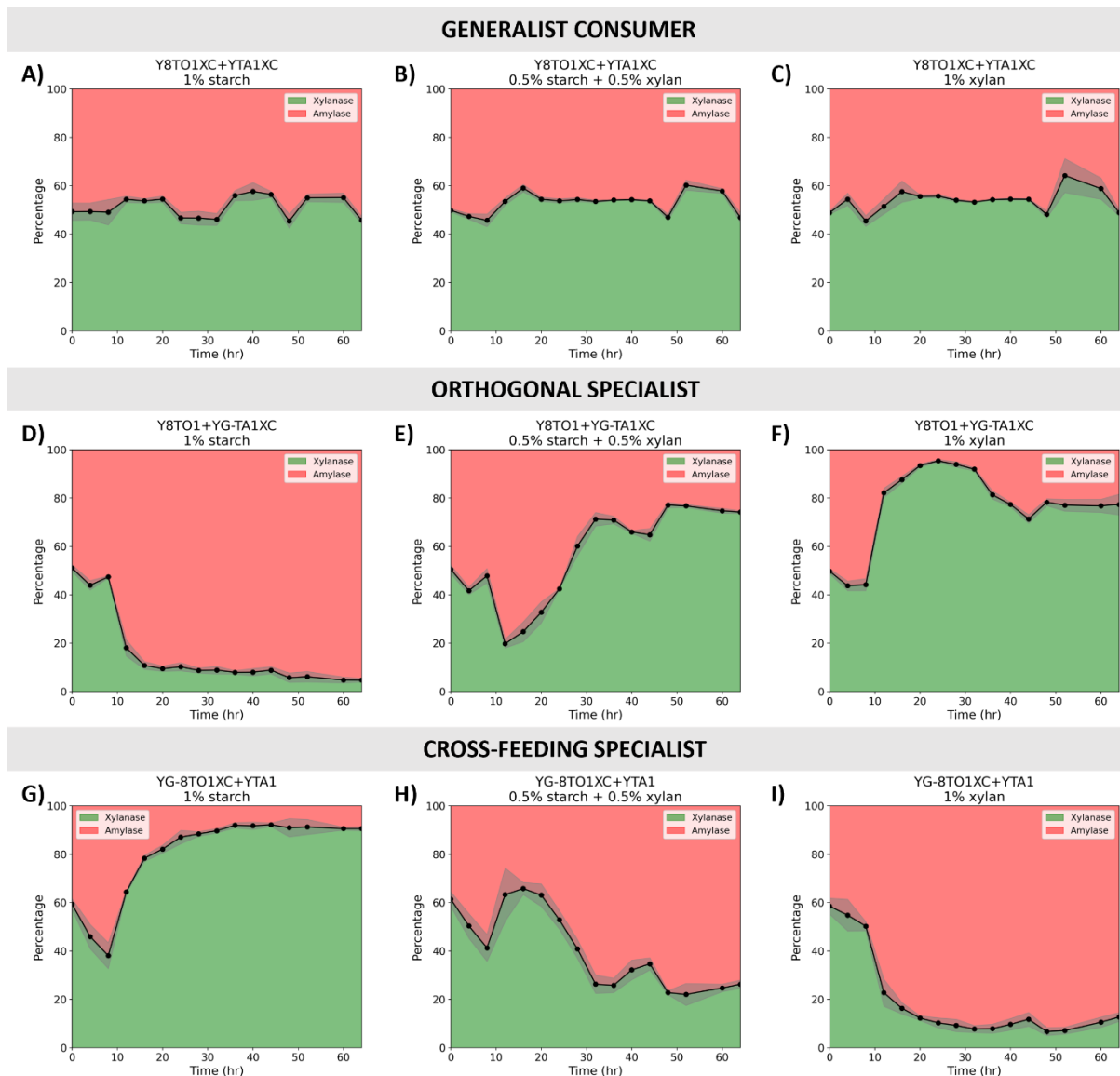


Figure 4.10 Population dynamics of different DOL strategies on starch and pre-treated xylan media

Xylan was pre-treated by first culturing with a WT strain to remove any free glucose. Consortia with a 1:1 ratio of amylase strain:xylanase strain were grown on starch + xylan YNB media in flasks over 68 hours. Amylase strains have an RFP marker gene and xylanase strains have a GFP marker gene. The percentage of each fluorescence was calculated out of the total fluorescence events. The generalist consumer consortium has a relatively stable 1:1 ratio in all media compositions (A,B,C). On 1% starch, both the orthogonal specialist consortium (D) and the cross-feeding consortium (G) have 90%+ of the glucose-consuming strain by the end of the culture. Similarly, on 1% xylan, both the orthogonal (F) and cross-feeding (I) specialist consortia have a majority xylose-consuming strain at the end of the culture, although the orthogonal specialist maintains slightly more of its glucose-consumer than the cross-feeder. In the 0.5% starch + 0.5% xylan media, both the orthogonal (E) and the cross-feeding (H) specialist consortia show a peak in the glucose-consumer at between 12 and 14 hours, and then begin to shift towards the xylose strain, with another slight peak of glucose-consumer at 44 hours. After 64 hours the orthogonal specialist consortium has a 1:3 ratio in favour of the YG-TA1XC strain and the cross-feeding consortium has a 3:1 ratio in favour of the YG-8TO1XC strain. Data is presented as the mean of $n=3$, where the grey region represents standard deviation about the mean.

4.8. How different DOL strategies respond to a change in the polysaccharide content midway through a culture

In the previous section I examined how the growth and population dynamics of the different DOL strategies varied depending on the composition of the media at the beginning of the culture. Only the generalist consumer consortium maintained an equal ratio of its two component strains at the end of the culture period on all media compositions. For the orthogonal and cross-feeding specialist consortia there was a sustained higher proportion of one strain than the other after around 30 hours. I wanted to see if this inequity could be recovered or would be exacerbated by adding either supplementary starch or supplementary xylan into the culture after the 30 hour mark.

The cultures were grown as before with 1:1 inoculation ratio of amylase strain : xylanase strain on 10 mL 0.5% starch + 0.5% xylan YNB media. A flow cytometry (Attune) measurement was taken at 40 hours, to confirm that the population composition achieved was equivalent to that previously attained at this timepoint in Section 4.7. Then 5 mL of either 1% starch YNB or 1% xylan YNB was added to the culture. While I am aware this diluted the culture, and indeed the OD600 reflects this, I wanted to add sufficient supplementary media to maximise any population shift.

The generalist consumer consortium, Y8TO1XC+YTA1XC, grew to the highest OD600 after 72 hours when more starch was added (Figure 4.11A), In contrast, the monoculture, Y8TO1TA1XC, achieved the highest OD600 with the addition of more xylan (Figure 4.11B), although it reached its peak OD600 at a later timepoint than the consortium. The generalist consumer consortium maintained its steady 1:1 population composition throughout the culture when either starch (Figure 4.11C) or xylan (Figure 4.11D) was added.

The orthogonal specialist consortium began the 40 hour timepoint having reached a 1:4 ratio of its component strains Y8TO1:YG-TA1XC, When starch is added it then showed a very small shift towards the amylase-expressing Y8TO1 strain (Figure 4.11E), so that the ratio by 72 and 144 hours was closer to a 2:3 ratio. In contrast, when xylan was added there was an increase in the xylanase-expressing YG-TA1XC strain at 44 hours, but this reverted back toward the 1:4 ratio as before (Figure 4.11F).

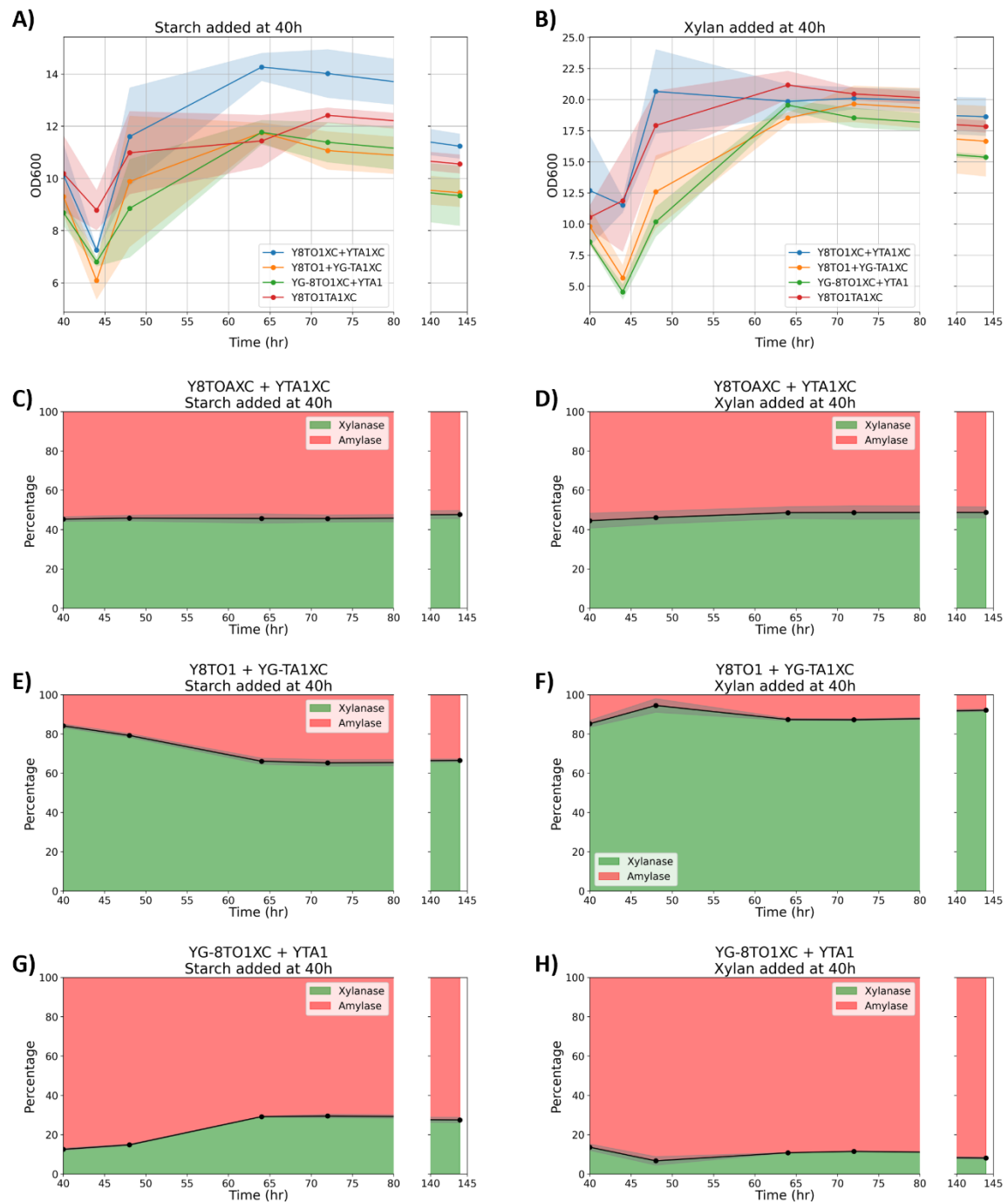


Figure 4.11 Changes in population dynamics after fresh polysaccharide medium is added

Consortia with a 1:1 ratio of amylase strain:xylanase strain were grown on 0.5% starch + 0.5% xylan YNB media for 40 hours. At the 40 hour timepoint a fresh batch of 1% starch (A,C,E,G) or 1% xylan (B,D,F,H) YNB was added. **A)** The growth of the different DOL strategies after 1% starch was added. **B)** The growth of the different DOL strategies after 1% xylan was added. **C)** and **D)** The generalist consumer consortium maintains its steady 1:1 ratio. **E)** The orthogonal specialist consortium, when starch is added, shifts towards Y8TO1. **F)** The orthogonal specialist consortium, when xylan is added, shifts slightly towards the YG-TA1XC. **G)** The cross-feeding specialist consortium, when starch is added, shifts towards YTA1. **H)** The cross-feeding specialist consortium, when xylan is added, shifts slightly towards the YG-8TO1XC strain. Data is presented as the mean of n=3, where the grey region represents standard deviation about the mean.

The cross-feeding specialist consortium began the 40-hour timepoint at a 9:1 ratio of YG-8TO1XC:YTA1. When starch is added this shifts to a 7:3 ratio in favour of the xylanase-expressing glucose-consuming YTA1 strain (Figure 4.11G). When xylan is added there is very little change in the population, with only a temporary and very slight shift towards the amylase-expressing xylose-consuming YG-8TO1XC strain at 44 hours (Figure 4.11H). So, after the addition of further xylan both specialist consortia do show a slight temporary variation in favour of their xylose-consumer 4 hours after supplementary xylan is added.

This was a smaller change in the population composition than I expected. However, I have repeatedly observed that over time the growth on starch is not as good as the growth on xylan, therefore it is possibly unsurprising that starch supply does not have a bigger impact on the population. I would have liked to try introducing a higher concentration of starch, but the potato starch became insoluble above 2% and began interfering with the OD600 measurements, and became difficult to wash out of the cells for the flow cytometry measurements.

4.9. How culture temperature affects the DOL strategies

Consortia are prized for their robustness to a variety of changing environmental conditions. I decided to explore the resilience of the monoculture and the DOL strategies to environmental stressors, firstly temperature. At temperatures diverging from the normal, metabolism may be obliged to be faster or slower, and additional proteins may be required to deal with heat or cold shock. This additional stress may make DOL more beneficial. I tested three different temperatures, 30°C the normal temperature for *Y. lipolytica* cultivation, one lower than normal at 23°C, and the other higher at 35°C (Materials and Methods 7.11.4).

At 23°C the monoculture, Y8TO1TA1XC, grew faster than any of the DOL strategies (Figure 4.12A), although the generalist consumer consortium, Y8TO1XC+YTA1XC, reached a similar final OD600 after 48 and 72 hours. At 30°C (Figure 4.12B) and 35°C (Figure 4.12C) the generalist consumer consortium grew to a significantly higher OD600 after 24 hours than the monoculture. Surprisingly it also grew to a higher OD600 after 72 hours at 35°C than at 30°C. In contrast, the growth of the orthogonal specialist consortium, Y8TO1+YG-TA1XC and cross-feeding specialist consortium, YG-8TO1XC+YTA1, was significantly worse at 35°C than at 30°C. At elevated temperatures, sugar transporters or metabolic enzymes may be differentially affected, which may exacerbate differences in the growth of the xylose-specialist and glucose-specialist. If one sugar metabolism is more significantly affected, the generalists can rely on their second sugar, whereas the specialists cannot change which metabolism they rely on.

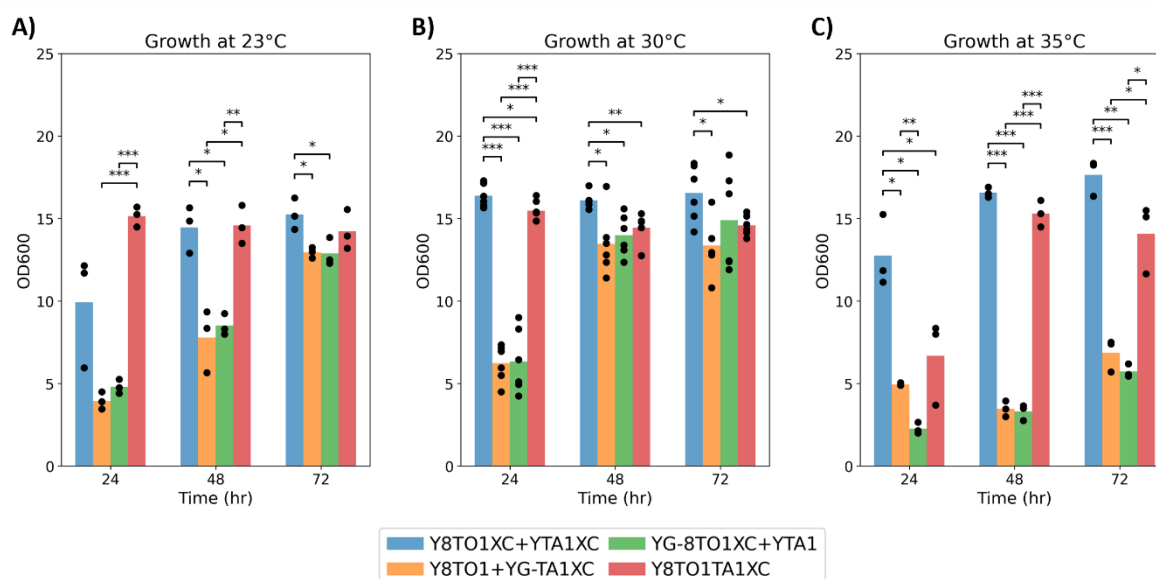


Figure 4.12 Different division of labour (DOL) strategies compared at different incubation temperatures

Consortia and the monoculture were incubated at **A) 23°C B) 30°C C) 35°C**. At 23°C the monoculture grew the fastest, reaching a higher OD600 than any DOL strategy at 24 hours. At 30°C the Y8TO1XC+YTA1XC generalist consumer consortium grew to a higher OD600 at all timepoints than the monoculture or either of the other DOL strategies. At 35°C the generalist consumer consortium achieved a higher OD600, but this was only a significant difference at 24 hours. Data is presented as the mean of $n=3$ (A,C) or $n=6$ (B). Statistical analysis by Welch's T-test; * represents a p -value < 0.05 , ** p -value < 0.005 and *** p -value < 0.0005 . Coloured region on the line graph represents the standard deviation about the mean at each timepoint.

4.10. How cells respond to oxidative stress caused by formate

Another stressor that consortia may provide a benefit for is toxicity. Currently the industrial use of lignocellulosic biomass involves pre-treatment with heat or chemicals, releasing several toxic byproducts such as acetic acid and formic acid (Du et al., 2022). Weak acids can disrupt cell growth and metabolism by inhibiting essential steps in the electron transport chain, causing oxidative and osmotic stress, and affecting the cellular pH (Zeng et al., 2022). I added sodium formate, to the 0.5% starch + 0.5% xylan YNB media to a final concentration of 35 g/L. All DOL strategies and the monoculture had significantly reduced growth with formate compared to without after 48 hours. With formate, the monoculture Y8TO1TA1XC had significantly higher OD600 than all three DOL strategies at all timepoints (Figure 4.13A). The monoculture also had the least change in growth with formate compared to growth without formate (Figure 4.13B). The generalist consumer consortium, Y8TO1XC+YTA1XC, had a lower change in growth at both 48 and 72 hours than either of the orthogonal or cross-feeding specialist consortia. The increased specialism towards one sugar consumption has reduced the robustness to toxicity stress. The

specialist consortia had the most unstable populations, and this may contribute to their lower robustness to perturbation.

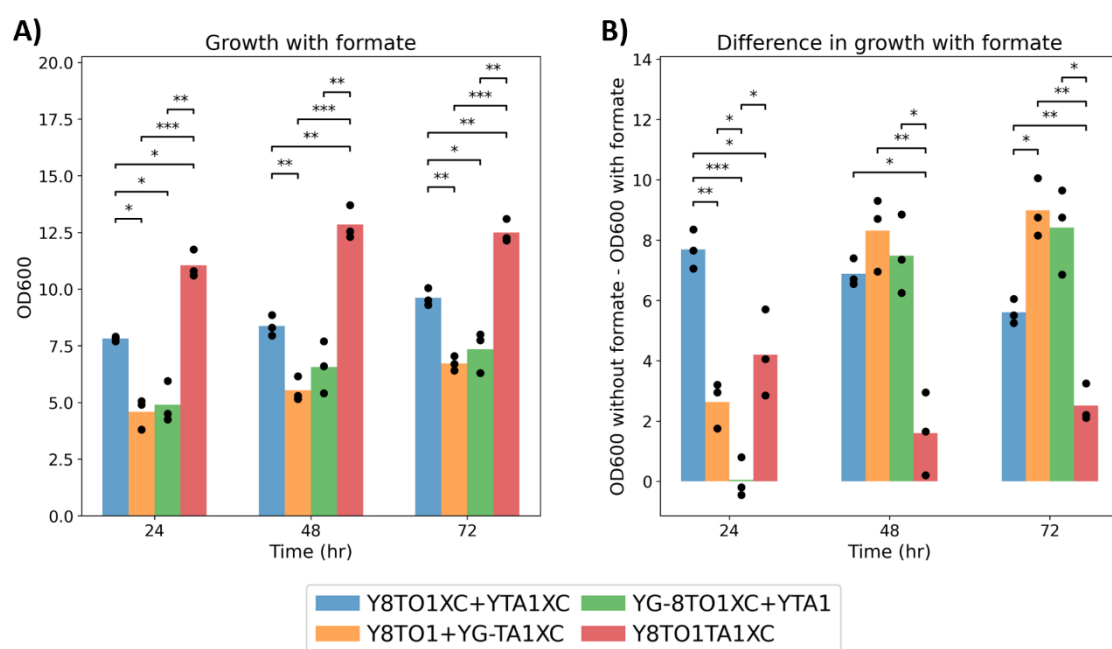


Figure 4.13 Different division of labour (DOL) strategies when under formate stress

Strains were grown for 72 hours in 0.5% starch 0.5% xylan YNB with sodium formate (35 g/L). **A)** The monoculture Y8TO1TA1XC achieved a higher OD600 than any of the DOL strategies. **B)** The difference in growth with formate was calculated by the OD600 without formate – OD600 with formate. The cross-feeding specialist consortium had the lowest difference after 24 hours, but the monoculture had the lowest difference at both 48 and 72 hours. The generalist consumer consortium had a lower difference than the two specialist DOL strategies. Data is presented as the mean of n=3. Statistical analysis by Welch's T-test; * represents a p-value < 0.05, ** p-value < 0.005 and *** p-value < 0.0005.

4.11. Using a stronger amylase may allow the strains to consume more starch, thus allowing similar growth in starch as in xylan

I have found that the xylanase strains on xylan grow better than the amylase strains on starch (Chapter 3, Section 3.5 compared to Section 3.10), and cultures with starch substrate contributed less to the final OD600 than those with xylan substrate (Section 4.6 and 4.7). So far in Chapter 4, I have exclusively used the amylase strains using OsAA, since I had not yet tested the AnGAMY strain developed in Chapter 3. In Chapter 3 Section 3.9 I showed that AnGAMY produced higher growth on starch than OsAA. I hypothesised that my observations of the DOL strategies developed in Chapter 4 would be different using AnGAMY.

Using the YTG1 strain, expressing AnGAMY, developed in Chapter 3, I engineered a strain YTG1XC, which expresses AnGAMY and metabolises both glucose (natively) and xylose (recombinantly). I combined YTG1XC+YTA1XC to make an AnGAMY+AnXlnD generalist consumer consortium, and I combined YTG1 + YG-TA1XC to make an AnGAMY+AnXlnD orthogonal specialist consortium (Figure 4.14). These consortia were grown on 0.5% starch + 0.5% xylan YNB with a 1:1 inoculation ratio of the two component strains, with OD600 and flow cytometry (Attune) measurements every 4 hours.

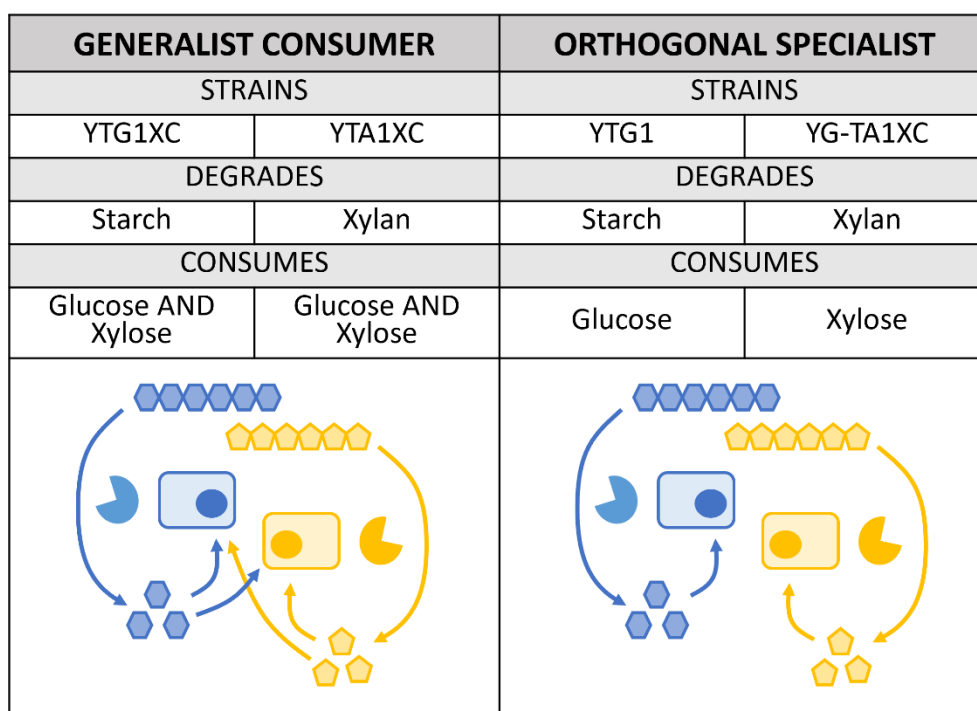


Figure 4.14 Division of labour (DOL) strategies for AnGAMY + AnXlnD consortia

The generalist consumer consortium and orthogonal specialist consortia perform the same function as the previous OsAA+AnXlnD consortia shown in Figure 4.2, but instead of the OsAA gene in the starch-degrader there is a stronger amylase AnGAMY gene.

Figure 4.15A shows the growth of the two AnGAMY+AnXlnD consortia. As in Sections 4.6 and 4.7, the generalist consumer consortium again grew better than the orthogonal specialist consortium. Compared to the growth, on the same mixed media, of the correspondent consortia with OsAA (Figure 4.9), the consortia with AnGAMY achieved a higher OD600. Indeed, at 60 mins, the ODs for the AnGAMY generalist consumer, YTG1XC+YTA1XC, and the AnGAMY orthogonal specialist, YTG1 + YG-TA1XC, is almost triple that for the correspondent OsAA consortia. So as I hypothesised the consortia with a component amylase-strain expressing AnGAMY do have better

growth on starch probably as a result of the improved degradation of starch achieved by the more active glucoamylase.

Figure 4.15B shows how the population composition of the two component strains in the AnGAMY+AnXlnD generalist consumer consortium, YTG1XC+YTA1XC, change over time. Unlike the generalist consumer with OsAA (Figure 4.10), the generalist consumer with AnGAMY does not maintain its 1:1 inoculation ratio (amylase strain : xylanase strain). Instead, it takes up and retains an approximately 3:7 ratio in favour of the xylanase-expressing YTA1XC strain after 12 hours, which is early in the exponential phase of growth. Increasing the activity of the amylase, and therefore the glucose available for both component strains to consume has caused the balance of the populations to shift. This movement in favour of the xylanase strain may be due to a difference in burden, which could affect the relative growth rates of the two strains. Expressing AnGAMY instead of OsAA may be more burdensome and therefore cause the amylase-strain to grow slower with AnGAMY.

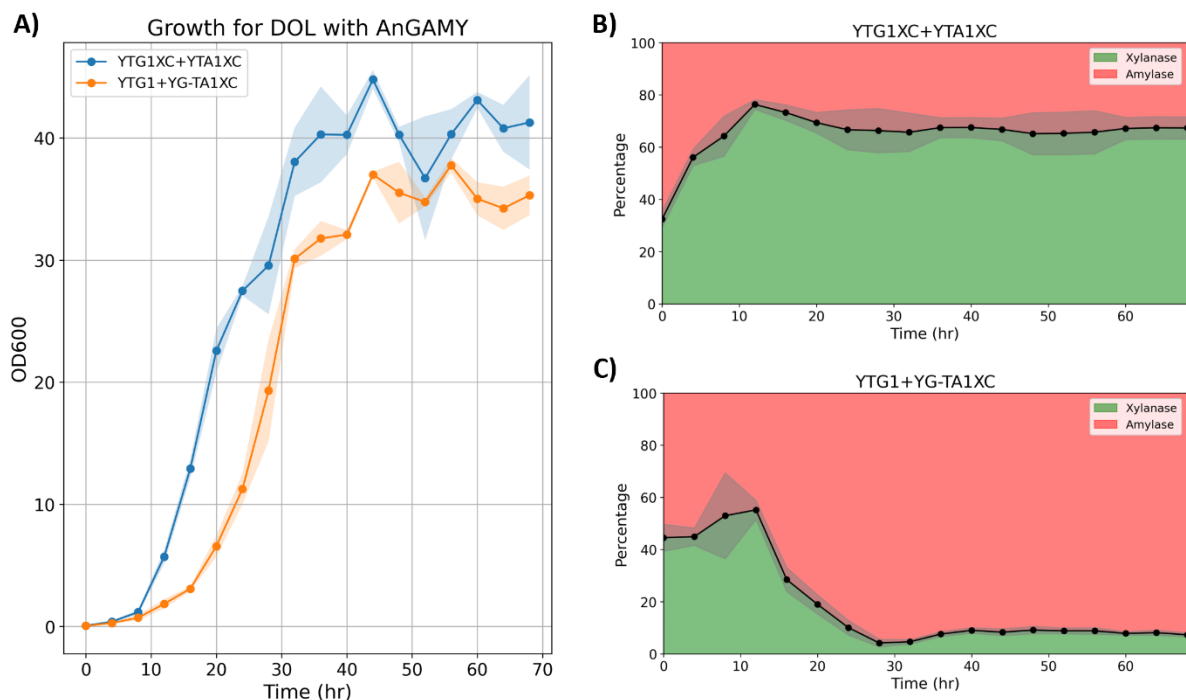


Figure 4.15 Growth of generalist consumer and orthogonal specialist consortia using AnGAMY for starch degradation

Two different division of labour (DOL) strategies were grown on 0.5% starch 0.5% xylan with a 1:1 ratio of the two strains. **A)** The growth of the two strategies over time. **B)** The generalist consumer consortium shifts the population ratio towards the xylanase YTA1XC strain. **C)** The orthogonal specialist consortium shifts the population ratio towards the amylase Y8TO1 strain. Data is shown as the mean of $n=3$, the shaded regions show the standard deviation around the mean.

Figure 4.15C shows how the population composition of the two component strains in the AnGAMY+AnXlnD orthogonal specialist consortium, YTG1+YG-TA1XC, change over time. Previously with OsAA, the orthogonal specialist ended with a dominance of the xylanase-expressing xylose-consuming YG-TA1XC strain by the end of the culture. Now with AnGAMY the opposite occurs, the amylase-expressing glucose-consuming YTG1 strain takes up and maintains 90% of the culture after 24 hours, which is about midway through exponential phase. With AnGAMY the glucose-consuming strain has access to more glucose than it did with OsAA, and since glucose is consumed first it has a growth advantage over the xylose-consuming YG-TA1XC strain. I might have expected that once the starch is exhausted the YG-TA1XC strain would begin to grow more than YTG1, but this does not seem to be reflected in the final population composition. It may be that more glucose is produced by AnGAMY than the strains require to reach stationary phase, and therefore the xylose is never required or consumed.

In order to determine a more specific cause for the population shifts in these consortia, I would want to repeat the experiments I performed with the OsAA consortia exploring cultures with different inoculation ratios of the two component strains, and also different ratios of the two substrates. This would help determine how much individually each strain and each substrate is contributing to growth. In addition I would need to measure the growth rate on either xylose or glucose alone, to see if a difference in growth rate accounts for the shift in population- if one strain has a higher growth rate it will naturally begin to take up more of the culture during the exponential phase.

4.12. Reducing catabolite repression may improve the growth of the orthogonal and cross-feeding strains

Earlier in Section 4.5 I introduced the possibility that catabolite repression is impacting the behaviour of the consortia, particularly the strains which cannot consume glucose. Here, I will be addressing that potential issue.

Carbon catabolite repression is a regulatory mechanism allowing the cell to take up a more favourable carbon source preferentially over a less favourable one. This is usually achieved by repressing genes for alternative substrate metabolism at the transcriptional level (Nair & Sarma, 2021). Catabolite repression has been demonstrated in *Y. lipolytica* (Zhou et al., 2020), however it is mainly attributed to transcriptional repression of xylose metabolism genes by glucose (Ryu, Hipp & Trinha, 2016). I have avoided transcriptional repression of the xylanase and xylose metabolism genes through the use of the pTEF constitutive promoter, which lacks the transcription factor binding sites required for catabolite repression.

As discussed, Figure 4.4 shows that, on mixed media, the xylanase-expressing xylose-consuming YG-TA1XC strain grown alone had significantly reduced growth compared to the xylanase-expressing xylose-and-glucose-consuming YTA1XC strain. However, I demonstrated that when YG-TA1XC was paired with an amylase strain in a consortium, its growth improved. I also established that there was some amount of free glucose present in the media from the beginning. One explanation for the poor growth of YG-TA1XC was because its growth was inhibited by the presence of glucose. To test this theory I looked at how YG-TA1XC and YG-8TO1XC, the two xylose-consuming strains which cannot metabolise glucose, grew on full xylose media and xylose-heavy media with variable amounts of added glucose.

After 1 week on 1% xylose YNB media alone, YG-TA1XC grew to a final OD₆₀₀ of 103.8, while YG-8TO1XC grew to a final OD₆₀₀ of 69.4. However, on a mixed media with 0.9% xylose + 0.1% glucose, YG-TA1XC and YG-8TO1XC grew to a final OD₆₀₀ of 7.28 and 16.54 respectively, on 0.7% xylose + 0.3% glucose they only grew to 0.78 and 2.22 respectively, and on a 0.5% xylose + 0.5% glucose they grew to 0.74 and 2.08 respectively. This demonstrates that glucose is inhibiting growth of these strains on xylose and supports the previous evidence for catabolite repression affecting the behaviour of the two populations in the DOL strategies. This means that the two consumptions are not truly orthogonal from one another- which means they will not have the advantages previously shown for DOL in sugar consumption (Verhoeven et al., 2018).

To eliminate catabolite repression, I attempted to improve both the xylose uptake and metabolism of all the xylose-consuming strains with directed evolution on media containing xylose as the sole carbon source. I grew the strains for eight passages and sampled them each week to determine if their growth on a mixture of xylose and glucose had improved. However, the growth of each strain on the mixed sugars decreased rather than increased. Potentially the directed evolution was reinforcing catabolite repression.

Having observed the phenotype of catabolite repression, with glucose inhibiting growth on xylose, but having eliminated repression of the xylose metabolism genes as the mechanism behind it, I looked at previous work reducing catabolite repression in yeast. Adaptive laboratory evolution has been used to improve growth on mixtures of xylose and glucose for *S. cerevisiae* (Lane et al., 2018) and *Y. lipolytica* (Zhou et al., 2020), but instead of glucose this research used a glucose analogue that could not be metabolised. Analysis of these evolved strains revealed mutations in glucose phosphorylating enzymes, and upregulation of xylose transport gene but no significant changes to xylose metabolism genes. This work informed my next steps. Since the parental strain (Po1d ΔHXT) I used to engineer the YG-TA1XC and YG-8TO1XC strains already has

glucose phosphorylating hexokinases deleted, that prevents glucose being metabolised, I identified the most likely mechanism for the catabolite repression I was observing was via the transport genes. By repressing transport genes, glucose can inhibit the uptake of xylose into the cell.

S. cerevisiae lacks specific pentose transporters, so to improve pentose metabolism, the hexose transporters can be mutated with one point mutation of an asparagine that reduces their affinity for glucose (Nijland & Driessen, 2020). I tried to express these *S. cerevisiae* mutated Hxt1 and mutated Gal2 in *Y. lipolytica*, but unfortunately was unsuccessful in attaining transformed colonies.

Unlike *S. cerevisiae* however, *Y. lipolytica* does have endogenous pentose transporters, most of which already have the same structural motifs as the mutated *S. cerevisiae* transporters (Ryu, Hipp & Trinha, 2016). Some of these transporters are transcriptionally repressed by glucose, however at least 2 of them, YALI0F19184g and YALI0A01958g, had no decrease in expression in the presence of glucose (Ryu, Hipp & Trinha, 2016). However, these transporters may not be sufficient to support growth on xylose when the other transporters are repressed; therefore expression of pentose transporters under a constitutive promoter may enable better utilisation of xylose in a mixture with glucose. Ryu & Trinh (2018) found that overexpressing *Y. lipolytica* TRP6 (YALI0C04730g) or TRP22 (YALI0B00396g) caused enhanced growth on xylose, with a 23% and 50% increase in the specific xylose uptake rates respectively. I successfully amplified YITRP22 from my Po1d strain, but I was not able to amplify YITRP6. YITRP22 was expressed under the control of a constitutive pTEF promoter to create strain YG-TRP22XC, thus removing catabolite repression from this pentose-specific transporter gene. This strain was compared to my negative control strain YG-XC, which cannot consume glucose, but can consume xylose, on a 9:1 ratio of xylose:glucose. After 72 hours YG-TRP22XC achieved a final OD600 of 4.42 compared to YG-XC with an OD600 of 3.28. However, this is still less than the OD600 achieved by the xylose-only-consumers on xylose alone, implying continued repression. It may require higher expression or multi-copies of this transporter gene to overcome the suppression of xylose uptake entirely. Unfortunately, this could not be completed within this project.

4.13. Conclusions for this chapter

In this chapter I applied DOL to degradation of two polysaccharides in *Y. lipolytica*, where one strain expresses amylase and the other expresses xylanase. I created three different designs of 2-strain consortia and tested to see how well they grew on mixtures of starch and xylan. I also observed the population dynamics of the two strains.

The cross-feeding specialist consortium achieved the lowest growth rate of all the DOL strategies tested. In this design, each strain is dependent on its consortium partner to derive its specific sugar substrate; one strain cannot grow without the other. Previous studies on cross-feeding systems with amino acids showed that this mutual dependence can increase consortium stability, preventing any single strain from dominating the culture, and often leads to higher growth rates than monocultures (Losoi, Santala & Santala, 2019; Pande et al., 2014). The mutual dependence of the cross-feeding specialist consortium was confirmed when neither strain could grow in isolation. However, when xylan feedstock alone was provided, unexpectedly some growth still occurred, likely due to xylanase enzyme being secreted into the media. Over 72 hours, however, unlike amino-acid based cross-feeding systems, this consortium was not stable. Instead, the populations fluctuated as the strains sequentially consumed the two substrates: the glucose consumer grew first, then the xylose consumer dominated.

A similar pattern of population dynamics was observed in the other specialist DOL strategy, the orthogonal specialist consortium. In this setup, each strain degrades and consumes its own specific sugar substrate, functioning independently of the other. The orthogonal consortium achieved higher growth than the cross-feeding consortium but overall behaved quite similarly. It showed a strong synergistic effect when the two strains were grown together compared to when each component strain was grown alone. The population dynamics mirrored those of the cross-feeding consortium, with the glucose-consumer initially increasing before the xylose-consumer eventually dominated.

Two main explanations account for the behaviour of these specialist consortia. First, the sequential substrate consumption likely results from catabolite repression of xylose transport genes: the presence of glucose suppresses the uptake of xylose. Both consortia adjusted their populations in response to this repression. This mechanism explains the synergy observed in the orthogonal specialist consortium, as small amounts of glucose in the xylan media caused immediate catabolite repression when the xylose-degrading strain was grown alone. Only when the glucose-consuming strain was present could this repression be relieved, enabling the xylose-consuming strain to grow. Though this interference limited the consortium's ability to achieve truly orthogonal sugar uptake, it demonstrated an advantage of consortia over monocultures: by removing the repressive glucose, the glucose-consuming strain enabled the growth of the xylose consumer. Engineering strategies to overcome catabolite repression can be time-consuming, and therefore DOL may provide a solution, even if the substrates must be consumed sequentially.

The second explanation specifically addresses why the xylose-consuming strain ultimately dominated the consortium populations. The xylanase enzyme was more potent than the amylase, as shown in Chapter 3; the YTA1XC strain reached an OD600 of around 25 on xylan (Figure 3.20), while the Y8TO1 strain reached an OD600 of around 5 on starch (Figure 3.12). Consistently, monocultures and all three DOL consortia achieved higher growth in xylan media than in starch media, with growth in mixed media falling between the two. When a stronger amylase enzyme (AnGAMY) was substituted, the dynamics shifted, and the glucose-consuming strain became dominant. Thus, the strength of the degradation enzyme being expressed determined which strain prevailed in the consortium.

The specialist consortia were also more susceptible to environmental perturbations in temperature and to the introduction of toxic byproducts than the generalist consortia or monoculture strategies. In nature, substrate specialists provide maximal efficiency in utilising a specific substrate and allow the consortium to thrive in environments where this substrate is plentiful. However, specialists are vulnerable to environmental changes or resource depletion since they rely heavily on specific resources being available or on consortium partners to support them in roles outside their metabolic scope. In contrast, substrate generalists may not be as efficient at degrading substrates, but they have flexible metabolic pathways that allow them to utilise multiple resources. In natural microbial consortia, generalist organisms may take on a more versatile role, performing a range of tasks that can complement the work of specialists. Generalists are therefore less susceptible to environmental changes (Xu et al., 2022). The results of Chapter 4 reflect this known ecological trade-off.

The generalist consumer strategy, where both strains could consume the products of both polysaccharides, glucose and xylose, proved to be the most promising DOL strategy overall. This strategy provided the highest final OD600 of all the DOL strategies and was the only one to provide higher growth than the equivalent monoculture co-expressing both amylase and xylanase. It was expected that this DOL strategy would not be stable over time, as the two strains compete for substrate. However, it was the most stable of the three DOL strategies, maintaining a 1:1 ratio consistently. Interestingly, when a stronger amylase (AnGAMY) was used this stability was not maintained- indicating that this stable ratio was a product of specifically OsAA+AnXlnD consortia. Since the OsAA+AnXlnD generalist consumer consortium maintained its stable ratio even when fresh media was added, and it grew to a higher density than the equivalent monoculture, it made a promising choice for bioproduction on mixtures of starch and xylan, which will be explored in the next chapter.

5. Chapter 5: Substrate degradation DOL applied to bioproduction of terpenoids

5.1. Introduction to this chapter

The previous chapters focussed purely on the degradation of alternative substrates. However, the final objective of this project was to identify if DOL in degradation would help improve bioproduction. In this chapter I focus on the bioproduction of two terpenoids, the sesquiterpene alcohol patchoulol and the tetraterpene β -carotene (Figure 5.1).

Patchoulol is a sesquiterpene alcohol and is the primary contributor to the distinctive scent of patchouli oil. The complexity and stereospecificity of fragrance molecules such as patchoulol, makes them ideal choices for biological or enzymatic production (Michailidou, 2023). In addition, patchoulol has been an ideal target for biomanufacturing, as the land and climate limitations of the patchouli plant (*Pogostemon cablin*) hinder the production of the oil from its natural source. Patchoulol production has been biologically engineered in *E. coli* (Zhou et al., 2021), *C. glutamicum* (Henke et al., 2018), and *S. cerevisiae* (Liu et al., 2021a). In 2014 Amyris and Firmenich launched Clearwood, a biotech-produced synthetic version of patchouli oil, where the patchoulol component was produced by engineered yeast (Firmenich, 2023; Michailidou, 2023).

β -carotene is a red-orange pigment, which can be used as a dye or food-colourant and is a precursor of vitamin A (retinol) in the human body, which is essential for vision, immune function, and skin health. There is significant market demand for new sources of β -carotene for use in dietary supplements, food fortification, and cosmetics. In response, bioengineering organisms to produce β -carotene has been successfully demonstrated in plants (Golden Rice), bacteria and yeast, including *Y. lipolytica* (Nair & Sarma, 2021; Larroude et al., 2018b). These successes provide a strong foundation and encourage further biotechnological developments and applications.

In this chapter, β -carotene and patchoulol are used as exemplar bioproduction molecules to test the different DOL strategies explored in the previous chapters. In Chapter 3, the starch-degrading consortia did not universally provide a significant benefit over their equivalent monoculture, but I questioned if the added burden of bioproduction would make DOL more favourable. In Chapter 4, I identified that the generalist consumer consortium of Y8TO1XC+YTA1XC could achieve higher growth than its equivalent monoculture and therefore may be capable of higher bioproduction. In addition, I wanted to ask if, independent of growth rate, reduced metabolic burden is in fact favourable for bioproduction. This is in response to my findings in Chapter 3 with

the 8UASpTEF-controlled strains (Section 3.2); where the strong 8UASpTEF promoter resulted in slower growth rate but also resulted in a higher carrying capacity. This chapter aims to address these questions and make a conclusion as to whether DOL in substrate degradation can achieve higher bioproduction.

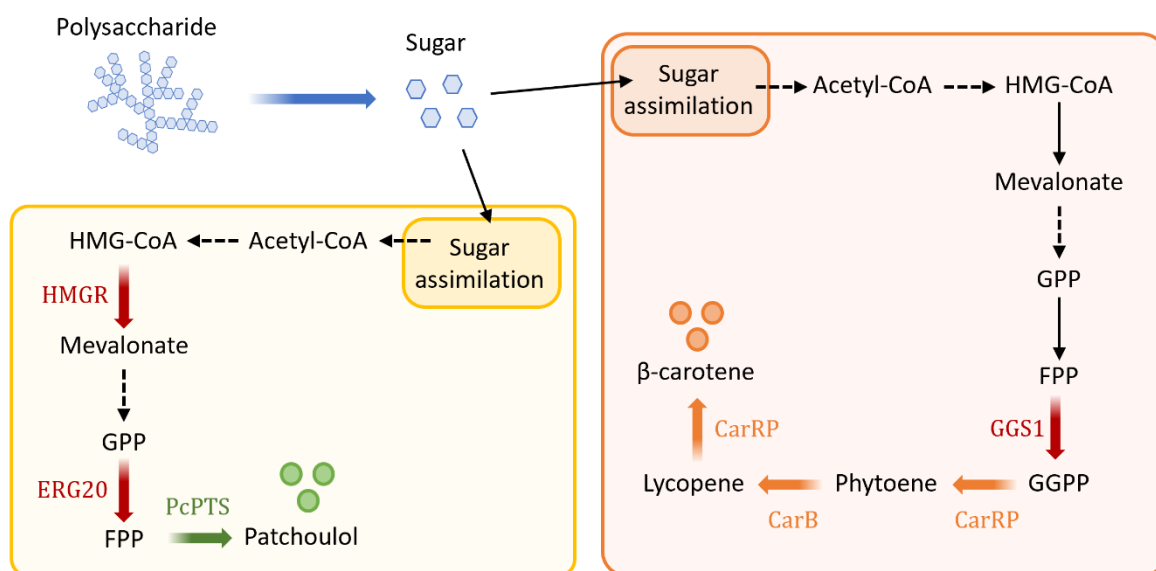


Figure 5.1 Metabolic pathways for patchoulol and β -carotene biosynthesis in engineered cells.

Glucose is converted into acetyl-CoA, which enters the mevalonate pathway through HMG-CoA to produce mevalonate via HMGR (3-hydroxy-3-methylglutaryl-CoA reductase). Mevalonate is further processed into GPP (geranyl pyrophosphate). The pathway branches at FPP (farnesyl pyrophosphate), where ERG20 (farnesyl pyrophosphate synthase) drives FPP formation. From FPP, patchoulol is synthesized (shown in green) by heterologous PcPTS (patchoulol synthase). For β -carotene synthesis (shown in orange), FPP is converted into GGPP (geranylgeranyl pyrophosphate) by GGS1. The pathway continues from GGPP through phytoene, lycopene, and β -carotene formation, mediated by the enzymes CarB and CarRP (carotenoid pathway enzymes).

5.2. Production of patchoulol on starch

In *Y. lipolytica*, to produce patchoulol, a single heterologous gene from the patchouli plant is required, a patchoulol synthase (PTS), however overexpression of genes upstream will improve production. PTS from *Pogostemon cablin* was codon-optimised for expression in *Y. lipolytica* and put under the control of the pTEF promoter (Materials and Methods 7.4.1 and 7.8). The patchoulol strains were grown on 2% glucose YNB in flasks for 72 hours. Patchoulol was extracted *in situ* with dodecane and sent for quantification by GC-MS (Materials and Methods 7.13.2). As shown in Figure 5.2A, the strain expressing a single copy of PTS produced 10.16 ± 0.31 (SD) mg/L of patchoulol. To improve the yield, I introduced multiple copies of the PTS gene as well as

increasing the mevalonate (MVA) pathway upstream by adding one or two extra copies of HMGR and ERG20, based on existing improvements in the literature for patchoulol production in *S. cerevisiae* (Liu et al., 2021a).

Figure 5.2A shows that on 2% glucose, the 2xHMGR-2xERG20-2xPTS (2xHEP) strain, which expresses two copies of each gene, was the most effective, showing the highest patchoulol concentration of 16.94 ± 1.16 mg/L and a high patchoulol/OD600 ratio. Of note, the 3xPTS strain, despite having the highest patchoulol/OD600 ratio, had a lower overall patchoulol concentration and biomass compared to 2xHEP. In fact, increasing the copy number of PTS, without any of the upstream genes, demonstrated a noticeable decrease in the OD600, indicating that the overexpression of this gene, or the production of patchoulol itself is toxic to the cells.

Next the HMGR-Erg20-PTS (HEP) and the 2xHEP strains were engineered further to contain various combinations of OsAA and TlGAMY for growth on starch. Unfortunately, the Y8TT1-2xHEP strain could not be transformed. Therefore, the Y8TO1-HEP and Y8TT1-HEP strains were used to compare the bioproduction of patchoulol from either single- or double-amylase monocultures or a consortium of amylase-expressing strains. The consortium Y8TO1-HEP+Y8TT1-HEP was prepared with 50:50 ratio of Y8TO1-HEP : Y8TT1-HEP in 10 mL of 1% starch, and compared to the equivalent monoculture Y8TT18TO1-HEP.

In the first experiment attempting to produce patchoulol with starch, I observed practically no growth. Since patchoulol is volatile, it is important to ensure the growth containers are sealed to prevent any of the product escaping. However, as discussed in Chapter 3, *Y. lipolytica* is an obligate aerobe, and sealing the growth container may be preventing sufficient oxygen for the growth previously seen. To combat this, I allowed the cultures 16 hours to grow prior to adding the dodecane and sealing the container.

Figure 5.2B shows that the double-amylase monoculture Y8TT18TO1-HEP had both higher patchoulol concentration, and higher patchoulol/OD600 ratio than the consortium of Y8TO1-HEP+Y8TT1-HEP, despite both achieving similar OD600. The monoculture of single-amylase Y8TO1-HEP alone had the highest OD600, consistent with previous results in Chapter 3, however its patchoulol concentration was lower than the monoculture Y8TT18TO1-HEP.

In all cases the concentration of patchoulol produced on starch was very small, only 0.72 ± 0.07 mg/L for the Y8TT18TO1-HEP strain. Therefore, in view of the findings on 2% glucose I transformed the 2xHEP strain to express OsAA and co-express OsAA and TlGAMY (Figure 5.2C). This helped improve the patchoulol concentration slightly, with the double-amylase

monoculture Y8TT18TO1-2xHEP strain producing 1.01 ± 0.0008 mg/L of patchoulol on starch as the sole carbon source. However, these are not close to being industrially relevant amounts. As previously stated, I was unable to obtain a Y8TT1-2xHEP strain, so I was unable to make direct comparisons with the relevant consortium.

Figure 5.2B and Figure 5.2C show that the OD600 of these strains was only 0.1-0.4 after 72 hours. This is more than a 10x reduction in growth on starch compared to that observed with no bioproduction in Chapter 3. This may be due in part to the toxicity of patchoulol, indicated in Figure 5.2A. Since the growth was so low, I decided to move onto β -carotene as an alternative molecule of interest, since it did not require the same anaerobic *in situ* extraction method as a volatile molecule like patchoulol.

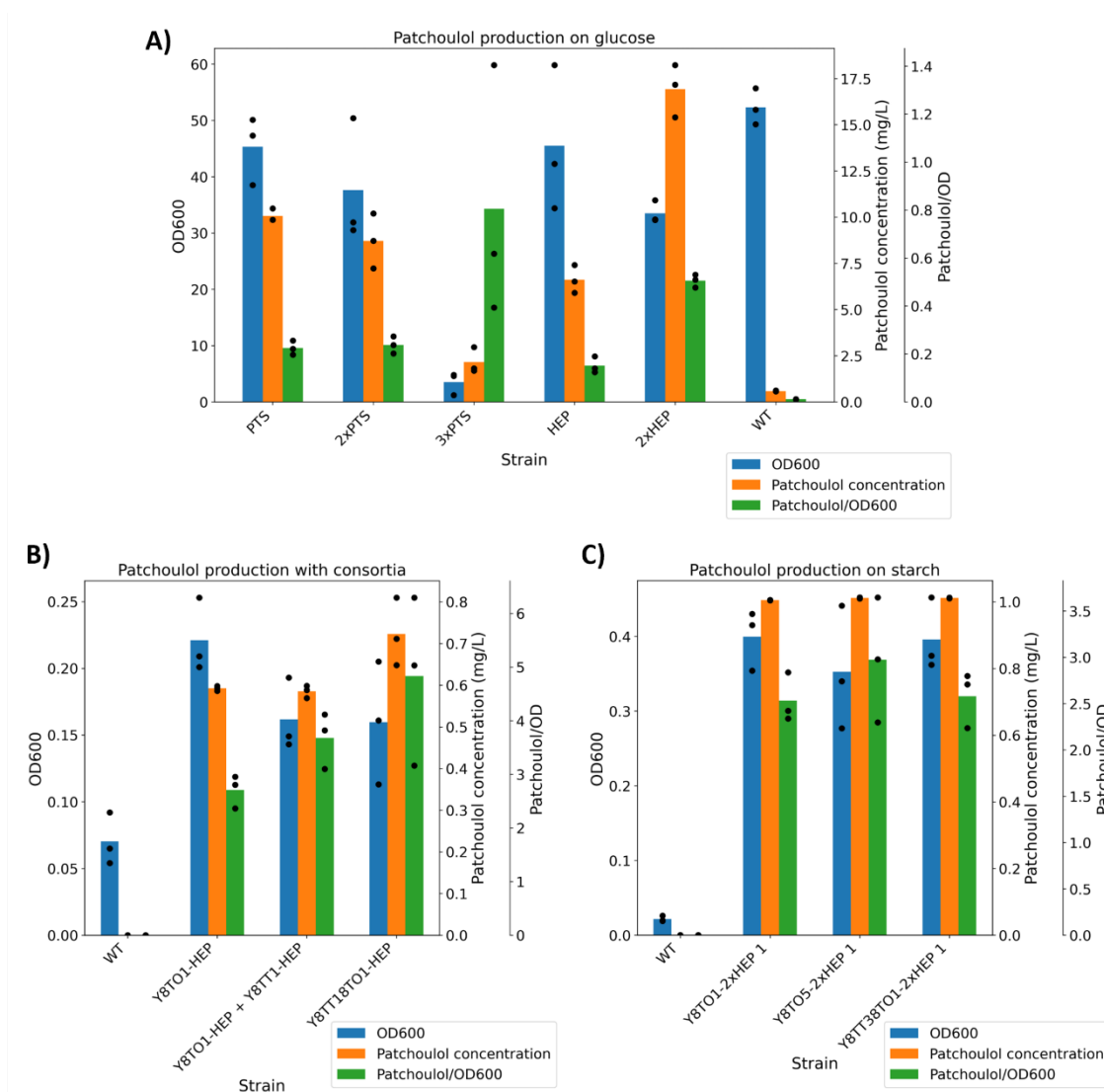


Figure 5.2 Patchoulol production

Strains were grown for 72 hours on A) 2% glucose YNB and B & C) 1% starch (from potato) YNB. Patchoulol was extracted *in vivo* using a dodecane layer and sent for GC-MS. **A)** Increasing the number of copies of PTS significantly decreases the OD600. Addition of one copy of upstream genes did not improve patchoulol concentration. The highest patchoulol concentration was produced by the strain expressing two copies of all 3 genes: HMGR, Erg20 and PTS. **B)** The T8TT1-HEP+Y8TO1-HEP consortium produced less patchoulol than the Y8TT18TO1-HEP monoculture. **C)** The best plasmid constructs for starch degradation were added to the 2xHMGR-ERG20-PTS (2xHEP) strain to create strains which can produce patchoulol using starch as the sole carbon source. n=3.

5.3. Consortia of one or multiple starch-degrading strains combined with an optimised β -carotene-producer strain

In Chapter 3, I observed that a consortium of OsAA+TlGAMY could achieve a higher growth rate and carrying capacity than an equivalent monoculture. However, I also observed that the TlGAMY strain contributed little to this growth, instead the OsAA supported growth while TlGAMY benefitted from the glucose provided. In effect the TlGAMY strain acts as a cheater exploiting the system to its advantage. While this was disappointing from the perspective of the synergy of these two enzymes, it did reveal an opportunity. If a cell which contributes nothing to starch degradation is combined with the OsAA strain, the resulting consortium may achieve a higher growth than a monoculture equivalent. The cheater cell in this case could perform a different function, such as bioproduction of β -carotene.

To test this hypothesis, I first assessed a selection of the single-amylase strains, double-amylase strains and consortia from Chapter 3, combining them with a high-producer of carotene, *Y. lipolytica* ob-CHC^{TEF}C^{TEF} (OB-C) published in Larroude *et al.*, 2018. This created starch-degrader + β -carotene producer consortia. In the case of one amylase strain + OB-C, they were inoculated in a 1:1 ratio. In the case of two amylase strains they were inoculated in a 2:1:1 ratio of OB-C:amylase:amylase, so the total amylase: β -carotene strain was 1:1. The strains were grown for 72 hours on 1% starch (potato) YNB, and β -carotene was extracted by homogenisation and quantified with HPLC (Materials and Methods 7.13.1).

Figure 5.3A shows that the combination of single-amylase (AnGAMY) and β -carotene strains, YTG1+OB-C, produced the highest amount of β -carotene at 6.493 mg/L and demonstrated the greatest growth, with an OD600 of 29.03, making it the best strain for overall production. However, when looking at efficiency in Figure 5.3B, the combination of double-amylase (TlGAMY+OsAA) Y8TT18TO1+OB-C yielded the highest β -carotene per cell density with a ratio of 0.527 β -carotene/OD600, indicating it produces β -carotene more efficiently relative to its growth.

To see if DOL within the starch-degradation pathway had any effect on bioproduction, I compared the consortia YTT3+YTO1 and Y8TT1+Y8TO1 with their single-amylase and double-amylase equivalents. The double-amylase strains (YTT3TO1 or Y8TT18TO1)+OB-C had a similar β -carotene/OD600 value as their equivalent OsAA-single-amylase strain (YTO1 or Y8TO1)+OB-C, while the consortia (YTT3+YTO1 or Y8TT1+Y8TO1)+OB-C had lower β -carotene/OD600 (Figure 5.3B). Unfortunately, I did not obtain the YTG18TO1 strain from Chapter 3 until later in the project, so there is no comparison between consortia and monoculture for the AnGAMY strains, but the

results from Chapter 3 (Figure 3.17) suggest that YTG18TO1 would have behaved similarly to YTG1.

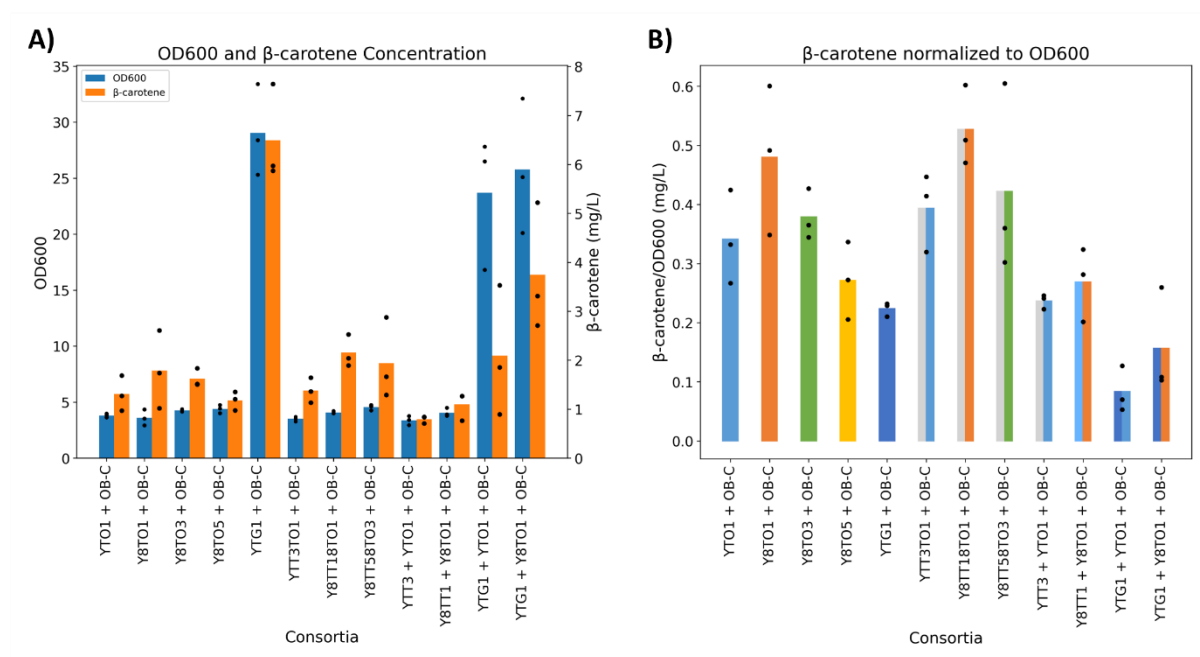


Figure 5.3 Bioproduction from consortia of amylase-expressing strains and a β -carotene overproducer

A) β -carotene production (mg/L) and OD600 (cell growth) for various strain combinations with OB-C. **B)** β -carotene production normalised to cell growth. YTG1 + OB-C exhibits the highest β -carotene production (6.493 mg/L) and OD600 (29.03), indicating strong carotene output and growth. In contrast, YTG1 + Y8TO1 + OB-C shows moderate β -carotene production (3.747 mg/L) with slightly lower OD600 (25.77), while Y8TO1 + OB-C has one of the lowest OD600 values (3.6) but higher β -carotene production (1.79 mg/L) relative to its growth. Data presented as the mean of n=3.

5.4. Comparing a degrader + producer consortium to an equivalent monoculture

I now needed to explore whether this starch-degrader + β -carotene-producer DOL strategy would be an improvement over a monoculture that both degrades starch and produces β -carotene.

B-carotene production in *Y. lipolytica* can be achieved by overexpression of three genes, the endogenous geranylgeranyl diphosphate synthase (GGs1), and the heterologous phytoene synthase/lycopene cyclase and phytoene dehydrogenase (carPR and carB from *Mucor circinelloides*) (Larroude et al., 2018a). I engineered the amylase strains created in Chapter 3 to express these β -carotene genes (CRT) under the control of the strong 8UASpTEF promoter. B-carotene strains engineered for this chapter are summarised in Table 5.1.

Table 5.1 β -carotene-producing strains engineered for this chapter

Strain	Insert/s
For TlGAMY+OsAA consortia	
WT-CRT	8UASpTEF-(GGs1-CarB-CarRP)
YTO5-CRT	1. pTEF-SP5-OsAA 2. 8UASpTEF-(GGs1-CarB-CarRP)
YTT5-CRT	1. pTEF-SP5-TlGAMY 2. 8UASpTEF-(GGs1-CarB-CarRP)
YTT5TO5-CRT	1. pTEF-SP5-TlGAMY-pTEF-SP5-OsAA 2. 8UASpTEF-(GGs1-CarB-CarRP)
Y8TO1-CRT	1. 8UASpTEF-SP1-OsAA 2. 8UASpTEF-(GGs1-CarB-CarRP)
Y8TT1-CRT	1. 8UASpTEF-SP1-TlGAMY 2. 8UASpTEF-(GGs1-CarB-CarRP)
Y8TT18TO1-CRT	1. 8UASpTEF-SP1-TlGAMY-8UASpTEF-SP1-OsAA 2. 8UASpTEF-(GGs1-CarB-CarRP)
OB-C	Engineered in Larroude et al., 2018
For OsAA+XlnD consortia	
Y8TO1XC-CRT	1. 8UASpTEF-SP1-OsAA 2. pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK 3. 8UASpTEF-(GGs1-CarB-CarRP)
YTA1XC-CRT	1. pTEF-SP1-XlnD 2. pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK 3. 8UASpTEF-(GGs1-CarB-CarRP)
Y8TO1TA1XC-CRT	1. pTEF-SP1-XlnD 2. 8UASpTEF-SP1-OsAA 3. pTEF-yIXDH-pTEF-yIXR-pTEF-yIXK 4. 8UASpTEF-(GGs1-CarB-CarRP)

First, I introduced the β -carotene-expression cassette into the single-amylase (OsAA) expressing Y8TO1 strain, creating strain Y8TO1-CRT, and I created an equivalent non-amylase-expressing β -carotene producer, WT-CRT. Figure 5.4A shows that the monoculture Y8TO1-CRT produces more β -carotene than the consortium of Y8TO1+WT-CRT. In the consortium the two strains must share resources and only half the cells produce β -carotene. So DOL in this way does not improve production.

Instead, I considered a consortium where both cells can produce β -carotene, but only one can degrade starch, which I achieved by combining Y8TO1-CRT+WT-CRT. In theory the WT-CRT strain in this consortium would act as a cheater cell, allowing it to focus solely on production, without having to contribute to starch-degradation. Figure 5.4B shows that in this consortium, as the proportion of WT-CRT increases, the more β -carotene is produced, but the OD600 remains the same. This allows the consortium to produce more β -carotene than the Y8TO1-CRT strain alone.

Next, I engineered the double-amylase Y8TT18TO1 strain, to create a consortium of Y8TT18TO1-CRT+WT-CRT. Figure 5.4C shows that in this case the Y8TT18TO1-CRT strain alone produces the most β -carotene and has the highest OD600. Here, as the proportion of WT-CRT increases the less β -carotene is produced and the OD600 declines. It may be in this case that the addition of the cheater WT-CRT reduces the starch-degradation that can be achieved, possibly by competing with Y8TT18TO1-CRT for glucose.

One issue with this experiment is that the β -carotene cassette is introduced with random integration, and therefore the β -carotene production from Y8TT18TO1-CRT might be more than the carotene production in WT-CRT, simply due to being integrated in a better site in the genome. The other issue is the stability of the carotene cassette over time. I noticed at the end of the culture when I spun the cells down, that there were white cells (not β -carotene producing) in the cell pellet and the proportion of white cells was different between the different strains. So, random integration may also affect how stable the carotene cassette is.

To try and combat the instability of the cassettes, in all future strains, I used directed evolution by growing three generations of each strain on starch and selecting cells which remained orange. This gave me improved versions of each strain which consistently remained orange after 72 hours of culture.

5.5. Applying the DOL strategy of a 2-strain starch-degrading consortium to bioproduction of β -carotene

The next step was to implement the 2-strain consortia designed in Chapter 3 to create a consortium where all cells are β -carotene producers but differ in the amylase they express. This means taking the strains from Chapter 3 and introducing the CRT cassette. I chose the YTT5+YTO5 and the Y8TT1+Y8TO1 combination of SPs, because YTT5+YTO5 had a lower growth rate than its equivalent monoculture (Figure 3.7), while Y8TT1+Y8TO1 had a higher growth rate than its equivalent monoculture (Figure 3.8). In some cases, for bioproduction, a lower growth rate might be desirable as it consumes the limited resources slower, leading to a higher accumulation of bioproducts.

Figure 5.4D shows that, like the results in Chapter 3, the YTT5TO5-CRT monoculture outperformed the consortium of YTT5-CRT+YTO5-CRT. However, unlike in Chapter 3, the Y8TT1-CRT+Y8TO1-CRT consortium did not reach a higher final OD600 than its equivalent monoculture, and both the consortium and monoculture had similar β -carotene production. Hence, DOL in this way did not improve bioproduction.

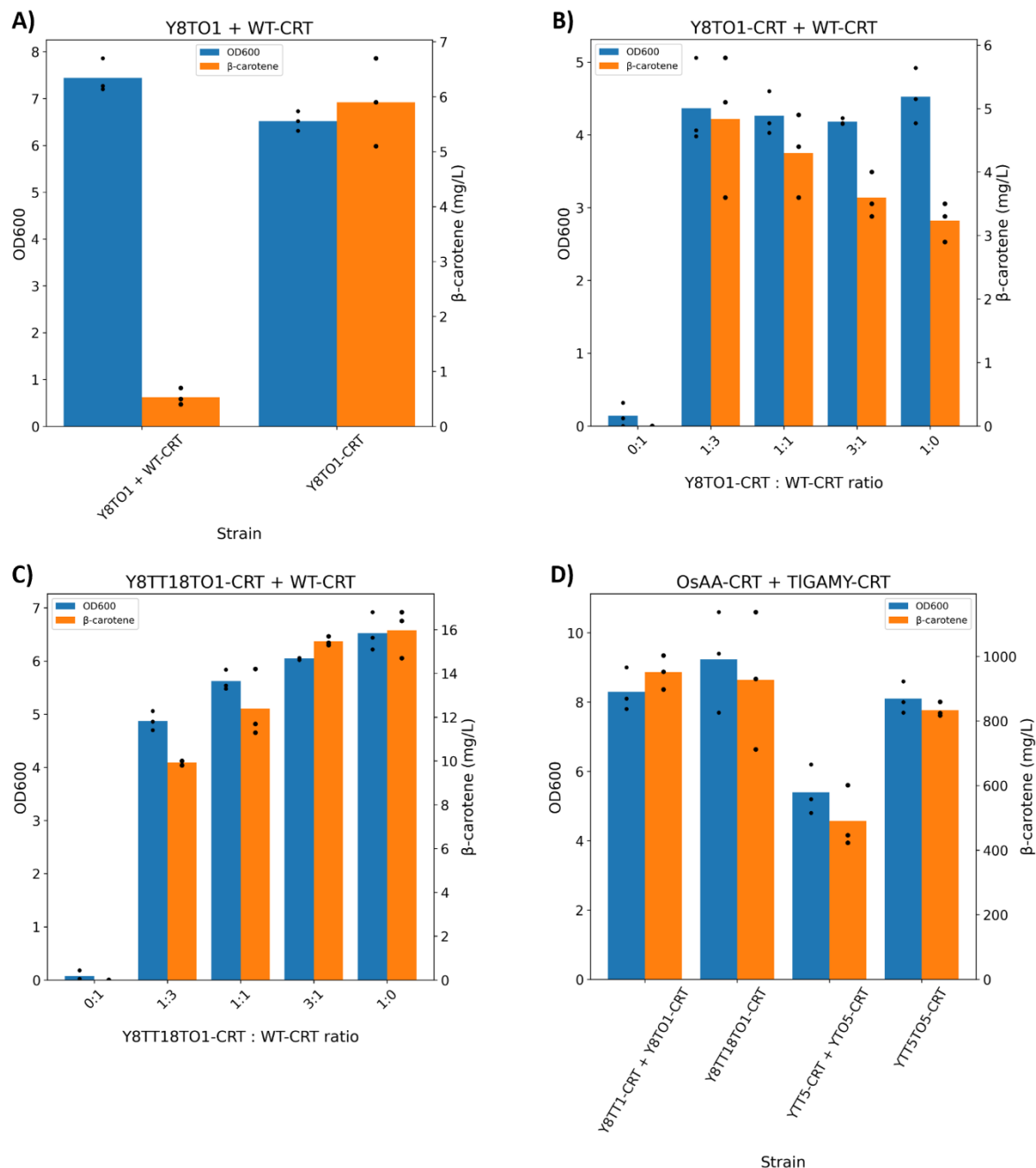


Figure 5.4 Division of labour (DOL) strategies between starch-degraders and β-carotene-producers

A) The strains in the consortium are specialists for their roles: Y8TO1 is a starch-degrader and WT-CRT is a β-carotene producer. In this case the monoculture Y8TO1-CRT has a worse OD600 but produces far more β-carotene. **B)** Both strains produce β-carotene, but only Y8TO1-CRT can degrade starch. In this case the Y8TO1-CRT monoculture produces the least carotene, and as more WT-CRT is added the carotene production increases, but the OD600 remains similar. **C)** Both strains produce β-carotene, but only Y8TT18TO1-CRT can degrade starch. In this case the Y8TT18TO1-CRT monoculture produces more carotene than any of the consortia of Y8TT18TO1-CRT+WT-CRT. **D)** Comparing monocultures co-expressing both OsAA and TIGAMY to 2-strain consortia where each cell type expresses a single amylase. Y8TT1-CRT+Y8TO1-CRT and Y8TT18TO1-CRT have similar β-carotene production. YTT5-CRT+YTO5-CRT has lower growth and lower β-carotene production than its equivalent monoculture. Data presented as the mean of n=3.

5.6. Applying the DOL strategy of a starch-specialist and xylan-specialist consortium to bioproduction of β -carotene

Next, I turned my attention to exploring a consortium where all cells are β -carotene producers but differ in the polysaccharide they can degrade. In Chapter 4 I established that a consortium of a starch-specialist Y8TO1XC and a xylan-specialist YTA1XC, where both cells can eat both glucose and xylose, led to the highest OD600, higher than an equivalent monoculture. The β -carotene biosynthesis pathway was introduced to Y8TO1XC, YTA1XC and Y8TO1TA1XC. The 2-strain consortium was inoculated at different ratios of Y8TO1XC-CRT : YTA1XC-CRT (Materials and Methods 7.11.5) and compared to Y8TO1TA1XC-CRT. The strains were grown on 1% starch (potato) YNB for 72 hours and carotene was extracted at the end of the culture (Materials and Methods 7.13.1),

Figure 5.5 shows that the generalist consumer consortium (Y8TO1XC-CRT+YTA1XC-CRT) attained both a higher OD600 and an increased production of β -carotene compared to its monoculture counterpart (Y8TO1TA1XC-CRT). Notably, the 1:1 ratio yields the highest β -carotene concentration, with a 14.11% increase compared to the monoculture (p-value of 0.0017). This demonstrates how DOL in polysaccharide degradation pathways can enable higher growth and bioproduction than current monoculture strategies.

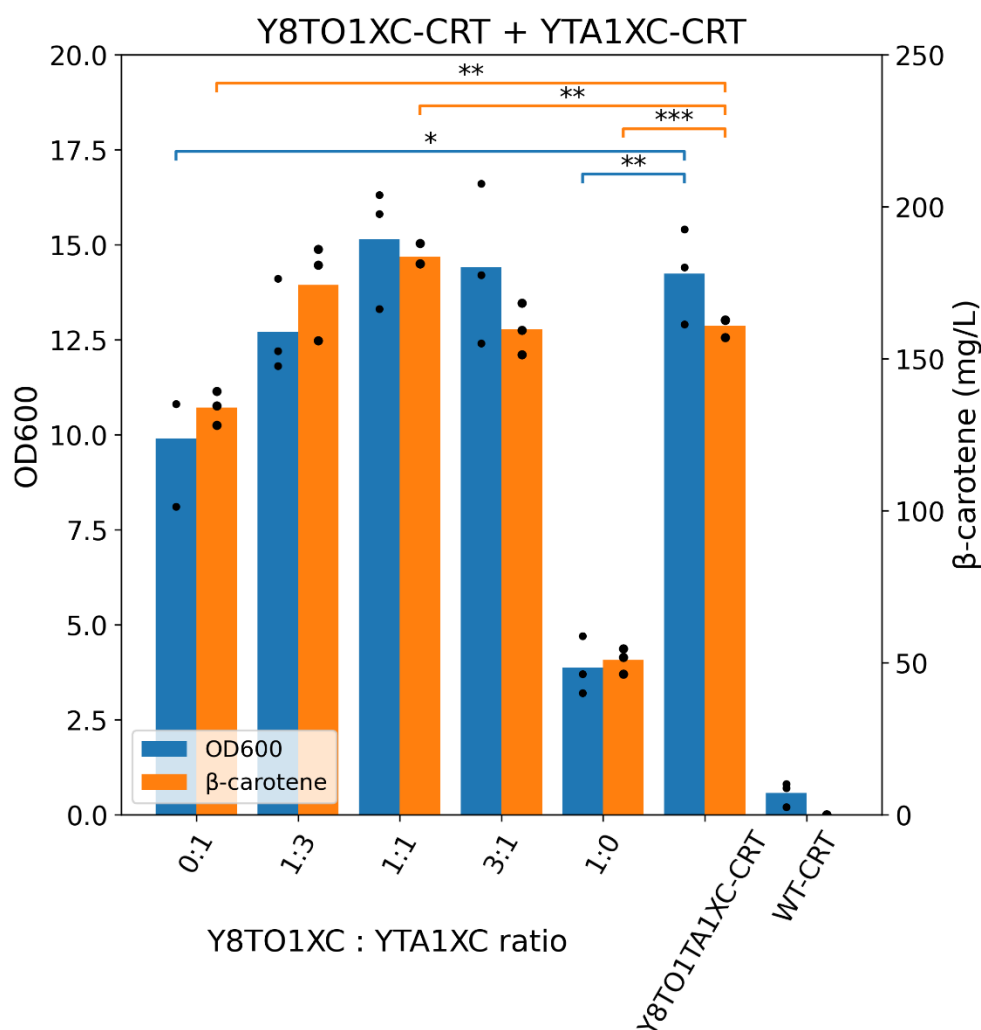


Figure 5.5 Production of β -carotene from a consortium of xylanase strain YTA1XC and amylase strain Y8TO1XC

The consortium with an equal ratio of the xylanase and the amylase strain has the highest OD600 and the highest production of β -carotene, significantly higher than the equivalent monoculture. Production decreased as the ratio of the two strains deviated further from equal, with the amylase strain alone (1:0) and the xylanase strain alone (0:1) having significantly lower production than the monoculture Y8TO1TA1XC-CRT strain. Data presented as the mean of $n=3$. Statistical analysis by Welch's T-test; * represents a p-value < 0.05 , ** p-value < 0.005 and *** p-value < 0.0005 (only the significant differences between the monoculture and the consortium are shown).

5.7. Conclusions for this chapter

This chapter used the strains designed in Chapters 3 and 4 to create a proof-of-concept for whether DOL would be advantageous for the bioproduction of value-added compounds. I investigated how DOL could provide a benefit to the production of patchoulol and β -carotene over a monoculture strategy.

My initial aim for this chapter involved the creation of a biosynthesis pathway in *Y. lipolytica* to produce patchoulol; this was at that time a novel value-added compound in *Y. lipolytica*. During the course of this PhD, Peng *et al.* (2023) produced 2.864 g/L of patchoulol from *Y. lipolytica* monoculture, thus proving a significant improvement over the 1.632 g/L produced in *S. cerevisiae* (Liu *et al.*, 2021a). To achieve this they followed the same metabolic engineering approach I employed, plus several other improvements to redirect flux towards patchoulol. However, in contrast to my work, they used a glucose rich media. This means the 1.01 mg/L produced in this chapter is the highest yield to date for patchoulol using starch as the sole carbon source. In the future, combining my work on amylase/xylanase expression with Peng *et al.*'s engineering strategy may enable higher yield on plant biomass derived substrates. However, the DOL strategy developed in Chapter 3, with a 2-strain *Y. lipolytica* consortium where each strain expresses one of two amylases, did not provide higher concentrations than a monoculture strategy for patchoulol.

Using β -carotene as a further proof-of-concept I found that the DOL strategy separating starch-degradation and β -carotene production did not achieve a higher yield of product than a monoculture that could both degrade and produce. However, this consortium strategy has merits owing to its flexibility, since many different producer strains could be combined with the amylase-expressing strain without the requirement to engineer a new strain. I also found that using a consortium where both cells produce β -carotene, but only one was a starch-degrader, achieved mixed results. The consortium using only OsAA achieved higher production than either component strain alone, while the consortium using OsAA+TIGAMY had lower production than the OsAA+TIGAMY strain alone. The monoculture expressing the most proteins, two amylases and three carotene genes (Y8TT18TO1-CRT), achieved the highest β -carotene overall, indicating that the metabolic burden of these genes was not limiting production. As with patchoulol, the DOL strategy designed in Chapter 3, with a 2-strain consortium where each strain expresses one of two amylases, did not provide any benefit over the double-amylase equivalent monoculture to β -carotene production.

The most significant result in this chapter was that the generalist-consumer DOL strategy for mixed polysaccharide degradation, developed in Chapter 4, did show improvement over its equivalent monoculture strategy for β -carotene production. This strategy produced 183 mg/L of β -carotene on 1% starch + 1% xylan YNB media. I envisage that future work using the improvements to this DOL strategy discussed in Chapter 4: employing a stronger amylase and reducing catabolite repression may further improve β -carotene production.

6. Conclusions and Future work

In Chapter 6 I summarise the conclusions of my thesis as aligned with my objectives, discuss what I have learnt from this work, its limitations and further applications for future research.

6.1. Summary of results

This thesis sought to investigate the application of DOL to the degradation of alternative substrates found in agricultural and food wastes, specifically the polysaccharides starch and xylan. It investigated whether DOL, and its associated reduction of metabolic burden, would enable synthetic microbial consortia to achieve higher growth than an equivalent monoculture. Within this project, using the host *Y. lipolytica*, I studied which DOL strategies would be most beneficial for maximising alternative substrate degradation and product of interest yields.

6.1.1. **Result of Objective 1: Modelling of metabolic burden in *E. coli* predicted the range of expression within which a 2-strain consortium could achieve a higher growth rate on starch than an equivalent monoculture**

In Chapter 2, I created a novel model to predict metabolic burden in a 2-strain consortium of *E. coli* engineered for the cooperative degradation of starch, with each cell expressing a single amylase. I then used this model to characterise the effect of heterologous protein expression on the resource pools of ribosomes and energy in the consortium versus an equivalent monoculture, which co-expressed two amylases (Objective 1.1). The model demonstrated that while the monoculture achieved higher total amylase production at the population level, the consortium produced more amylase per cell due to reduced diversion of essential resources. Further, I used this model to identify the range of expression within which DOL was predicted to provide a higher growth rate on starch (Objective 1.2). My findings revealed a critical threshold in substrate availability where DOL shifted from a disadvantage to an advantage. Beyond this threshold the consortium's reduced metabolic burden allowed it to achieve superior growth rates than the monoculture.

This work provides a quantitative framework for evaluating resource trade-offs in microbial consortia, offering insights that could inform the design of synthetic microbial systems with optimized metabolic efficiency.

6.1.2. Result of Objective 2: DOL in *Y. lipolytica* between two cells that both contribute to the degradation of the same substrate can achieve a higher growth rate and final OD600 than an equivalent monoculture

In Chapter 3 I engineered a series of *Y. lipolytica* strains to express either a single α -amylase or glucoamylase, or both amylases simultaneously. By characterising metabolic burden through growth measurements under different promoters and SP configurations, I demonstrated that stronger promoters and/or co-expression of two amylases imposed a greater metabolic burden, but this effect plateaued beyond a certain threshold (Objective 2.1). I then compared a 2-strain consortium, combining an α -amylase strain and a glucoamylase strain, with a monoculture strain co-expressing both amylases. I identified five different 2-strain consortia which could achieve a higher growth rate on starch than their equivalent monoculture (Objective 2.2). However, there was no predictive link between the strains which had the greatest metabolic burden on glucose (Objective 2.1) and the strains which performed best in a DOL strategy on starch (Objective 2.2). Instead, I found that the strength of the SP for the α -amylase strain appeared to correlate with the fastest growing consortia.

To further explore these dynamics, I scaled up the fastest-growing consortia from microplates into flasks and investigated the impact of different initial inoculation ratios on growth and final population distributions (Objective 2.3). I determined that when one amylase had significantly stronger activity than the other, higher initial ratios of the stronger strain improved growth on starch. However, a strain expressing only the strongest amylase exhibited the fastest growth overall, surpassing both the equivalent consortia and co-expressing monoculture. My finding has highlighted a challenge of DOL within a single degradation pathway, as competition for the resultant substrate, glucose, between the two strains reduces the overall growth compared to a strong single-amylase strain alone.

This work provides critical insights into the trade-offs and limitations of DOL strategies in synthetic biology, emphasising the importance of balancing cooperative behaviour with competitive dynamics for efficient consortium design. It also suggests that in some cases, the monoculture strategy can be beneficial and should be chosen over DOL.

6.1.3. Result of Objective 3: DOL between two *Y. lipolytica* cells that degrade different substrates achieves a higher growth when both cells are generalists for both substrates

In Chapter 4, I developed three distinct DOL strategies by designing degradation and substrate specificities: generalist consumer, orthogonal specialist and cross-feeding specialist. I determined that the generalist consumer consortium, composed of amylase- and xylanase-

expressing strains that could consume both glucose and xylose, outperformed an equivalent co-expressing monoculture in terms of final growth (Objective 3.1). This consortium maintained a stable population ratio over time and under varying media conditions (Objective 3.2). My finding demonstrates how DOL between two different substrate degradation pathways can provide a growth advantage compared to a monoculture. This strategy worked better than the DOL strategy in Chapter 3, since both strains were required for full degradation of both carbon sources, which highlighted an important design rule for microbial consortia for polysaccharide degradation.

In contrast, I found that the orthogonal and cross-feeding specialist consortia, where each strain specialised in metabolising either glucose or xylose, showed reduced growth compared to co-expressing monoculture and exhibited dynamic population shifts in response to media changes. I demonstrated that the generalist consortium was also resilient to increased temperatures, whereas the specialist consortia experienced significant growth inhibition under higher temperatures due to their dependence on precise sugar metabolism (Objective 3.3). However, I did determine that while they underperformed compared to the generalists, the specialist consortia did grow better than either specialist component strain alone, therefore demonstrating how DOL can be beneficial to overcoming the limitations of catabolite repression.

By comparing DOL approaches, this work advances our understanding of how substrate utilisation strategies influence consortia performance. It highlighted that generalist consumer strategies gave more stable consortia, and specialist consortia resulted in dynamic population changes due to substrate preferences.

6.1.4. Result of Objective 4: DOL between two specialist degrader and generalist consumer *Y. lipolytica* cells could achieve higher bioproduction of β -carotene than a monoculture

In Chapter 5 I applied the DOL strategies of Chapters 3 and 4 to bioproduction (Objective 4). I produced 1.01 mg/L of patchoulol using starch as the sole carbon source, but the DOL strategies did not show any improvement over monoculture. With β -carotene production, I demonstrated that the DOL strategies developed in Chapter 3, where growth rate was improved over monoculture, did not show any improvement in production over monoculture. However, the generalist consumer DOL strategy developed in Chapter 4 did show improved performance on mixed polysaccharide media, with a maximum β -carotene production 14.11% higher than the equivalent monoculture.

This work highlighted the potential for enhancing value-added product yields through DOL in synthetic microbial consortia.

The overall summary of results as they relate to the original objectives in Figure 1.4 is shown in Figure 6.1.

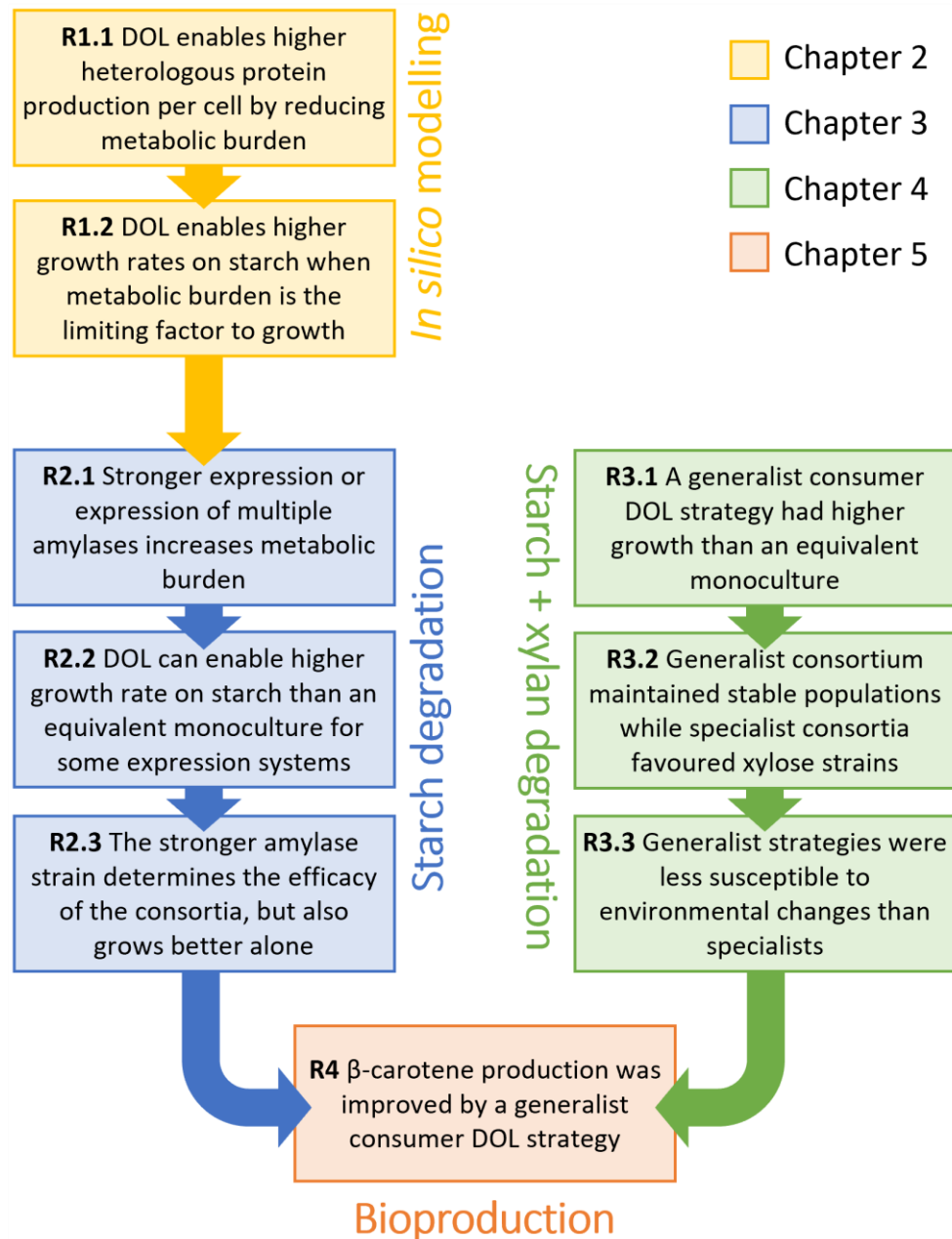


Figure 6.1 Summary of results

6.2. Reflections and limitations

6.2.1. Matching the *in silico* and *in vivo* data would have improved the accuracy of the model to predict the behaviour of the starch-degrading consortia

The modelling in Chapter 2 was parametrised for *E. coli* but I chose not to pursue the re-parametrisation to match *Y. lipolytica*. I determined that identifying the fundamental parameters would have been too time and labour intensive (during an uncertain period of restricted laboratory access due to COVID) and would not have significantly advanced the project's overall goal.

Since, based on the literature available, the underlying assumptions for the parameters matched in bacteria and yeast, it could be assumed the same trend in the results would be seen in *Y. lipolytica* in Chapter 3. This held true for the prediction of metabolic burden; in both the model and in the *Y. lipolytica* strains, the greater expression levels provided by the stronger promoter induced greater effects on growth, indicating higher burden.

When grown on starch however, the *Y. lipolytica* strains showed different behaviour *in vivo* to that predicted by the model *in silico*. Firstly, the model assumed that both cells were required to ensure sufficient glucose for either cell to grow. However, some of the amylase strains could provide enough glucose from starch to grow without any input from their consortium partner. Secondly, based on the predictions of the model, the 8UASpTEF promoter which created higher burden, should have shown greater benefits for the DOL strategy than the pTEF promoter. While some of the 8UASpTEF-controlled consortia did show greater benefits, there was no direct correlation between metabolic burden and higher growth rates for the consortia *in vivo*. Part of the explanation for this behaviour in the consortia is likely hidden within the supply of released glucose. In the model the threshold at which the DOL outperformed the monoculture was met when glucose availability was no longer limiting to growth. It was only at this point that metabolic burden became the limiting factor, and therefore DOL became favourable. I did not measure the glucose concentration during exponential phase, which in retrospect, would have identified if the consortia that achieved a higher growth rate than their equivalent monoculture, did so because they had an excess of glucose. This could have assisted in matching the *Y. lipolytica* data to the model predictions, at least at a phenomenological level.

6.2.2. Limitations to the quantification of metabolic burden in *Y. lipolytica*

In this thesis, the effect of metabolic burden has been measured assuming decreases in growth parameters are solely due to the differences in the expression cassette. However, this may have

limitations, as consideration should be given to other factors potentially influencing growth and metabolic burden.

The most important factor to consider is integration site. The use of random integration in the engineering of the strains in this thesis may have confounded the link between expression and metabolic burden. Depending on exactly where in the genome a gene was integrated, the level of expression or its impact on cellular metabolism may differ. In fact, I did observe differences in growth parameters between transformants for some strains, highlighting the potential for the integration site to act as another expression modifier. At the beginning of my project, our in-house specific integration protocol was still in development, hence I used random integration and tested multiple transformants for metabolic burden to account for different integration sites. However, employing specific integration would have facilitated a more controlled measurement of how the expression and secretion levels affected burden, particularly for matching *in vivo* data from Chapter 3 to the model in Chapter 2.

As introduced in Chapter 1, in *E. coli* quantitative methods exist to measure the reallocation of endogenous resources within cells, such as *in vivo* cellular capacity monitors (Ceroni et al., 2015) and single-cell metabolomics (Rouches et al., 2022). In the future, adapting and implementing these tools in *Y. lipolytica* may visualise hidden burden that could not be measured in growth parameters, and help elucidate why certain expression systems delivered more effective DOL.

6.2.3. Competition for substrate limited the ability of a consortia to outperform its component strains

The results from Chapter 3 identified a key obstacle in the DOL strategy, where the two component strains expressed amylase but competed for the same substrate. This resource competition hindered their performance in a consortium, as one single-amylase strain alone performed better than both the equivalent double-amylase monoculture and 2-strain consortium. In contrast, the generalist consumer DOL strategy from Chapter 4 overcame this limitation. Although the two component strains were still competing for the same substrates, they performed different degradation functions, which effectively contributed more to overall sugar availability.

This does not necessarily imply that consortia cannot be successful if the component strains perform the same function, as previous DOL strategies using two amylases or two xylanases have shown improved growth compared to monoculture (Wang et al., 2014, 2019a). However,

my results suggest that each cell's cooperative degradation function must outweigh its competitive consumption for DOL to be appropriate, which was not the case for the strains created in Chapter 3. It is possible that further work improving the function of the weaker amylase would reduce substrate competition and allow DOL to become more advantageous.

6.2.4. Extracellular hydrolysis limited the mutual dependence that could be achieved

In this thesis I observed different pairwise interactions between the amylase and xylanase strains, which were controlled by the substrate preferences of the strains. The component strains in the cross-feeding specialist consortium were mutualists, with the aim to make them dependent on each other to survive. However, since hydrolysis is an extracellular function, the actual cells did not have to be functionally active for the whole duration of the culture.

A potential solution to increase dependence on the cell's active presence would be to use cell-surface display as an alternative method of expression. However, this may not be sufficient mitigation as hydrolysis still occurs extracellularly and the release of proteins upon cell death would still be problematic. Alternatively, syntrophy could be implemented with other established amino acid cross-feedings (Introduction 1.5.2).

6.2.5. Single-organism consortia limit the adaptability to environmental fluctuations

In nature, functional redundancy in polysaccharide degradation is very common, with multiple species from diverse taxa performing similar degradation functions. In these natural consortia, if one specialist is lost due to an unfavourable environment, there are other specialists for the same function that can take over the role for the community (Li et al., 2024a). The synthetic consortia designed in this project contained only one species, *Y. lipolytica*. This limits the adaptability of the consortia to different environmental conditions.

However, using one species avoids the difficulty of co-culturing multiple microorganisms with different environmental preferences, which usually results in compromised conditions and suboptimal performance. When planning a consortium for use on agricultural and food waste feedstocks, a decision must be made early in the design process if the environment is likely to fluctuate significantly. If it is, then functional redundancy between cells with different environmental preferences will be necessary. However, even for bioreactors, where environments are tightly controlled, functional redundancy may still need to be integrated. This is a particularly important consideration as a plethora of different media compositions can exist

in agriculture and food waste. Therefore, for example, species or strains may need to be included which are more resilient to diverse toxic byproducts.

6.2.6. Catabolite repression limited the simultaneous consumption of glucose and xylose

One of the limitations highlighted in Chapter 4 was the catabolite repression in *Y. lipolytica* when consuming mixed starch and xylan, with sequential sugar consumption: glucose-consuming strains grew first, followed by xylose-consumers. As a consequence, strains that could not metabolize glucose failed to grow alone in mixed media. This repression restricted the growth of the specialist consortia, since only one strain could grow while glucose was available, while the other strain had to wait for repression to be overcome. If catabolite repression was not present, through deletion or decoupling of the regulatory pathway, the specialist consortia may improve their performance, as the two strains may be active simultaneously.

Simultaneous sugar consumption has shown advantages in *E. coli* and *S. cerevisiae* (Verhoeven et al., 2018; Xia, Eiteman & Altman, 2012; Eiteman, Lee & Altman, 2008). As mentioned in Section 4.12, Zhou et al. used adaptive laboratory evolution to overcome catabolite repression in *Y. lipolytica*, allowing it to metabolise xylose even in the presence of 10 g/L of glucose. They also identified several key gene targets for future work to rationally engineer reduced catabolite repression (Zhou et al., 2020).

However, overcoming catabolite repression by rationally designed engineering or directed evolution is time-consuming. When considering the vast array of different substrates available in agriculture and food waste, it may be unrealistic to overcome all the complex variations of catabolite repression by extensive engineering. Therefore, DOL with sequential substrate uptake may still provide an easier alternative avenue and an effective workable solution to catabolite repression.

6.3. Future opportunities

6.3.1. Building up the consortia toolbox for alternative feedstock utilisation

In natural consortia, behaviour is shaped by complex functional diversity, cell-to-cell interactions and cross-feeding. This dissertation establishes the first step, or the first module, of building a bottom-up synthetic consortium for polysaccharide degradation in *Y. lipolytica*. By adding other novel modules for degradation and metabolism, *Y. lipolytica* consortia may be expanded and amplified to effectively degrade further components of agriculture and food waste. As discussed in Chapter 1, there is increasing work focussed on engineering *Y. lipolytica*

toward utilisation of the individual components of lignocellulose (Celińska, Nicaud & Białas, 2021; Spagnuolo et al., 2018). These engineered strains could be components of ligninolytic, cellulolytic and hemicellulolytic modules in a large lignocellulose degrading *Y. lipolytica* consortium. I envisage that as more building blocks are constructed, synthetic consortia will progress beyond minimalist designs and reconstruct the complexity observed in natural consortia. However, it will undeniably require a consortium of synthetic biologists to necessitate this realisation.

One opportunity for expanding the complexity of synthetic consortia is through the addition of more complex substrates. This thesis has highlighted that DOL which creates more than one sugar product, glucose and xylose, is potentially a more promising approach than DOL which creates a single product, glucose. This observation highlights the promise of heterogeneous polysaccharides, such as xylan, over homogeneous polysaccharides like cellulose or starch in DOL processes. By exploring the xylose backbone of xylan in this thesis, I have only utilised part of its full sugar content. Xylan also contains diverse subunits, including galactose, arabinose, glucuronic acid, and galacturonic acid, each offering additional opportunities for DOL. There are three critical synthetic control points that can be engineered to control sugar utilisation: 1) the expression of different degradation enzymes, 2) the presence of specialised monomer transporters, and 3) the ability to metabolise these monomers intracellularly. These factors could be leveraged in DOL to generate distinct strains, each specialised for the degradation of specific xylan components. Building upon the findings in Chapter 4, exploring xylan and starch consortia, it will be crucial to further investigate whether generalist, orthogonal or cross-feeding consumer strategies offer superior growth and substrate utilisation in more-than-pairwise interactions between strains. The diverse structure of xylan, and other heterogeneous macromolecules such as lignin, presents an exciting avenue for future research, and DOL offers a promising approach to harness the full potential of these alternative substrates.

Another avenue for expanding complexity is the addition of functional redundancy. As highlighted in Section 6.2.5, minimal synthetic consortia, as created in this thesis, often exhibit limited robustness to environmental perturbations. Functional redundancy, the inclusion of multiple microbial species or strains capable of performing the same or similar functions, plays a crucial role in maintaining the stability and resilience of natural ecosystems. This redundancy ensures that when one species is removed or underperforms, others can compensate, preventing a loss in efficiency. While incorporating functional redundancy may seem contrary to the ideals of simplified bottom-up consortia design, it is, in fact, essential for optimizing the performance of synthetic consortia. In *S. cerevisiae* Vidal et al. showed that communities with greater functional

redundancy are more likely to survive exploitation by cheater cells than simplified communities (Vidal et al., 2020). Embracing redundancy will not only enhance the robustness of the system but also unlock its full potential, enabling DOL approaches to function stably under a wider range of conditions.

I believe that in the future, rationally designed bottom-up consortia for CBP will begin to look more like natural consortia in terms of their complexity, but with a better understanding of the function of each member of the consortia and each relationship between them.

6.3.2. Self-regulating or externally controlled cell populations

Precise control of cell populations will be advantageous for the high variability of feedstocks from agricultural and food wastes. These wastes will depend on the source, the type of crop, the maturity of the crop, the geographical location, and the season the waste was produced, and will thus vary greatly in their macromolecular composition. As shown in this thesis, stable populations can be created with correctly balanced generalist consumers, and dynamic populations can be created with specialist consumers. However, while the observations of this thesis may be helpful for the prediction of how specialist degraders may behave when in specific media compositions, there was no direct programmable control over this behaviour.

The ideal microbial consortium using DOL will be able to adapt its population composition to increase the number of cells producing the enzymes relevant to the most abundant macromolecules. Thus, the consortium would be able to efficiently utilise any waste it is presented with. As introduced in Chapter 1, there are many synthetic biology tools for passively and actively controlling cell populations to ensure optimal function.

Self-regulating consortia can be created using rationally designed genetic control circuits or cell-to-cell communication (Introduction 1.5.1). Biosensor circuits specific for each macromolecular component coupled with activators and repressors within cells, promoting growth or death, and population controls, such as quorum sensing and other methods of cell-to-cell communication, will allow consortia to detect the macromolecular composition of the media and act autonomously to adjust their populations. Creating control circuits to match the wide range of polysaccharide compositions which exist in waste is an exceptionally large undertaking. Specific receptors for complex polysaccharides are not well-characterised, but there is already an interest in creating biosensors for their monomer components in screening for microorganisms with lignocellulolytic functions (Alvarez-Gonzalez & Dixon, 2019). Biosensors for lignin-derived aromatics are already used for functional metagenomic screens to identify lignin-degraders (Ho

et al., 2018). In nature, consortia can achieve very complex responses to their changing environment, so synthetic consortia may yet be able to emulate this.

Alternatively, consortia could be engineered to self-regulate independently of the exact composition of the waste, but instead in a pre-programmed fashion that focuses on the known structure of lignocellulose: first degrading the lignin, then the hemicellulose, then the cellulose. Or, instead, there could be a switching circuit to transition from a degradation-population to a bioproduction-population (Honjo et al., 2019). I predict that self-regulating synthetic consortia will mainly be used to implement small scale consortia, or for few-step processes where a bistable switch or a controlled oscillation between functions is required.

Another approach is to use *in silico* controls of populations. Here the cells respond to an external input- whether that be light, or a change in temperature, or a specific inducer molecule. This would rely on an external analysis of the waste feedstock composition, which could then be fed into a computer model that could calculate the optimal population composition of the consortia to deal with that specific waste. The computer could actively observe the bioreactor and continually feed inputs to adjust the population.

Tight and specific control of cell populations has been a significant challenge in utilising natural consortia in CBP. Synthetic cells, with rationally designed genetic control circuits will be capable of regulation in CBP to more efficiently utilise agricultural and food wastes.

6.3.3. Impact on the cost and sustainability of bioproduction

As introduced in Chapter 1, utilising agricultural and plant biomass as feedstock for biorefineries will address several key global challenges: increasing resource efficiency, reducing waste, and creating cleaner industrial processes.

In terms of bioproduction, the aim of using a CBP is to reduce the costs from commercial enzymes and additional infrastructure and the aim of using agriculture and food waste is to reduce costs of feedstock. CBP has been estimated to provide a twofold reduction in overall production costs compared to current SSF, which relies on a separate enzyme-production step (Lynd et al., 2002). The price for pure glucose is around \$91-272 USD per ton (£82-246 GBP per tonne) (Alibaba.com, 2025), although biorefineries are likely to have commercial deals with specific sugar suppliers. The exact value of the waste feedstock is very variable, as expected due to the diversity of wastes, however as an example, the minimum selling price of corn stover (stalks, leaves, husks) with delivery to the biorefinery included, was estimated to be \$28.49 USD per ton (£23.08 GBP per tonne) (Becerra-Pérez, Rincón & Posada-Duque, 2022). In many

commercial settings, disposal of agricultural and food waste has to be paid for by the waste producer, hence waste could provide a very affordable feedstock.

Responsible research innovation requires that the potential applications of any technology are considered in their wider societal context. One key consideration is ensuring that use in a biorefinery is the best possible application of the feedstock. What might be considered “waste” could be highly competitive for other processes such as animal feed, biogas production and compost, which may be an eco-friendlier use for it than running a bioreactor. Dumping organic waste into landfill creates nearly 400 kg CO₂eq/tonne in greenhouse gas emissions (Nordahl et al., 2020). While any alternative that diverts waste from landfill will result in reduced GHG emissions, existing alternatives such as composting and anaerobic digestion to biogas can result in net negative emissions of -41 CO₂eq/tonne and -36 CO₂eq/tonne respectively (Nordahl et al., 2020). To reduce carbon emissions overall, biorefineries must focus on reducing the fraction of food and agricultural waste destined for landfill. Another key consideration is the location of the biorefinery – if the feedstock must be transported a long distance, then any environmental benefits will be reduced. Building a new, more sustainable bioeconomy will require robust collaborations between agriculture and food waste producers and the biotechnology sector.

The starch and xylan degrading consortia designed in this thesis act as a first step toward improving the valorisation of low-cost waste, and as discussed in this Future Work section, can be built upon to create more complex synthetic consortia. These synthetic consortia will be highly controllable, due to the bottom-up approach used to create them, and this thesis has demonstrated how they can show improvements over an equivalent monoculture strategy. By improving the performance of industrial hosts on waste feedstocks in CBP, microbial consortia will ensure progress towards more sustainable bioproduction.

Concluding statement

In the introduction to this thesis, I highlighted the significant advantages of DOL in reducing metabolic burden and enhancing the growth of microorganisms on sustainable and inexpensive feedstocks derived from agricultural and food waste. Through my work with *Y. lipolytica*, I have explored a variety of DOL strategies for hydrolytic enzyme production for the degradation of polysaccharides, starch and xylan, and identified the most effective approaches. Notably, I developed a generalist-consumer DOL strategy, which significantly improved β -carotene production compared to its monoculture counterpart, underscoring the transformative potential of DOL. This achievement demonstrates that, when correctly optimized, DOL strategies can not only alleviate the limitations of monoculture systems but also exceed their performance in bioproduction applications, paving the way for more efficient, affordable and sustainable industrial biotechnology.

7. Materials and Methods

7.1. Computational methods

7.1.1. ODE solver and simulation methods

The `scipy.integrate.solve_ivp` function from the `scipy` package was used to solve ODEs, with the LSODA method (Adams/BDF method with automatic stiffness detection and switching). In all simulations the time span was set to 0 – 20,000 mins, and the absolute tolerance (atol) and relative tolerance (rtol) were set to 1e12. Steady state was taken as the value for each intracellular molecule at the end of a numerical simulation of 20,000 mins.

For simulations where glucose is supplied externally the ODEs were initialised with 1000 energy units (a) and 10 ribosomes (r), as in the original Weiße model. For simulations on starch, a simulation was first run for growth on glucose, then the steady-state values reached were used as initial conditions for a simulation on starch.

Data was generated on a Windows 10 PC with Intel® Core™ i7-1065G7 CPU @ 1.30GHz, 16 GB RAM using Python 3.7 with a Spyder 4.1 (as part of Anaconda) IDE. Where multiple processors were available, the computational labour was divided between processors using the `dask.distributed` package (`distributed.dask.org`) for Python.

7.1.2. ODEs used for the basis of the single-cell model

The whole-cell model published by (Weiße et al., 2015) was used as the framework for the consortia and monoculture models in Chapter 3.

Table 7.1 Modelling framework from Weiße et al. (2015)

Where $x \in \{r, et, em, q\}$

Rates	
Transcription	$\omega_x(a) = \frac{\omega_x a}{\theta_x + a}$ For housekeeping (q) genes only: $\omega_q(a) = \frac{\omega_x a}{\theta_x + a} \times \frac{1}{1 + \left(\frac{q}{K_q}\right)^{hq}}$
Import	$v_{imp}(et, s) = \frac{v_t et s}{s + K_{M_t}}$
Metabolism	$v_{cat}(em, si) = \frac{v_m em si}{si + K_{M_m}}$
Rate of amino acid incorporation	$\gamma(a) = \frac{\gamma_{max} a}{K_\gamma + a}$

Rate of translation	$v_x(c_x, a) = \frac{\gamma(a)}{n_x} c_x$
Intracellular molecules	
Imported glucose (si)	$\frac{dsi}{dt} = v_{imp}(et, s) - v_{cat}(em, si) - \lambda si$
Energy molecules (a)	$\frac{da}{dt} = n_s v_{cat}(em, si) - \sum_{x \in \{r, et, em, q\}} \gamma(a) c_x - \lambda a$
mRNA (m_x)	$\frac{dm_x}{dt} = \omega_x(a) - k_b m_x r + k_u c_x + v_x(c_x, a) - d_m m_x - \lambda m_x$
Ribosome:mRNA complex (c_x)	$\frac{dc_x}{dt} = k_b m_x r - k_u c_x - v_x(c_x, a) - \lambda c_x$
Ribosomes (r)	$\frac{dr}{dt} = v_r(c_r, a) - \lambda r + \sum_{x \in \{r, et, em, q\}} (v_x(c_x, a) - k_b r m_x + k_u c_x)$
Transport protein (et)	$\frac{det}{dt} = v_{et}(c_{et}, a) - \lambda et$
Metabolic protein (em)	$\frac{dem}{dt} = v_{em}(c_{em}, a) - \lambda et$
Housekeeping protein (q)	$\frac{dq}{dt} = v_q(c_q, a) - \lambda q$

7.1.3. ODEs for consortia and monoculture model

Table 7.2 ODEs for starch degradation and glucose consumption in a 2-strain consortium expressing one amylase (ea or eb) per cell, or a monoculture expressing both amylases

Two-strain consortium	
Loss of glucose from the environment	$v_{uptake}(et, s) = \frac{v_{et} s (N_a et_{cell_a} + N_b et_{cell_b})}{s + K_{met}}$
Glucose s (if glucose is supplied externally)	$\frac{ds}{dt} = s_{in} - v_{uptake}(et, s) - d_s s$
Glucose s (if starch is supplied externally)	$\frac{ds}{dt} = N_b \frac{v_{eb} eb s_1}{s_1 + K_{meb}} - v_{uptake}(et, s) - d_s s$
Starch s0	$\frac{ds_0}{dt} = s_{0in} - N_a \frac{v_{ea} ea s_0}{s_0 + K_{mea}} - d_{s_0} s_0$
Intermediate s1	$\frac{ds_1}{dt} = N_a \frac{v_{ea} ea s_0}{s_0 + K_{mea}} - N_b \frac{v_{eb} eb s_1}{s_1 + K_{meb}} - d_{s_1} s_1$
Imported glucose in an average Cell A	$\frac{dsi_{cell_a}}{dt} = \frac{v_{et} et_{cell_a} s}{s + K_{met}} - \frac{v_{em} em_{cell_a} si_{cell_a}}{si + K_{Mem}} - \lambda_{cell_a} si_{cell_a}$
Imported glucose in an average Cell B	$\frac{dsi_{cell_b}}{dt} = \frac{v_{et} et_{cell_b} s}{s + K_{met}} - \frac{v_{em} em_{cell_b} si_{cell_b}}{si + K_{Mem}} - \lambda_{cell_b} si_{cell_b}$
Monoculture	
Loss of glucose from the environment	$v_{uptake}(et, s) = \frac{v_{et} s N et}{s + K_{met}}$
Glucose s (if glucose is supplied externally)	$\frac{ds}{dt} = s_{in} - v_{uptake}(et, s) - d_s s$
Glucose s (if starch is supplied externally)	$\frac{ds}{dt} = N \frac{v_{eb} eb s_1}{s_1 + K_{meb}} - v_{uptake}(et, s) - d_s s$

Starch s_0	$\frac{ds_0}{dt} = s_{0in} - N \frac{v_{ea} ea s_0}{s_0 + K_{m_{ea}}} - d_{s_0} s_0$
Intermediate s_1	$\frac{ds_1}{dt} = N \frac{v_{ea} ea s_0}{s_0 + K_{m_{ea}}} - N \frac{v_{eb} eb s_1}{s_1 + K_{m_{eb}}} - d_{s_1} s_1$
Imported glucose in an average monoculture cell	$\frac{ds_i}{dt} = \frac{v_{et} et s}{s + K_{m_{et}}} - \frac{v_m em si}{si + K_{M_m}} - \lambda si$
Species: s: glucose, s1: intermediate, s0: starch, si: imported glucose, ea: heterologous protein A (α -amylase), eb: heterologous protein B (glucoamylase), et: endogenous transport protein, em: endogenous metabolic protein (subscript denotes Cell A or Cell B which may differ in the number of intracellular molecules)	
All species are given as a concentration in molecules/cell assuming a constant cell volume of $1\mu m^3$ and a constant bioreactor volume. It is assumed all species are stable with no degradation except for that provided by the desired enzyme activity.	

7.1.4. Parameters

Table 7.3 Parameters for an *E. coli* host cell

Parameter description	Name	Value	Unit	Reference
Translational threshold	K_v	7	molecs/cell	(Weiße et al., 2015)
Km for metabolic enzyme	$K_{m_{em}}$	1000	molecs/cell	
Km for transport enzyme	$K_{m_{et}}$	1000	molecs/cell	
Ki for housekeeping autoregulation	K_q	152219	molecs/cell	
Total proteome content of cell	M	1E+08	amino acids	
Degradation rate of mRNA	d_m	0.1	min^{-1}	
Maximum translation rate	γ_{max}	1260	aa/min molecs	
Rate of ribosome binding	k_b	1	cell/min molecs	
Rate of ribosome unbinding	k_u	1	min^{-1}	
Length of ribosome	n_r	7549	amino acids	
Length of non-ribosomal endogenous protein	n_x	300	amino acids	
Hill coefficient for housekeeping autoregulation	n_q	4	none	
Nutrient efficiency	n_s	0.5	none	
Transcription threshold for ribosomal genes	θ_r	426.8693	molecs/cell	
Transcription threshold for non-ribosomal genes	θ_x	4.379733	molecs/cell	
kcat of metabolic enzyme	v_{em}	5800	min^{-1}	
kcat of transport enzyme	v_{et}	726	min^{-1}	
Transcription rate of metabolic gene	ω_{em}	4.139172	molecs/min	
Transcription rate of housekeeping gene	ω_q	948.935	molecs/min	
Transcription rate of ribosomal gene	ω_r	929.9679	molecs/min	
Transcription rate of transport gene	ω_{et}	4.139172	molecs/min	
Length of α -amylase	n_a	434	amino acids	Uniprot: P17654
Length of glucoamylase	n_b	617	amino acids	Uniprot: Q58HN1

Km for enzyme a (α -amylase)	$K_{m_{ea}}$	1140000	molecs/cell	(Fujii & Kawamura, 1985) (with units converted to molecs/cell)
Km for enzyme b (glucoamylase)	$K_{m_{eb}}$	68200	molecs/cell	
kcat of α -amylase reaction	v_{ea}	271	min ⁻¹	
kcat of glucoamylase reaction	v_{eb}	403	min ⁻¹	
Ki for glucose inhibition of α -amylase	K_i	2980000	molecs/cell	
Fraction conversion of s0	ξ	1	none	
Molecular weight glucose	Mw_{glc}	180	g/mol	
Molecular weight starch	Mw_{s0}	19000	g/mol	
Ratio of cell A	N_a	1	cells	Can be adjusted based on reactor set up
Ratio of cell B	N_b	1	cells	
Input of starch	$s0_{in}$	1E+21	molecs/min	
Input of glucose	s_{in}	1E+21	molecs/min	
Dilution rate of glucose	d_s	0.001	molecs/min	
Dilution rate for starch	d_{s0}	0.001	molecs/min	
Dilution rate for maltodextrins	d_{s1}	0.001	molecs/min	
Dilution rate for α -amylase	d_{ea}	0.001	molecs/min	
Dilution rate for glucoamylase	d_{eb}	0.001	molecs/min	Values screened
Transcription rate of α -amylase	w_{ea}	-	molecs/min	
Transcription rate of glucoamylase	w_{eb}	-	molecs/min	

Table 7.4 Parameters for a yeast host cell

Parameter description	Name	Value	Unit	Reference
mRNA degradation	d_m	0.03	min ⁻¹	(Wang et al., 2002)
Ribosome length	n_r	15477	amino acids	Calculated from 79 ribosomal proteins (Woolford & Baserga, 2013) with an average molecular weight of 21550 g/mol (Kruiswijk, Planta & Mager, 1978).
Non-ribosomal protein length	n_x	379	amino acids	(Brocchieri & Karlin, 2005)
Max translational elongation	γ_{max}	1200	aa/min molecs	(Futcher et al., 1999)
Nutrient import rate	v_t	9.94E+15	molecs/min	(Maier et al., 2002)
Nutrient import threshold	K_{Mt}	2.29E+08	molecs/cell	
Max metabolism rate	v_m	3.88E+07	molecs/min	(Chen et al., 2012)
Metabolism threshold	K_{Mm}	1.75E+07	molecs/cell	
Relative rate of ribo:mRNA binding	k_b	0.00022	n/a	(Siwiak & Zielenkiewicz, 2010)
Relative rate of ribo:mRNA unbinding	k_u	0.00022	n/a	
Total cell mass	M	2.24E+10	amino acids	Calculated from a protein mass of 4.00E-12 g/cell (Futcher et al., 1999) and 5.60E+21 amino acids per 1g of protein (Scott et al., 2010a).

7.1.5. Use of generative AI for Figures

I would like to acknowledge the use of ChatGPT, an AI language model developed by OpenAI, for providing coding assistance and debugging during the development of scripts for presenting data during Chapters 3, 4 and 5 of this project. No generative AI was used in the development of the model in Chapter 2.

7.1.6. Statistical analysis

Statistical differences between growth parameters were determined by Welch's T-test using scipy stats. A p-value < 0.05 was considered significant, with further significance specified in each graph.

7.2. Materials

7.2.1. *Yarrowia lipolytica* strains

JMY2101 (Po1d U-L+) (RLA S93) was used as the parental strain for all amylase and xylanase strains in Chapter 3. Po1d (U-L-) (RLA S3) was used as the parental strain for all glucose-consuming strains in Chapter 4. Po1d with hexokinase knock-out (RLA S1074), kindly obtained from Hapeta *et al.* (Hapeta *et al.*, 2021) was used as the parental strain for all non-glucose-consuming strains. The control strain for xylose consumption JMY5814, was obtained from Niehus *et al.* (Niehus *et al.*, 2018).

Table 7.5 Full list of strains

RLA Strain No.	Name	Plasmids
STARCH DEGRADATION (CHAPTER 3)		
1806	YTO1	pZUS1.1-pTEF-SP1-OsAA-Lip2T
1807	YTO2	pZUS1.1-pTEF-SP2-OsAA-Lip2T
1808	YTO3	pZUS1.1-pTEF-SP3-OsAA-Lip2T
1809	YTO4	pZUS1.1-pTEF-SP4-OsAA-Lip2T
1810	YTO5	pZUS1.1-pTEF-SP5-OsAA-Lip2T
1811	YTT1	pZUS1.1-pTEF-SP1-TIGAMY-Lip2T
1812	YTT2	pZUS1.1-pTEF-SP2-TIGAMY-Lip2T
1813	YTT3	pZUS1.1-pTEF-SP3-TIGAMY-Lip2T
1814	YTT4	pZUS1.1-pTEF-SP4-TIGAMY-Lip2T
1815	YTT5	pZUS1.1-pTEF-SP5-TIGAMY-Lip2T
1826	YPO1	pZUS1.1-pPGM-SP1-OsAA-Lip2T
1827	YPO2	pZUS1.1-pPGM-SP2-OsAA-Lip2T

1828	YPO3	pZUS1.1-pPGM-SP3-OsAA-Lip2T
1829	YPO4	pZUS1.1-pPGM-SP4-OsAA-Lip2T
1830	YPO5	pZUS1.1-pPGM-SP5-OsAA-Lip2T
1831	YPT1	pZUS1.1-pPGM-SP1-TIGAMY-Lip2T
1832	YPT2	pZUS1.1-pPGM-SP2-TIGAMY-Lip2T
1833	YPT3	pZUS1.1-pPGM-SP3-TIGAMY-Lip2T
1834	YPT4	pZUS1.1-pPGM-SP4-TIGAMY-Lip2T
1835	YPT5	pZUS1.1-pPGM-SP5-TIGAMY-Lip2T
1846	Y8TO1	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T
1847	Y8TO2	pZUS1.1-8UASpTEF-SP2-OsAA-Lip2T
1848	Y8TO3	pZUS1.1-8UASpTEF-SP3-OsAA-Lip2T
1849	Y8TO4	pZUS1.1-8UASpTEF-SP4-OsAA-Lip2T
1850	Y8TO5	pZUS1.1-8UASpTEF-SP5-OsAA-Lip2T
1851	Y8TT1	pZUS1.1-8UASpTEF-SP1-TIGAMY-Lip2T
1852	Y8TT2	pZUS1.1-8UASpTEF-SP2-TIGAMY-Lip2T
1853	Y8TT3	pZUS1.1-8UASpTEF-SP3-TIGAMY-Lip2T
1854	Y8TT4	pZUS1.1-8UASpTEF-SP4-TIGAMY-Lip2T
1855	Y8TT5	pZUS1.1-8UASpTEF-SP5-TIGAMY-Lip2T
1867	YTT3TO1	pZUA2.II-pTEF-SP3-TIGAMY-Lip2T-pTEF-SP1-OsAA-Lip2T
1868	YTT3TO2	pZUA2.II-pTEF-SP3-TIGAMY-Lip2T-pTEF-SP2-OsAA-Lip2T
1869	YTT3TO5	pZUA2.II-pTEF-SP3-TIGAMY-Lip2T-pTEF-SP5-OsAA-Lip2T
1870	YTT5TO1	pZUA2.II-pTEF-SP5-TIGAMY-Lip2T-pTEF-SP1-OsAA-Lip2T
1871	YTT5TO2	pZUA2.II-pTEF-SP5-TIGAMY-Lip2T-pTEF-SP2-OsAA-Lip2T
1872	YTT5TO5	pZUA2.II-pTEF-SP5-TIGAMY-Lip2T-pTEF-SP5-OsAA-Lip2T
1883	YTO7	pZUS1.1-pTEF-SP7-OsAA-Lip2T
1884	YTT7	pZUS1.1-pTEF-SP7-TIGAMY-Lip2T
1887	Y8TT18TO1	pZUA2.II-8UASpTEF-SP1-TIGAMY-Lip2T-8UASpTEF-SP1-OsAA-Lip2T
1888	Y8TT18TO3	pZUA2.II-8UASpTEF-SP1-TIGAMY-Lip2T-8UASpTEF-SP3-OsAA-Lip2T
1889	Y8TT38TO1	pZUA2.II-8UASpTEF-SP3-TIGAMY-Lip2T-8UASpTEF-SP1-OsAA-Lip2T
1890	Y8TT38TO3	pZUA2.II-8UASpTEF-SP3-TIGAMY-Lip2T-8UASpTEF-SP3-OsAA-Lip2T
1891	Y8TT58TO3	pZUA2.II-8UASpTEF-SP5-TIGAMY-Lip2T-8UASpTEF-SP3-OsAA-Lip2T
1892	Y8TT58TO5	pZUA2.II-8UASpTEF-SP5-TIGAMY-Lip2T-8UASpTEF-SP5-OsAA-Lip2T
2409	YTG1	pZUS1.1-pTEF-SP1-AnGAMY-Lip2T
2796	YTO1-RFP	pZUS1.1-pTEF-SP1-OsAA-Lip2T, pZLS1.1-pTEF-RFP-Lip2T
2798	YTT3-GFP	pZUS1.1-pTEF-SP3-TIGAMY-Lip2T, pZLS1.1-pTEF-GFP-Lip2T
2920	Y8TT1-GFP	pZUS1.1-8UASpTEF-SP3-TIGAMY-Lip2T, pZLS1.1-pTEF-GFP-Lip2T
3489	YTG18TO1	pZUS1.1-pTEF-SP1-AnGAMY-Lip2T, pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T
3492	Y2x8TO1	2x pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T
3495	Y2x8TT1	2x pZUS1.1-8UASpTEF-SP1-TIGAMY-Lip2T
3498	Y2x8TT18TO1	2x pZUA2.II-8UASpTEF-SP1-TIGAMY-Lip2T-8UASpTEF-SP1-OsAA-Lip2T
4265	YTG2	pZUS1.1-pTEF-SP2-AnGAMY-Lip2T
4267	YTG3	pZUS1.1-pTEF-SP3-AnGAMY-Lip2T
XYLAN DEGRADATION (CHAPTER 3)		
2385	YTA1XC	PZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK
2386	YTA2XC	PZUS1.1-pTEF-SP2-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK

2387	YTA3XC	PZUS1.1-pTEF-SP3-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK
2388	YTA4XC	PZUS1.1-pTEF-SP4-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK
2389	YTA5XC	PZUS1.1-pTEF-SP5-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK
2390	YTH1XC	PZUS1.2-pTEF-SP1-HlXyn2-Lip2T, pSB1A3-XDH-XR-XK
2391	YTH2XC	PZUS1.2-pTEF-SP2-HlXyn2-Lip2T, pSB1A3-XDH-XR-XK
2392	YTH3XC	PZUS1.2-pTEF-SP3-HlXyn2-Lip2T, pSB1A3-XDH-XR-XK
2393	YTH4XC	PZUS1.2-pTEF-SP4-HlXyn2-Lip2T, pSB1A3-XDH-XR-XK
2394	YTH5XC	PZUS1.2-pTEF-SP5-HlXyn2-Lip2T, pSB1A3-XDH-XR-XK
2524	YXC	pSB1A3-XDH-XR-XK
4259	YTA1TH1XC	pZUA2.II-pTEF-SP1-AnXlnD-T-pTEF-SP1-HlXyn2-T, pSB1A3-XDH-XR-XK
XYLAN AND/OR STARCH DEGRADATION (CHAPTER 4)		
1846	Y8TO1	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T
1913	YTA1	pZUS1.1-pTEF-SP1-AnXlnD-Lip2T
2385	YTA1XC	PZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK
2524	YXC	pSB1A3-XDH-XR-XK
2800	YTA1-GFP	pZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pZLS1.1-pTEF-GFP-Lip2T
2881	YG-8TO1XC	ΔH XK, pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pSB1A3-XDH-XR-XK
2883	YG-TA1XC	ΔH XK, pZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK
2891	Y8TO1XC	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pSB1A3-XDH-XR-XK
2893	YG-8TO1XC+RFP	ΔH XK, pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-RFP-Lip2T
2895	YG-TA1XC+GFP	ΔH XK, pZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-GFP-Lip2T
2897	YTA1XC-GFP	PZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-GFP-Lip2T
2899	Y8TO1-RFP	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pZLS1.1-pTEF-RFP-Lip2T
2963	Y8TO1XC + RFP	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-RFP-Lip2T
1177	YGFP	pZLS1.1-pTEF-GFP-Lip2T
1259	YRFP	pZLS1.1-pTEF-RFP-Lip2T
3095	YG-XC	ΔH XK, pSB1A3-XDH-XR-XK
3098	YG-XC-GFP	ΔH XK, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-GFP-Lip2T
3101	YG-XC-RFP	ΔH XK, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-RFP-Lip2T
3683	Y8TO1TA1XC	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK
3693	Y8TO1TA1XC+GFP	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-GFP-Lip2T
4263	YG-TRP22XC	ΔH XK, pZUS1.1-pTEF-TRP22, pSB1A3-XDH-XR-XK
4243	YTG1-RFP	pZUS1.1-pTEF-SP1-AnGAMY-Lip2T, pZLS1.1-pTEF-RFP-Lip2T
4269	YTG1XC-RFP	pZUS1.1-pTEF-SP1-AnGAMY-Lip2T, pZLS1.1-pTEF-RFP-Lip2T, pSB1A3-XDH-XR-XK
PATCHOULOL BIOPRODUCTION (CHAPTER 5)		
3305	Y8TT1-HEP	pZUS1.1-pTEF-SP1-TIGAMY-Lip2T, pZLA2.II-pTEF-tHMGLip2T-pTEF-ERG20m-Lip2T, pZUS1.1-pTEF-PcPTS-Lip2T1-3,
3308	Y8TT18TO1-HEP	pZUA2.II-8UASpTEF-SP1-TIGAMY-Lip2T-8UASpTEF-SP1-OsAA-Lip2T, pZLA2.II-pTEF-tHMGLip2T-pTEF-ERG20m-Lip2T, pZUS1.1-pTEF-PcPTS-Lip2T1-3,
3310	YPTS	pZUS1.1-pTEF-PcPTS-Lip2T

3313	Y2xPTS	pZUS1.1-pTEF-PcPTS-Lip2T, pZLS1.1-pTEF-PcPTS-Lip2T
3292	Y3XPTS	pZT4A2.III-3xPcPTS
3556	Y2xHE3xP	2x (pZLA2.II-pTEF-tHMGlip2T-pTEF-ERG20m-Lip2T), 3x (pZUS1.1-pTEF-PcPTS-Lip2T1-3)
3860	Y8TO1-2xHEP	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, 2x (pZLA2.II-pTEF-tHMGlip2T-pTEF-ERG20m-Lip2T, pZUS1.1-pTEF-PcPTS-Lip2T1-3)
3862	Y8TO5-2xHEP	pZUS1.1-8UASpTEF-SP5-OsAA-Lip2T, 2x (pZLA2.II-pTEF-tHMGlip2T-pTEF-ERG20m-Lip2T, pZUS1.1-pTEF-PcPTS-Lip2T1-3)
3864	Y8TT38TO1-2xHEP	pZUA2.II-8UASpTEF-SP3-TIGAMY-Lip2T-8UASpTEF-SP1-OsAA-Lip2T, 2x (pZLA2.II-pTEF-tHMGlip2T-pTEF-ERG20m-Lip2T, pZUS1.1-pTEF-PcPTS-Lip2T1-3)
4261	Y8TA1-2xHEP-XC	pZUS1.1-8UASpTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK, 2x (pZLA2.II-pTEF-tHMGlip2T-pTEF-ERG20m-Lip2T, pZUS1.1-pTEF-PcPTS-Lip2T1-3)
CAROTENE BIOPRODUCTION (CHAPTER 5)		
3640	Y8TO1-RFP-CRT	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pZLS1.1-pTEF-RFP-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3643	Y8TO1XC-RFP-CRT	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-RFP-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3646	YG-8TO1XC-RFP-CRT	ΔHXK, pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-RFP-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3649	YTA1-GFP-CRT	pZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pZLS1.1-pTEF-GFP-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3651	YTA1XC-GFP-CRT	pZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-GFP-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3653	YG-TA1XC-GFP-CRT	ΔHXK, pZUS1.1-pTEF-SP1-AnXlnD-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-GFP-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3703	YTO2CRT	pZUS1.1-pTEF-SP2-OsAA-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3763	Y8TT18TO1CRT	pZUA2.II-8UASpTEF-SP1-TIGAMY-Lip2T-8UASpTEF-SP1-OsAA-Lip2T, ZUA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3764	Y8TO1CRT	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, ZUA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3765	Y8TT1CRT	pZUS1.1-8UASpTEF-SP1-TIGAMY-Lip2T, ZUA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3766	YTT5TO5CRT	pZUA2.II-pTEF-SP5-TIGAMY-Lip2T-pTEF-SP5-OsAA-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3767	YTO5CRT	pZUS1.1-pTEF-SP5-OsAA-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
3768	YTT5CRT	pZUS1.1-pTEF-SP5-TIGAMY-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T
4237	Y8TO1TA1XC-GFP-CRT	pZUS1.1-8UASpTEF-SP1-OsAA-Lip2T, pZUS1.1-pTEF-SP1-XlnD-Lip2T, pSB1A3-XDH-XR-XK, pZLS1.1-pTEF-GFP-Lip2T, ZHA2.III-8UAS TEF-(GGs1-CarB-CarRP)-Lip2T

7.2.2. Plasmids

The plasmid for TRP22 was assembled with a Gibson protocol (7.4.2). All other plasmids were assembled following Golden Gate protocol (7.4.1). The plasmid for xylose consumption was kindly donated by Niehus *et al.* (Niehus et al., 2018).

Table 7.6 Full list of plasmids

RLA Plasmid No.	Name
GOLDEN GATE BIOPARTS	
49*	TOPO T1-3 Lip2
120*	TOPO P1PGM_new (no palindromic)_colony1
235*	TOPO P1 8UASxpr2 x TEF
280*	pYaTK001
524*	pZUS1.1
525*	pZUS1.2
526*	pZUS1.3
528*	pZUA2.III
540*	pYTK001-pTEF
541*	pYTK001-RFPTurbo
675*	pYTK001-hrGFP
684*	pZUA2.II
800*	pZLS1.1
801*	pZLA2.III
802*	pZLA2.II
806	pYTK001-SP1
807	pYTK001-SP2
808	pYTK001-SP3
809	pYTK001-SP4
810	pYTK001-SP5
813	pYTK001-TIGAMY-no SP
814	pYTK001-OSAA-no SP
1092	pYTK001-spLip2pre-3xLA
1103	pYTK001-Xyn2
1104	pTWIST-XlnD
2204	pYTK001-PcPTS
2301*	pZT4A2.III
2332	pYTK001-AnGAMY
FLUORESCENT MARKERS	
1475	pZLS1.1-pTEF-hrGFP-Lip2T1-3
1503	pZLS1.1-pTEF-RFPTurbo-T1/3Lip2
AMYLASE PLASMIDS	
0815	pZUS1.1-pTEF-SP1-OSAA-T1-3Lip2
0816	pZUS1.1-pTEF-SP2-OSAA-T1-3Lip2
0817	pZUS1.1-pTEF-SP3-OSAA-T1-3Lip2

0818	pZUS1.1-pTEF-SP4-OSAA-T1-3Lip2
0819	pZUS1.1-pTEF-SP5-OSAA-T1-3Lip2
0820	pZUS1.1-pTEF-SP1-TIGAMY-T1-3Lip2
0821	pZUS1.1-pTEF-SP2-TIGAMY-T1-3Lip2
0822	pZUS1.1-pTEF-SP3-TIGAMY-T1-3Lip2
0823	pZUS1.1-pTEF-SP4-TIGAMY-T1-3Lip2
0824	pZUS1.1-pTEF-SP5-TIGAMY-T1-3Lip2
1012	pZUS1.1-pPGM-SP1-OSAA-T1-3Lip2
1013	pZUS1.1-pPGM-SP2-OSAA-T1-3Lip2
1014	pZUS1.1-pPGM-SP3-OSAA-T1-3Lip2
1015	pZUS1.1-pPGM-SP4-OSAA-T1-3Lip2
1016	pZUS1.1-pPGM-SP5-OSAA-T1-3Lip2
1017	pZUS1.1-pPGM-SP1-TIGAMY-T1-3Lip2
1018	pZUS1.1-pPGM-SP2-TIGAMY-T1-3Lip2
1019	pZUS1.1-pPGM-SP3-TIGAMY-T1-3Lip2
1020	pZUS1.1-pPGM-SP4-TIGAMY-T1-3Lip2
1021	pZUS1.1-pPGM-SP5-TIGAMY-T1-3Lip2
1082	pZUS1.1-8UAS-pTEF-SP1-OSAA-T1-3Lip2
1083	pZUS1.1-8UAS-pTEF-SP2-OSAA-T1-3Lip2
1084	pZUS1.1-8UAS-pTEF-SP3-OSAA-T1-3Lip2
1085	pZUS1.1-8UAS-pTEF-SP4-OSAA-T1-3Lip2
1086	pZUS1.1-8UAS-pTEF-SP5-OSAA-T1-3Lip2
1087	pZUS1.1-8UAS-pTEF-SP1-TIGAMY-T1-3Lip2
1088	pZUS1.1-8UAS-pTEF-SP2-TIGAMY-T1-3Lip2
1089	pZUS1.1-8UAS-pTEF-SP3-TIGAMY-T1-3Lip2
1090	pZUS1.1-8UAS-pTEF-SP4-TIGAMY-T1-3Lip2
1091	pZUS1.1-8UAS-pTEF-SP5-TIGAMY-T1-3Lip2
1105	pZUA2.II-pTEF-SP3-TIGAMY-pTEF-SP1-OSAA
1106	pZUA2.II-pTEF-SP3-TIGAMY-pTEF-SP2-OSAA
1107	pZUA2.II-pTEF-SP3-TIGAMY-pTEF-SP5-OSAA
1108	pZUA2.II-pTEF-SP5-TIGAMY-pTEF-SP1-OSAA
1109	pZUA2.II-pTEF-SP5-TIGAMY-pTEF-SP2-OSAA
1110	pZUA2.II-pTEF-SP5-TIGAMY-pTEF-SP5-OSAA
1156	pZUS1.2-pTEF-SP1-OSAA-Lip2T1-3
1157	pZUS1.2-pTEF-SP2-OSAA-Lip2T1-3
1158	pZUS1.2-pTEF-SP3-OSAA-Lip2T1-3
1159	pZUS1.2-pTEF-SP4-OSAA-Lip2T1-3
1160	pZUS1.2-pTEF-SP5-OSAA-Lip2T1-3
1161	pZUS1.2-8UAS-pTEF-SP1-OSAA-Lip2T1-3
1162	pZUS1.2-8UAS-pTEF-SP2-OSAA-Lip2T1-3
1163	pZUS1.2-8UAS-pTEF-SP3-OSAA-Lip2T1-3
1164	pZUS1.2-8UAS-pTEF-SP4-OSAA-Lip2T1-3
1165	pZUS1.2-8UAS-pTEF-SP5-OSAA-Lip2T1-3
1254	pZUS1.1-pTEF-SP1-AnGAMY-T1-3 (1)

1255	pZUS1.1-pTEF-SP1-AnGAMY-T1-3 (5)
1256	PZUA2.II-8UASpTEF-SP1-TIGAMY-T1/3-8UASpTEF-SP1-OSAA
1257	PZUA2.II-8UASpTEF-SP1-TIGAMY-T1/3-8UASpTEF-SP3-OSAA
1258	PZUA2.II-8UASpTEF-SP3-TIGAMY-T1/3-8UASpTEF-SP1-OSAA
1259	PZUA2.II-8UASpTEF-SP3-TIGAMY-T1/3-8UASpTEF-SP3-OSAA
1260	PZUA2.II-8UASpTEF-SP5-TIGAMY-T1/3-8UASpTEF-SP3-OSAA
1261	PZUA2.II-8UASpTEF-SP5-TIGAMY-T1/3-8UASpTEF-SP5-OSAA
1474	pZUA2.II-pTEF-SP1-AnXlnD-T-pTEF-SP1-HlXyn2-T
1500	pZUS1.1-pTEF-spLip23xLA-TIGAMY-T1/3Lip2
1501	pZUS1.2-pTEF-spLip23xLA-OsAA-T1/3Lip2
1515	pZUA2.II-pTEF-SP3-TIGAMY-pTEF-SP1-OSAA
3155	pZUS1.1-pTEF-SP2-AnGAMY-Lip2T
3156	pZUS1.1-pTEF-SP3-AnGAMY-Lip2T
XYLANASE PLASMIDS	
1122	PZUS1.1-pTEF-SP1-XlnD-T1-3
1123	PZUS1.1-pTEF-SP2-XlnD-T1-3
1124	PZUS1.1-pTEF-SP3-XlnD-T1-3
1125	PZUS1.1-pTEF-SP4-XlnD-T1-3
1126	PZUS1.1-pTEF-SP5-XlnD-T1-3
1127	PZUS1.2-pTEF-SP1-Xyn2-T1-3
1128	PZUS1.2-pTEF-SP2-Xyn2-T1-3
1129	PZUS1.2-pTEF-SP3-Xyn2-T1-3
1130	PZUS1.2-pTEF-SP4-Xyn2-T1-3
1131	PZUS1.2-pTEF-SP5-Xyn2-T1-3
1272	PZUS1.1-8UASpTEF-SP1-XlnD-T1-3
1273	PZUS1.1-8UASpTEF-SP2-XlnD-T1-3
1274	PZUS1.1-8UASpTEF-SP3-XlnD-T1-3
1275	PZUS1.1-8UASpTEF-SP4-XlnD-T1-3
1276	PZUS1.1-8UASpTEF-SP5-XlnD-T1-3
1277	PZUS1.2-8UASpTEF-SP1-Xyn2-T1-3
1278	PZUS1.2-8UASpTEF-SP2-Xyn2-T1-3
1279	PZUS1.2-8UASpTEF-SP3-Xyn2-T1-3
1281	PZUS1.2-8UASpTEF-SP5-Xyn2-T1-3
2331**	pSB1A3-XDH-XR-XK
3123	pZUS1.1-pTEF-YlTRP22-Lip2T
PATCHOULOL PLASMIDS	
1440***	pZLA2.II-tHMG-ERG20m
2329	pZUS1.1-pTEF-PcPTS-Lip2T1-3
2330	pZLS1.1-pTEF-PcPTS-Lip2T1-3
2362	pZUS1.3-pTEF-PcPTS-T (1)
2676	pZUS1.2-pTEF-PcPTS-T
2679	pZT4A2.III-3xPcPTS
CAROTENE PLASMIDS	
2309***	ZUA2.III-8UAS TEF-carotene (GGS1-CarB-CarRP)

2310***	ZLA2.III-8UAS TEF-carotene (GGS1-CarB-CarRP)
2997	ZHygA2.III-8UAS TEF-carotene (GGS1-CarB-CarRP)

* These plasmids were assembled by members of RLA lab for the YaTK toolkit

** Kindly donated by the lab of Dr Jean-Marc Nicaud (Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay).

*** These plasmids were assembled by Dr Young-Kyoung Park (RLA lab).

7.2.3. Genes

Genes were codon optimized for *Y. lipolytica* using Thermo Fisher's GeneArt online portal. For degradation genes the native signal peptides, identified from the literature and confirmed with SignalP, were removed from the 5' end of the sequence.

Table 7.7 Gene sequences

Name	Sequence 5'-3'
OsAA	CAGGTGCTGTTCCAGGGCTTCAACTGGGAGTCTTGGAAGGAAAACGGCGGCTGGT ACAACTTCCTGATGGGCAAGGTGGACGACATTGCCGCCGCTGGCATCACCCACG TGTGGCTGCCTCCACCTTCTCACTCTGTGGGCGAGCAGGGCTACATGCCCGGAC GACTGTACGACCTGGACGCCTCTAAGTACGGCAACGAGGCCAGCTGAAGTCTCT GATCGAGGCCCTCCACGGCAAGGGCGTGCAGGTCATTGCCGACATCGTGATCAA CCACCGAACC GCCGAGCACAAGGACGGCCGAGGCATCTACTGCCTGTT CGAAG GCGGCACCCCTGACTCTCGACTGGACTGGGGACCCACATGATCTGCCGAGATG ACCCCTACGGCGACGGCACTGGCAACCCGACACCGGCGCTGACTTCGCCGCT GCTCCCGACATCGACCACCTGAACAAGCGAGTGCAGCGAGAGCTGATCGGCTGG CTGGACTGGCTGAAGATGGACATCGGCTTCGACGCCTGGCGACTCGACTTCGCC AAGGGCTACTCTGCCGACATGGCCAAGATCTACATCGACGCTACCGAGCCTTCGT TCGCCGTGGCCGAGATCTGGACCTCTATGGCCAACGGCGGCGACGGAAAGCCC AACTACGACCAGAACGCCCACCGACAAGAGCTGGTGAAGTGGGTCGACCGAGTC GGCGGAGCCAACTCTAACGGCACCGCTTTCGACTTCACCACCAAGGGCATCCTG AACGTGGCCGTGCGAGGGCGAGCTGTGGCGACTGCGAGGCGAGGACGGCAAGG CTCCCGGCATGATCGGATGGTGGCCCGCCAAGGCCACCACCTTCGTGGACAAC CATGACACCGGCTCTACCCAGCACCTGTGGCCTTTTCCATCTGACAAGGTGATGC AGGGATACGCCTACATCCTGACTCACCTGGAAACCCCTGTATCTTCTACGACCA CTTCTTCGACTGGGGCCTGAAGGAAGAGATCGAGCGACTGGTGTCTATCCGAAAC CGACAGGGCATTACCCCGCCTCTGAGCTGCGAATCATGGAAGCCGACTCTGAC CTGTACCTGGCTGAGATTGACGGAAAGGTGATTACCAAGATCGGACCCCGATACG ACGTCGAGCATCTGATCCCCGAGGGCTTCCAGGTGGTTGCCACGGTGACGGCT ACGCCATCTGGGAGAAGATCTAG
TIGAMY	AGACCAGAAACAACCGTTCCTCACGCAACCGGTTCTTTGGATGATTTCTTGGCTGC ACAATCACCAATCGCTTTCCAAGGTATTTGAACAACATTGGTCCATCTGGTGCATAT TCAGAAGGTGTTAATCCAGGTGTTGTTATTGCTTCACCATCTAAGCAAGATCCAGATT ACTTCTACACTTGGGTTAGAGATGCTGCATTGACAGTTCAATATTTGGTTGAAGAATT GGTTGCTGGTAATGCTTCTTTGCAATTCTTGATCCAAGATTACATCTTTCACAAGCTA GATTGCAAACCTGTTGAAAATCCATCAGGTTCTTTGTCTTCAGGTGGTTTAGGTGAACC AAAGTTCCATGTTGATGAAACTGCTTTTACTGATTCTTGGGGTAGACCACAAAGAGAT GGTCCACCATTGAGAGCTATTGCAATGATCTCTTTCGCTAACTATTTGATCGATAACG GTCATCAATCAACAGTTGAAGATATCATCTGGCCAATCGGTAGAAACGATTTGTCATA CGTTTCTCAACATTGGAACGAAACTACATTGATATCTGGGAAGAAGTTCACTTCA TCTTTCTTACTACAGCAGTTCAATATAGAGCTTTAGTTCAAGGTTCTGCTTTGGCATC

	AAAGTTGGGTCATACTTGTGATAACTGTGGTTCTCAAGCACCACAAATTTGTGTTTCT TGCAATCTTACTGGACTGGTTCACATATTTTGGCTAATACTGGTGGTGGTAGATCTGG TAAAGATGTTTCAACTATTTTAGGTGTTATTGGTTCTTTTCGATCCAAATGCTGATTGTGA TGATGTTACATTTCAACCATGTTTACAGCTAGAGCATTAGCTAACCATAAGCAAGTTGTT GATTCTTTTAGATCTATCTATGCAATTAATGCTGGTATTCCATCAGGTTCTGCAGTTGC TGTGGTAGATATCCAGAAGATGTTTACCAAGGTGGTCATCCATGGTACTTGACTAC AGCTGCAGCTGCAGAACAATTGTACGATGCAATCTATCAATGGAACCATGTTGGTCA TATCGATATCAACGCTGTTAATTTGGATTTCTTTAAATCTATCTATCCATCAGCTGCAG AAGGTACATACACTTCTGATTCATCTACTTTCCAAGATATCATCTCAGCAGTTAGAAC ATATGCTGATGGTTTCTTGTCTGTTATTGAAAAGTACACTCCACCAGATAATTTGTTGG CTGAACAATTCCATAGAGAAACAGGTATTCCATTGTCAGCTGCATCTTTAACTTGGTC TTATGCTGCATTGAATACAGCTGCACAAAGAAGAGCTTCTATTGTTCCATCACCATG GAACTCAAACCTCTACAGATTGCTGATAAGTGTTTACGCAACTTCTGCTACAGGTCC ATACGCAACACCAACTAATACAGCTTGGCCAACTACAACCTCAACCACCAGAAAGA CCAGCTTGTACTCCACCATCTGAAGTTACTTTGACTTTTAATGCATTGGTTGATACAG CTTTCGGTCAAACATATATTTGGTTGGTTCTATTCCAGAATTAGGTTTCATGGGACCC AGCAAATGCTTTGTTAATGTCAGCAAATCTTGGACTTCAGGTAATCCAGTTTGGACA TTGTCAATTTCTTACCAGCTGGTACTTCTTTCGAATACAAGTTTATTAGAAAGGATGAT GGTTCATCTGATGTTGTTTGGGAATCTGATCCAAATAGATCATAAATGTTCCAAAAG ATTGCGGTGCTAACACAGCTACAGTAAACTCTTGGTGGAGACATCATCATCATCATC ATTGA
AnGAMY	AACGTGATCTCTAAGCGAGCTACCCTGGACTCTTGGCTGTCTAACGAGGCCACCG TGGCTCGAACCGCCATCCTGAACAACATCGGAGCCGACGGCGCCTGGGTGTCTG GCGCCGACTCTGGCATCGTGGTGGCTTCTCCCTCTACTGACAACCCCGACTACTT CTACACTTGGACCCGAGACTCTGGCCTGGTGTCTAAGACCCTGGTGGACCTGTTC CGAAACGGCGACACCTCTCTGCTGTCTACCATCGAGAACTACATCTCTGCCCAGG CCATCGTGCAGGGCATCTCTAACCCTCTGGCGACCTGTCTCTGGCGCTGGACT GGGCGAGCCCAAGTTCAACGTGGACGAGACTGCCTACACCGGCTCTTGGGGACG ACCCAGCGAGATGGCCCCGCTCTGCGAGCCACCGCCATGATCGGCTTCGGCC AGTGGCTGCTGGACAACGGATACACCTCTACCGCCACCGACATCGTGTGGCCCC TGGTGCAGAACGACCTGTCTTACGTGGCTCAGTACTGGAACCAGACCGGCTACGA CCTGTGGGAAGAAGTGAACGGATCTTCTTCTTACCATTGCCGTGCAGCATCGAG CCCTGGTTCGAGGGCTCTGCCTTCGCTACCGCCGTGGGCTCCTCTTGCTCTTGGTG CGACTCTCAGGCTCCCGAGATCCTGTGCTACCTGCAGTCTTTCTGGACCGGATCTT TCATCCTGGCCAACTTCGACTCTTCTCGATCTGGCAAGGACGCTAACACCCTGCTG GGCTCTATCCACACTTTCGACCCCGAGGCCGCTGCGACGACTCTACCTCCAG CCTTGCTCTCCCCGAGCACTGGCCAACCACAAGGAAGTGGTGGACTCCTTCCGAT CTATCTACACCCTGAACGACGGCCTGTCTGACTCTGAGGCCGTGGCCGTGGGAC GATACCCCGAGGACACCTACTACAACGGCAACCCCTGGTTCCTGTGCACCCTGG CCGCTGCCGAGCAGCTGTACGACGCCCTGTACCAGTGGGACAAGCAGGGCTCT CTCGAGGTGACCGACGTGTCTCTGGACTTCTTCAAGGCCCTGTACTCTGACGCCG CTACCGGAACCTACTCTTCTTCTTCTGCTACCTACTCCTCTATCGTGGACGCCGTCA AGACCTTCGCCGACGGCTTCGTGTCTATCGTCGAGACTCACGCCGCTCTAACGG CTCTATGTCTGAGCAGTACGACAAGTCTGACGGCGAGCAGCTCTCCGCTCGAGAT CTCACCTGGTCTTACGCCGCTCTGCTGACCGCCAACAACCGACGAACTCTGTGG TGCCCGCCTCTTGGGGCGAGACTTCTGCCTCTTCTGTGCCCGGCACCTGTGCCG CCACCTCTGCTATCGGCACCTACTCGTCTGTGACCGTGACCTCTTGGCCCTCCAT CGTGGCCACCGGCGGAACCACCACCACCGCTACTCCACCGGATCTGGCTCTG TGACCTCTACCTCTAAGACCACCGCCACTGCCTCCAAGACCTCCACCTCGACCTC TTCTACCTCTTGCACCACTCCTACCGCTGTGGCCGTACCTTCGACCTGACCGCT ACCACCACCTACGGCGAGAACATCTACCTGGTGGGATCTATCTCTCAGCTCGGCG

	ACTGGGAGACTTCCGACGGAATCGCCCTGTCTGCCGACAAGTATACCTCTTCTGA CCCTCTGTGGTACGTGACTGTGACCCTGCCTGCCGGCGAGTCTTTCGAGTACAAG TTCATCCGAATCGAGTCTGACGACTCTGTGAGTGGGAGTCTGACCCCAACCGAG AGTACACCGTGCCTCAGGCCTGCGGAACCTCCACTGCCACCGTGACCGACACCT GGCGATAA
AnXlnD	CAGGCCAACACCTCTTACGTGGACTACAACATCGAGGCTAACCCCGATCTGTACC CTCTGTGTATCGAGACTATTCCCCTGTCTGTTCCCCGACTGCCAGAACGGACCCCT GCGATCTCACCTGATCTGCGACGAGACTGCTACCCCTTACGACCGAGCCGCCTC TCTGATCTCTCTGTTACCCCTGGACGAGCTGATCGCCAACACCGGAAACACCGGC CTGGGCGTGTCTCGACTGGGACTGCCCGCCTACCAGGTGTGGTCTGAGGCCCTG CACGGCCTGGACCGAGCCAACCTTCTCTGACTCTGGCGCCTACAACCTGGGCCACC TCGTTTCCCCAGCCTATCCTGACCACCGCCGCTCTGAACCGAACTCTGATCCACC AGATCGCCTCTATCATCTCTACCCAGGGCCGAGCCTTCAACAACGCCGGACGATA CGGACTGGACGTGTACGCTCCCAACATCAACACCTTTCGACACCCCGTGTGGGG CCGAGGCCAAGAGACTCCCGGGCGAGGACGTGTCTCTGGCCGCGGTGTACGCCT ACGAGTACATCACCGGCATTACAGGGCCCCGATCCTGAGTCTAACCTGAAGCTGGC CGCCACCGCCAAGCACTACGCCGGCTACGACATCGAGAACTGGCACAACCACT CGCGACTGGGCAACGACATGAACATCACCCAGCAGGACCTGTCTGAGTACTACA CCCCTCAGTTCACGTGGCCGCTCGAGATGCCAAGGTGCAGTCTGTGATGTGTGC CTACAACGCCGTGAACGGCGTGCCCGCCTGCGCCGACTCTTACTTCCTGCAGAC CCTGCTGCGAGACACCTTCGGCTTCGTGGACCACGGCTACGTGTCTCTGACTGC GACGCCGCTTACAACATCTACAACCCCTCACGGATACGCCTCTTCTCAGGCCGCTG CCGCCGCTGAGGCCATCCTGGCCGGAACCGACATCGACTGCGGCACCACCTAC CAGTGGCACCTGAACGAGTCTATCGCCGCTGGCGACCTGTCTCGAGATGACATCG AGCAGGGCGTGATCCGACTGTACACCACTCTGGTGCAGGCCGGCTACTTCGACT CTAACACCACCAAGGCTAACAAACCCCTACCGAGATCTGTCTTGGTCTGACGTGCT CGAGACTGACGCCTGGAACATCTCTTACCAGGCCGCCACTCAGGGCATCGTGCT GCTGAAGAACTCTAACAAACGTGCTGCCCCTGACCGAGAAGGCTTACCCTCCTTCC AACACCACCGTGGCTCTGATCGGCCCTGGGCCAACGCCACCACTCAGCTGCTG GGAACTACTACGGCAACGCTCCCTACATGATCTCGCCCCGAGCCGCTTTCGAAG AGGCCGGATACAAGGTGAACCTCGCCGAAGGCACCGGCATCTCTTACCTCTAC CTCTGGCTTCGCCGCTGCTCTGTCTGCCGCTCAGTCTGCCGACGTGATCATCTAC GCTGGCGGCATCGACAACACCCTCGAGGCCGAGGCTCTCGACCGAGAGTCTATT GCTTGGCCCGGCAACCAGCTGGACCTGATCCAGAAGCTGGCTTCTGCTGCCGGA AAGAAGCCCCTGATCGTCCTGCAGATGGGCGGAGGCCAGGTGGACTCTTCGTCC CTGAAGAACAACACCAACGTGTCTGCTCTGCTGTGGGGCGGCTACCCCGGCCAG TCTGGCGGATTGCCCCTCCGAGACATCATTACCGGCAAGAAGAACCCCGCTGGC CGACTGGTGACCACTCAGTACCCCGCCTCTTACGCCGAGGAATTCCCCGCCACT GACATGAACCTGCGACCTGAGGGCGACAACCCCGGACAGACCTACAAGTGGTAC ACCGGCGAGGCCGTCTACGAGTTCGGCCACGGCCTGTTCTACACTACCTTCGCC GAGTCCTCTTCTAACACTACCACTAAGGAAGTGAAGCTGAACATCCAGGACATCCT GTCTCAGACCCACGAGGACCTGGCTTCTATTACCCAGCTGCCTGTGCTGAACTTCA CCGCCAACATTGAAACACTGGCAAGCTCGAGTCTGACTACACCGCCATGGTGTT CGCTAACACCTCTGACGCTGGCCCCGCTCCTTATCCTAAGAAGTGGCTGGTCGGC TGGGACCGACTCGGAGAGGTGAAGGTGGGCGAGACTCGAGAGCTGCGAGTGCC CGTCGAGGTGGGCTCTTTCGCCCGAGTGAACGAGGACGGCGACTGGGTGCTGTT CCCCGGCACCTTCGAGCTGGCCCTGAACCTCGAGCGAAAGGTGCGAGTGAAGGT CGTCCTCGAGGGCGAAGAGGAAGTGGTGTGCTGAAGTGGCCCGGAAAGGAATAG
HLXyn2	GCTCCTACCGAGTCCTCTGTCTGAGGTGCGAGAAGCGACAGACCATCGGACCCGGC ACCGGCTACTCTAACGGCTACTACTACTCTTACTGGAACGACGGCCACGCTGGCG TGACCTACACCAACGGCGGAGGCGGCTCTTTCACCGTGAACCTGGTCTAACTCTGG

	CAACTTCGTCGGCGGCAAGGGCTGGCAGCCCGGCACCAAGAACAAGGTGATCAA CTTCTCTGGCTCTTACAACCCCAACGGCAACTCTTACCTGTCTATCTACGGCTGGT CGCGAAACCCTCTGATCGAGTACTACATCGTCGAGAACTTCGGCACATACAACCC CTCTACCGGCGCCACCAAGCTCGGCGAGGTGACCTCTGACGGCTCTGTGTACGA CATCTACCGAACTCAGCGAGTGAACCAGCCTTCTATCATCGGCACCGCCACCTTC TACCAGTATTGGTCTGTGCGACGAAACCACCGATCTTCCGGATCTGTGAACACCGC CAACCACTTCAACGCCTGGGCCTCTCACGGACTGACCCTGGGCACCATGGACTA CCAGATCGTCGCCGTCGAGGGCTACTTCTCTTCTGGCTCTGCCTCTATCACCGTGT CTTAA
PcPTS	ATGGAAGTGTACGCCCAGTCTGTGCGCGTGGGAGCCGCTCTCGACCCCTGGCC AACTTTCACCCCTGCGTGTGGGGCGACAAGTTCATCGTGTACAACCCTCAGTCTTG CCAGGCCGGCGAGCGAGAGGAAGCCGAGGAACTGAAGGTGAGCTGAAGCGA GAGCTGAAGGAAGCCTCTGACAACTACATGCGACAGCTGAAGATGGTGGACGCC ATCCAGCGACTGGGCATCGACTACCTGTTCTGTCGAGGACGTGGACGAGGCCCTG AAGAACCTGTTTCGAGATGTTTCGACGCCTTCTGCAAGAACAACCACGACATGCACG CCACCGCTCTGTCTTCCGACTGCTGCGACAGCACGGCTACCGAGTGTCTTGCGA GGTGTTCGAGAAGTTAAGGACGGCAAGGACGGATTCAAGGTGCCCAACGAGGAC GGCGCTGTGGCCGTGCTCGAGTTCCTCGAGGCTACCCACCTCCGAGTGCACGGT GAGGACGTGCTGGACAACGCCTTCGACTTCACCCGAAACTACCTCGAGTCTGTGT ACGCTACCCTGAACGACCCACCGCCAAGCAGGTCCACAACGCTCTGAACGAGT TCTCGTTCGACGAGGACTGCCCCGAGTCGAGGCCCGAAAGTACATCTCTATCTA CGAGCAGTACGCCTCTCACCACAAGGGCCTGCTGAAGCTGGCCAAGCTGGACTT CAACCTGGTGCAGGCCCTGCATCGACGAGAGCTGTCTGAGGACTCTCGATGGTG GAAGACCCTGCAGGTCCCCACCAAGCTGTCTTTCGTGCGAGATCGACTGGTTCGAG TCTTACTTCTGGGCCTCTGGCTCTTACTTCGAGCCCAACTACTCTGTGGCCCGAAT GATCCTGGCCAAGGGCCTCGCCGTCCTGTCTCTGATGGACGACGTGTACGACGC CTACGGCACCTTTGAGGAACTGCAGATGTTACCGACGCCATCGAACGATGGGAC GCCTCTTGCTGGACAAGCTGCCCCGACTACATGAAGATTGTGTACAAGGCTCTGCT GGACGTGTTTGAAGAGGTGACGAGGAACTCATCAAGCTGGGCGCTCCCTACCG AGCCTACTACGGAAGGAAGCCATGAAGTACGCCGCTCGAGCCTACATGGAAGA GGCTCAGTGGCGAGAGCAGAAGCACAAGCCCACCACCAAGGAATATATGAAGCT CGCCACCAAGACCTGCGGCTACATCACCTGATCATCCTGTCTTGTCTGGGCGTC GAAGAGGGCATCGTGACCAAGGAAGCTTTCGACTGGGTTTTCTCTCGACCTCCATT CATCGAGGCCACTCTGATCATTGCCCGACTGGTGAACGACATCACCGGCCACGA GTTTGAGAAGAAGAGAGAGCACGTCCGAACCGCCGTCGAGTGTATATGGAAGAA CACAAGGTGGGCAAGCAAGAGGTGGTGTCTGAGTTCTACAACCAGATGGAATCTG CCTGGAAGGATATCAACGAGGGCTTCCTGCGACCTGTGAGTTCCCCATTCTCT GCTGTACCTGATCCTGAACTCTGTGCGAACCCTCGAGGTGATCTACAAGGAAGGC GACTCTTACACCCACGTGCGACCCGCCATGCAGAACATCATTAAGCAGCTGTACC TCCATCCTGTGCCTTACTAA

7.3. Media and buffers

All reagents were supplied by Sigma Aldrich and enzymes were supplied by New England Bioscience unless otherwise stated.

7.3.1. E. coli Media (LB) and antibiotics

LB was prepared with 25 g/L of LB, and for solid media 20 g/L agar was added. Antibiotics were prepared as in Table 7.8.

Table 7.8 Antibiotic preparation

Antibiotic	Stock concentration	Prepared in	Dilution
Ampicillin	100 mg/mL	Water	1000x
Chloramphenicol	34 mg/mL	100% Ethanol	1000x
Spectinomycin	50 mg/mL	25% Glycerol	1000x
Kanamycin	50 mg/mL	Water	1000x

7.3.2. Yeast Rich/Complex Media (YPD)

YPD medium was prepared with a final concentration of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. For solid media, 20 g/L agar was added. After autoclaving, the desired sugar substrate was added: glucose to a final concentration of 20 g/L, xylose to a final concentration of 20 g/L, or glycerol to a final concentration of 40 g/L (for glucose-non-consuming strains).

7.3.3. Yeast Minimal Media (YNB)

YNB medium with ammonium sulfate was prepared with a final concentration of either 1.7 g/L (Section 3.2-3.5) or 6.7 g/L yeast nitrogen base (YNB) (for all other YNB media) and 50 mM phosphate buffer (pH 6.8). The desired sugar or polysaccharide was added to these concentrations unless otherwise stated in the text; glucose (20 g/L), xylose (20 g/L), soluble starch (10 g/L), starch from potato (10 g/L), xylan (abcr) (10 g/L). For solid media, 20 g/L agar was added. Where necessary for selection, uracil, leucine or tryptophan were added to a final concentration of 0.1 g/L, or hygromycin (Life Technologies) to a final concentration of 300 µg/mL.

7.4. DNA assembly methods

7.4.1. YaTK toolkit

Inserts for transformation were assembled using an in-house Golden Gate cloning method, called YaTK, based on the one published by Larroude *et al.*, 2018. Golden Gate reaction mix was

made up in 200 μ L PCR tubes as follows: 1.5 μ L of type IIS restriction enzyme, 1.5 μ L T7 ligase, 2 μ L T4 ligase buffer, 1 μ L of the vector backbone (25 fmol/ μ L), 1 μ L of each biopart (50 fmol/ μ L), and made up to a total volume of 20 μ L with nuclease-free H₂O. This mix was incubated in a thermocycler (Applied Biosystems Proflex) running the program: 30 cycles of restriction digestion at 42°C for 2 mins and ligation at 16°C for 5 mins, followed by a final digestion at 60°C for 10 mins and heat inactivation at 80°C for 5 mins. Constructs were assembled in final plasmids containing zeta insertion elements (InsUP-zeta/InsDOWN-zeta) for random integration into the *Y. lipolytica* genome and a URA3, LEU2 or HygR marker gene. The full volume of the assembly mix was then used in an *E. coli* transformation.

To switch the markers for already assembled plasmids I used I-SceI digestion and ligation. To prepare the donor and acceptor fragments, the donor fragment was digested for 3 hours with 5 units of I-SceI (Thermo) and the acceptor plasmid was digested with I-SceI and FastAP phosphatase (Thermo) for 3 hours, using 5 units of I-SceI and FastAP to prevent plasmid re-ligation. In both cases, enzymes were heat-inactivated at 75°C for 15 mins post-digestion. The digested fragments were run on an agarose gel, and bands of the correct size were excised and purified using a gel extraction kit (Monarch). The purified donor fragment was ligated to the acceptor plasmid using T4 DNA ligase at a 1:3 molar ratio and incubated at 16°C for more than 8 hours. The ligation reaction was used directly in an *E. coli* transformation.

7.4.2. Gibson Assembly

The Gibson assembly master mix was prepared at a 1.33x final concentration using 100 μ L of 5x isothermal reaction buffer, 2 μ L of T5 exonuclease (1.0 U/ μ L), 6.25 μ L of Phusion DNA polymerase (2 U/ μ L), 50 μ L of Taq DNA ligase (40 U/ μ L), and 216.75 μ L of nuclease-free water.

The 5x isothermal reaction buffer was composed of 0.75 g of PEG-8000, 1.5 mL of 1 M Tris-HCl (pH 7.5), 75 μ L of 1 M MgCl₂, 150 μ L of 2 M DTT, 30 μ L each of 100 mM dATP, dTTP, dCTP, and dGTP, and 300 μ L of 50 mM NAD⁺.

For the Gibson reaction, a total volume of 20 μ L was used, with 5 μ L of PCR fragments added to 15 μ L of the 1.33x master mix. The reaction was performed at 50°C for 60 mins. The volume of DNA added was adjusted according to the number of fragments and fragment size, with a 3:1 ratio of insert to backbone.

Table 7.9 Primers used for Gibson assembly of TRP22 level 1 plasmid

RLA Oligo No.	Name	Sequence 5'-3'
2119	EA_TRP22-(pTEF-Lip2T)_F	GAGTATAAGAATCATTCAAAATGTCGCACCGGCCCTGG GA
2120	EA_TRP22-(pTEF-Lip2T)_R	AGCTTAGATACCACAGACACTCACCTATCAGCATCTTCA CCCATTTC
2123	EA_pTEF-(TRP22)-Lip2T_F	GTGAAGATGCTGATAGGTGAGTGTCTGTGGTATCTAAGC T
2124	EA_pTEF-(TRP22)-Lip2T_R	TCCCAGGGCCGGTGCGACATTTTGAATGATTCTTATACT CAGAAGG

7.5. Preparation of competent cells

7.5.1. Turbo Competent E. coli (NEB)

For Turbo competent cell preparation, TSS (Transformation Stock Solution) was prepared by combining 85 mL LB, 10 g PEG-3350 dissolved in 5 mL H₂O (10%), 5 mL DMSO (5%), and 2 mL of 1M MgCl₂ (where 1.017 g of hydrous or 0.476 g of anhydrous MgCl₂ was dissolved in 5 mL H₂O). KCM (Potassium Calcium Magnesium Solution) was prepared by dissolving 18.64 g KCl (500 mM), 8.32 g CaCl₂ (150 mM), and 11.90 g MgCl₂ (250 mM) in 500 mL H₂O.

On Day 1, Turbo bacteria were streaked onto LB plates with appropriate antibiotics from a -80°C stock. On Day 2, a single colony was grown in 10 mL LB. On Day 3, 10 mL of the overnight culture was added to pre-warmed 500 mL LB in a 1 L flask (1:100 dilution). The culture was grown to an OD₆₀₀ of 0.5–1.0, which took 2–3 hours, and growth was halted by shaking the culture on ice.

The cells were then spun at 3000 rpm for 10 mins or 4700 rpm for 5 mins in a pre-chilled centrifuge. The pellets were resuspended in 50 mL ice-cold TSS (25 mL per 500 mL culture batch) and aliquoted for storage at -80°C.

7.5.2. One Shot™ TOP10 Chemically Competent E. coli (Thermo)

Competent cells were prepared by inoculating 5 mL of LB medium with a loop TOP10 cells from a glycerol stock stored at -80°C and incubating overnight at 37°C. The following morning, 100 mL of LB medium was inoculated with 0.1 mL of the overnight culture and incubated at 30°C until the culture reached an OD₆₀₀ of 0.3-0.35, typically within 5 hours.

Upon reaching the desired OD₆₀₀, the cells were rapidly cooled in a box of ice. They were then transferred to 50 mL tubes and centrifuged at 2500 rpm at 4°C for 10 mins. The supernatant was

discarded, and the cell pellets were resuspended in 20 mL of ice-cold CCMB80 buffer. After a 20-minute incubation on ice, the cells were centrifuged again at 4°C, and the supernatant was discarded. The cells were then resuspended in 2 mL of ice-cold CCMB80 buffer and aliquoted into 30 µL portions in PCR tubes. These aliquots were immediately placed on dry ice and stored at -70°C.

Competence was tested by transforming 50 µL of competent cells with 1 µL of a standard plasmid (1-10 ng/µL) and plated on selective agar plates. Competent cells typically yielded approximately 100-400 colonies.

7.6. Escherichia coli transformation

7.6.1. Turbo Competent *E. coli* (NEB)

200 µL of competent Turbo *E. coli* cells (pre-prepared in house) was thawed on ice and mixed with 50 µL of 5x KCM (500 mM KCl, 150 mM CaCl₂, 250 mM MgCl₂). 50 µL of competent cell-KCM cocktail was added to the Golden Gate reaction mix. Cells were transformed with 10 min at 4°C, 1 min at 42°C, 1 min at 4°C and 60 mins at 37°C, performed in a thermocycler. The transformed *E. coli* was selected by plating on antibiotic LB plates.

7.6.2. One Shot™ TOP10 Chemically Competent *E. coli* (Thermo)

50 µL of One Shot™ TOP10 Chemically Competent *E. coli* were added to the Golden Gate reaction mix. Cells were heat-shocked with 10 min at 4°C, 1 min at 42°C and 1 min at 4°C. The competent cells were then added to 900 µL of LB and incubated at 37°C for 1 hour. The cells were spun down in the centrifuge and 800 µL of LB was removed. The cells were resuspended and plated on antibiotic LB selection plates.

7.7. Plasmid verification

Plasmids were extracted using the QIAGEN Miniprep Kit, following the manufacturer's instructions. The size of the assembly was confirmed by NotI digestion: 0.3 µL of NotI, 1 µL CutSmart Buffer, 100-200 ng DNA, made up to 10 µL with water, incubated at 37°C for 1 hour, and run in DNA gel electrophoresis with 1% agarose at 110 V for 40 mins.

For new bioparts developed during his project, the level 0 and level 1 plasmids were confirmed with sequencing by Source BioScience.

Table 7.10 Primers used for sequencing level 0 and level 1 plasmids

RLA Oligo No.	Name	Sequence 5'-3'
99*	(99TY) YTK001-URAseq-F	GGCCTTTTACGGTTCCTGG
184*	(184TY) YTK001-seqNew-R	TTGATATCGAGCTCGCTTGG
383	EA-SEQ-pZUS1.1-F	GTAGTTGATTTCATAGTGACTGCATATGTTGTG
384	EA-SEQ-pZUS1.1-R	TTACGCAGAGTTAATAAGGTGTTGGGAG

*Designed by Dr Tigran Yuzbashev (RLA lab)

7.8. *Y. lipolytica* transformation

Y. lipolytica was transformed using 100 µL of DTT (Thermo Scientific) transformation mixture (90 µL 50% PEG, 5 µL 2M LiAc pH 6.0, 5 µL 2M DTT), 5 µL of boiled DNA carrier (salmon sperm DNA) and 10 µL linearised DNA (600-800 ng DNA, 0.3 µL NotI, 1 µL buffer, made up to 10 µL with nuclease-free H₂O, incubated at 37°C overnight). The transformation mixture was incubated at 30°C for 30 mins, followed by 39°C for 30 mins. Transformed *Y. lipolytica* cells were washed with sterile water, plated onto selective YNB agar plates with 2% glucose and cultured at 30°C until prototrophic colonies appeared on the plate. For the xylose-utilisation gene cassette, selection was done using YNB with 2% xylose.

The amino acid marker genes are flanked by loxP sites. To recycle marker genes, the strain was transformed with an un-digested pUB4-Hygro-Cre plasmid, as outlined in Fickers *et al.* (2003). Auxotrophic strains were identified by plating on selective plates with and without amino acids.

7.9. *Y. lipolytica* colony PCR

Transformants were verified with colony PCR. 1 µL of colony (suspended in water) was combined with 5 µL 2x Plant Phire Master Mix and 2 µL of each 10 mM construct-specific forward and reverse primer. PCR in the thermocycler followed the protocol: 98°C for 5 mins, followed by 28 cycles of 98°C for 5 mins, T_m for 5 mins, 72°C for 20 s/kb, followed by a final step of 72°C for 1 min. The presence of the correct PCR fragment was confirmed by gel electrophoresis (1% agarose, 110 V, 40 mins).

Table 7.11 Primers used for colony PCR

RLA Oligo No.	Name	Sequence 5'-3'
385	EA-PCR-pTEF-F	GCCAAGGGTATAAAAGACCACCGTCCCCG
386	EA-PCR-OSAA-R	GTTACCAGCTCTTGTCTGGTGGGCG
387	EA-PCR-TIGAMY-R	CACAAGTATGACCCAACCTTTGATGCCAAAGCAGAACC
415	EA-PCR-pPGM-F	GGGTAGGGCAAAGCGCTCAACGGG
452	EA-PCR-XlnD-R	CGTACACGGCGGCCAGAGACACG
453	EA-PCR-Xyn2-R	GCCAGAAGAGAAGTAGCCCTCGACGGC
456	EA-PCR-HXK1-R	GGATGTTCTTTCGCTCGAGAGCGGCC
961	EA-PCR-hrGFP-R	CTTTTCCAGGCGGTGCTGGATGAAGTGG
1574	EA-PCR-yIXDH-F	ATGTCTTCTAACCCGTCATTTGTTCTTCGAAAGCCATTGG
1575	EA-PCR-yIXDH-R	CTACTCCTCCTCGGGACCGTCAATGATGATCTTG
1576	EA-PCR-yIXR-F	ATGTATCTCGGACTGGATCTTTCGACTCAACAGCTCAAG
1577	EA-PCR-yIXR-R	TTATTTCTCCAGGCAGGCGTTTTCGGCCAG
1578	EA-PCR-yIXK-F	ATGTCCTTCAAGCTCGCCTCCGGAAAGTC
1579	EA-PCR-yIXK-R	TTAGGCGAAAATGGGAAGGTTAGCGTAGACACCG
1763	EA-PCR-PcPTS-R	CCCAGAAGTAAGACTCGACCAGTCGATCTCGC
1780	EA-PCR-AnGAMY-R	CGGCCTCGGGGTCGAAAGTGTGG
1839	YP-RFPTurbo500-Rev	CGAGCTTCAGGGCCATCTGGC
1840	EA-PCR-HMGR-F	ATGACCCAGTCTGTGAAGGTGGTCGAGAAG
1841	EA-PCR-HMGR-R	CGACGCCCTTAGAGATCATGTTTCATGCCC
1843	EA-PCR-Erg20-F	ATGTCTAAGGCCAAGTTCGAGTCTGTGTTCCCT
1844	EA-PCR-Erg20-R	GGGGTAGCCTTCTGCAGGGCCTTG
2133	EA-PCR-TRP6-R	CGAGGGCAGCAAAGACTCCGGC
2134	EA-PCR-TRP22-R	GTCTGGTCAGAACTTGTCTGGCGC

7.10. Amylase activity assays

7.10.1. Micro-SIT

To obtain cellular supernatants, cells were seeded to a starting OD600 of 0.1 in 3 mL 2% glucose YNB media in a 15 mL culture flask and grown for 48 hours. Final OD600 was measured, and the cells and supernatant separated by centrifugation.

25 μ L of supernatant was mixed with 25 μ L of 1% starch (Sigma) in solution and incubated at 30°C for 5 to 120 mins. The reaction was stopped by adding 10 μ L of 1 M HCl, and the remaining starch was stained by adding 50 μ L of iodine solution. Absorbance at 580 nm was measured and compared to a standard curve of known concentrations of starch.

7.10.2. Micro-GOPOD

To obtain cellular supernatants, cells were seeded to a starting OD600 of 0.1 in 10 mL 4% glycerol YNB media in a 15 mL culture flask and grown for 72 hours. Final OD600 was measured, and the cells and supernatant separated by centrifugation.

The GOPOD assay was carried out by mixing 10 μ L of 2% starch (Sigma) in solution with 10 μ L of supernatant and incubating at 30°C for 15 mins (For AnGAMY strains) to 48 hours (For TIGAMY strains). 10 μ L of the resulting reaction mix was mixed with 10 μ L of assay buffer and 150 μ L of GOPOD reagent (Megazyme Glucose/Sucrose kit), incubated at 50°C for 20 mins. The glucose produced was measured by absorbance at 510 nm and compared to a standard curve of known concentrations of glucose.

7.11. Growth assays

7.11.1. Growth assays in 96-well plates

Growth of single strains and consortia was determined in 96-well plate format using a Biotek Synergy plate reader (for non-fluorescent strains) or a TECAN plate reader (for GFP/RFP-expressing strains). Strains were grown to stationary phase in 2% glucose YPD and then seeded in 200 μ L of the desired media to an initial absorbance at 600nm (Abs600) of 0.01, as measured in the plate reader. For consortia, both strains were seeded at 0.01, making the total starting inoculation 0.02, unless otherwise stated. Abs600 was measured every 30 mins for 48-72 hours with medium double-orbital shaking.

7.11.2. Predicting desirability of each promoter and signal peptide (SP) for growth

The calculated growth parameters for each replicate growth curve were fitted to a standard least squares model to give the relationship between the factors (promoter and SP) and the responses (growth rate, inflection point and carrying capacity), using JMP Pro 15. This model was used to predict the desirability of each promoter-SP combination. For each of the three responses the desirability function assigns a desirability value between 0 and 1, where 0 is a completely undesirable value and 1 is the ideal value. With multiple responses the overall desirability is calculated as a weighted geometric mean of the desirability functions for the individual responses. Desirability was defined as achieving the maximal growth rate and carrying capacity and the minimal inflection point and the three responses were weighted equally in the overall desirability.

7.11.3. Growth assays in different formats

Strains were cultured overnight in 2% glucose YPD media and then grown overnight in media with volumes corresponding to one-fifth of the maximum capacity of the culture vessel: 600 μ L for 24-well plates, 3 mL for 15 mL tubes, and 10 mL 50 mL flasks. The 24-well plates were sealed with a Breathe-easy film to prevent contamination and evaporation. Cultures were inoculated at an initial absorbance at 600nm (Abs600) of 0.1 and incubated at 30°C in a shaking incubator set to 250 rpm. Growth was monitored by measuring Abs600 every 24 hours over a period of 72 hours using a Byonoy plate reader.

7.11.4. Growth assays in flasks - timepoints

Strains were grown overnight: for the strains designed in Chapter 3 the overnight media was 2% glucose YPD, while for the strains designed in Chapter 4 the overnight media was 4% glycerol YP media, since some of these strains were unable to consume glucose. From these overnight cultures, monocultures were seeded at an initial optical density (OD600) of 0.1 and consortium cultures seeded at an OD600 of 0.05 for each cell type (to a total of 0.1 for the full culture), in 10 mL of media in 50 mL flasks. Cultures were incubated at 30°C with shaking at 250 rpm. OD600 was measured at selected timepoints.

7.11.5. Testing different inoculation ratios

For testing different inoculation ratios, the initial OD600 values were set as follows. For the lower-difference ratios the initial OD600 was calculated for 0.04 monoculture: The initial OD600 values were 0.04 and 0 for the 1:0 ratio, 0.03 and 0.01 for the 3:1 ratio, and 0.02 for the 1:1 ratio. For the higher difference ratios, the initial OD600 was calculated for a 0.1 monoculture: the initial OD600 values were 0.009 and 0.091 for a 1:10 ratio, 0.017 and 0.083 for a 1:5 ratio, and 0.05:0.05 for a 1:1 ratio.

7.11.6. Growth curves in flasks

To obtain full growth curves, three replicates of each sample were seeded at the 0-hour time point, and the cultures were stored in the refrigerator overnight. Subsequently, three additional replicates were set up from the same overnight cultures at the 12-hour time point, resulting in a total of six replicates with a 12-hour delay between the two sets. OD600 (and in some cases flow cytometry) was measured every 4 hours throughout the growth period. The cultures were diluted to remain within the range 0.1-1 OD600 in the spectrophotometer. This occasionally resulted in artificial dips in the overall OD600 when the dilution was increased.

7.11.7. Fitting growth parameters

Growth parameters were obtained using curve-fitting to a four-parameter Gompertz curve (Tjørve & Tjørve, 2017):

$$Y(t) = Y_0 + (Y_s - Y_0) \cdot \exp(-\exp(-r(t - t_i)))$$

where $Y(t)$ is the Abs600/OD600 at time t , Y_0 is the initial Abs600/OD600, Y_s is the carrying capacity (Abs600 at stationary phase), r is the growth rate and t_i is time of the inflection point.

For curves which showed biphasic growth a biphasic logistic model was used (Zwietering et al., 1990):

$$A(t) = \frac{A_1}{1 + e^{-(k_1(t - t_1))}} + \frac{A_2}{1 + e^{-(k_2(t - t_2))}}$$

Where $A(t)$ is the Abs600/OD600 at time t , A_1 and A_2 are the carrying capacity for the first and second phase of growth respectively, k_1 and k_2 are the growth rates for the first and second phase, and t_1 and t_2 are the inflection points (midpoints) of the two phases.

Curve-fitting was done using the `scipy.optimize.curve_fit` function. Fitting was done for each replicate curve individually and adjusted R^2 was calculated for each. For the plate reader assay any fits with adjusted R^2 less than 0.5 were removed for poor fit. For the flask assay any fits with adjusted R^2 less than 0.8 were removed for poor fit. The mean and standard deviations were calculated from the replicates.

7.12. Population tracking

7.12.1. Flow cytometry measurements

Flow cytometry was performed using either:

1. Guava EasyCyte (Cytex). RFP fluorescence detected with RED-G-H and GFP fluorescence detected with GRN-B-H. Gains were set to 6.73 FSC, 1.04 SSC, 1.04 GRN-B, 2.18 RED-G. FSC threshold was set to 76. Flow rate was set to Medium. In Chapter 3, doublet exclusion was not performed, but events which were both GFP+ and RFP+ were excluded. In Chapter 4, doublet exclusion was performed with FSC-A vs FSC-H.
2. Attune Nxt flow cytometer (Invitrogen). RFP fluorescence detected with YL2-H (yellow laser, 561 nm ex./620(15) nm em.) with voltage set to 415 V. GFP fluorescence detected with BL1-H (blue laser, 488 nm ex./530(30) nm em.), with voltage set to 510 V. FSC voltage was set to

100 V and SSC set to 355 V, with a threshold for FSC set as OR 10 x1000. Doublet exclusion was performed with FSC-A vs FSC-H. Flow rate was set to 100 μ L/min.

10000 total events were recorded. Red and green singlets populations were determined by plotting histograms of BL1-H/GRN-B-H and YL2-H/RED-G-H, and exported as a percentage of total singlets. The percentages presented in the data were calculated as GFP or RFP singlet population divided by GFP+RFP singlet population, to get a result out of 100% of total fluorescent events.

7.12.2. Microscopy measurements

Cells were imaged with a Nikon Ti Eclipse fitted with a Rolera EM-C2 EMCCD camera and a p300 coolLED light. Cells were imaged at 200x using the GFP-B filter (469/525 nm) for GFP+ cells and Cy3 filter (618/652 nm) for RFP+ cells. Exposure time was 500 ms.

GFP and RFP images were analysed using Fiji/ImageJ 2.9.0 using the following macro:

- `setAutoThreshold("Triangle no-reset");`
- `setOption("BlackBackground", false);`
- `run("Convert to Mask", "method=Triangle background=Light calculate");`
- `run("Analyze Particles...", "size=5-Infinity show=Nothing exclude include summarize stack");`

The resulting numbers for GFP+ and RFP+ cells were collected for each image and the percentages calculated as GFP+ or RFP+ count divided by GFP+RFP count, to get a result out of 100% of total fluorescent cells. The average and standard deviation were calculated from 5 images.

7.13. Product extraction and downstream analysis methods

7.13.1. Carotene production, extraction and analysis

Strains were grown overnight and then seeded to an initial OD₆₀₀ of 0.1 in 10 mL of the desired media in 50 mL conical flasks. Incubated at 30°C, 250 rpm for 72 hours. 1 mL of cell culture was centrifuged at max speed and the supernatant removed. 500 μ L of 425-600 μ m glass beads (Sigma) were added to the cell pellet, with 1 mL of HPLC-grade acetone. The cells were homogenised using the Precellys Evolution tissue homogeniser, with 2 rounds of 60 s at 6000 rpm. The homogenised mix was centrifuged for 2 mins at max speed, and 600 μ L of the sample

was pipetted into HPLC vials. Standards of β -carotene (Sigma) were prepared to concentrations of 1, 10, 100, 200, 500 and 1000 mg/L.

HPLC was performed by Dr Piotr Hapeta or Dr Jing Fu (Imperial College London, Department of Bioengineering). The analysis was performed using a Vanquish Core HPLC system (Thermo Scientific) equipped with a YMC Carotenoid C30 column (150 x 4.6 mm, 3 μ m particle size, TMC) and a UV detector. The column was maintained at a temperature of 25°C. The flow rate was set at 1 mL/min, and an injection volume of 10 μ L was used. Detection was carried out at a wavelength of 455 nm. The mobile phases consisted of the following: phase A was acetonitrile (ACN), phase B was methanol (MeOH), and phase C was methyl tert-butyl ether (MTBE). The gradient elution profile was as follows:

Time (min)	%A	%B	%C
0.01	3	97	0
3	3	97	0
5	10	85	5
15	15	75	10
30	40	40	20
30.01	3	97	0
40	3	97	0

The samples were analysed with Chromeleon 7.3.1 CDS SE, comparing the area of the peaks with the standard curve to obtain β -carotene concentration.

7.13.2. Patchoulolol production, extraction and analysis

Strains were grown overnight and then seeded to an initial OD600 of 0.1 in 10 mL of the desired media in 50 mL conical flasks. Media composition was 6.7 g/L YNB, 50 mM phosphate buffer plus 2% glucose or 1% starch. Initially, in glucose media, 2 mL of dodecane were added to each flask at the beginning of the culture, and the flasks were sealed with foil and parafilm to prevent evaporation. For the amylase-expressing strains on starch the strains were grown in a fully aerobic environment overnight, and the dodecane layer was added at the 12 hour or 20 hour mark.

After 72 hours, the entire culture was centrifuged and the top layer of dodecane was removed and sent for gas chromatography–mass spectrometry. The pelleted cells were resuspended in 10 ml of water and OD600 was measured with a spectrophotometer.

Samples were analysed by GC-MS by Dr David Bell (Department of Infectious Disease, Imperial College London) using an Agilent 5977A GC-MS operating in electron impact mode, with an Agilent DB-5MS column (30m length, 0.250mm id, 0.25um film thickness). 1 µL of sample was injected in splitless mode. A constant flow of 1.2 mL/min nitrogen was used as carrier gas. The injector temperature was 250 °C. The initial oven temperature was 80 °C. After 1 min, the oven temperature was increased to 120 °C at the rate of 10 °C/min, subsequently increased to 160 °C at the rate of 3 °C/min, and further to 270 °C at the rate of 10 °C/min and was held for 5 min. Patchoulol standards (Merck, 05690595) of 100, 50 and 20 mg/mL were used to identify the substance and calculate concentration.

8. Bibliography

Aditya, C., Bertaux, F., Batt, G. & Ruess, J. (2021) A light tunable differentiation system for the creation and control of consortia in yeast. *Nature Communications* 2021 12:1. 12 (1), 1–10. doi:10.1038/s41467-021-26129-7.

Ahamed, A. & Vermette, P. (2008) Enhanced enzyme production from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA grown as fed batch in a stirred tank bioreactor. *Biochemical Engineering Journal*. 42 (1), 41–46. doi:10.1016/J.BEJ.2008.05.007.

Airoldi, E.M., Huttenhower, C., Gresham, D., Lu, C., Caudy, A.A., Dunham, M.J., Broach, J.R., Botstein, D. & Troyanskaya, O.G. (2009) Predicting Cellular Growth from Gene Expression Signatures A. Rzhetsky (ed.). *PLoS Computational Biology*. 5 (1), e1000257. doi:10.1371/journal.pcbi.1000257.

Alibaba.com (2025) *Anhydrous Glucose High Purity 99.9% Sweeteners Glucose Food Grade Free Sample Cas 50-99-7*. 2025. https://www.alibaba.com/product-detail/Hot-Selling-Product-White-Powder-Anhydrous_1601245423021.html?spm=a2700.galleryofferlist.p_offer.d_title.391f13a0iY6xgt&s=p [Accessed: 11 January 2025].

Alper, H. & Stephanopoulos, G. (2004) Metabolic engineering challenges in the post-genomic era. *Chemical Engineering Science*. 59 (22–23), 5009–5017. doi:10.1016/J.CES.2004.09.027.

Alvarez-Gonzalez, G. & Dixon, N. (2019) Genetically encoded biosensors for lignocellulose valorization. *Biotechnology for Biofuels* 2019 12:1. 12 (1), 1–14. doi:10.1186/S13068-019-1585-6.

Anandharaj, M., Lin, Y.J., Rani, R.P., Nadendla, E.K., Ho, M.C., Huang, C.C., Cheng, J.F., Chang, J.J. & Li, W.H. (2020) Constructing a yeast to express the largest cellulosome complex on the cell surface. *Proceedings of the National Academy of Sciences of the United States of America*. 117 (5), 2385–2394. doi:10.1073/PNAS.1916529117/-/DCSUPPLEMENTAL.

Atkinson, E., Boo, A., Peng, H., Stan, G. & Ledesma-Amaro, R. (2022a) Principles, Tools, and Applications of Synthetic Consortia Toward Microbiome Engineering. *Principles in Microbiome Engineering*. 195–218. doi:10.1002/9783527825462.CH7.

Atkinson, E., Tuza, Z., Perrino, G., Stan, G.B. & Ledesma-Amaro, R. (2022b) Resource-aware whole-cell model of division of labour in a microbial consortium for complex-substrate degradation. *Microbial Cell Factories*. 21 (1), 1–12. doi:10.1186/S12934-022-01842-0/FIGURES/5.

Aulakh, S.K., Sellés Vidal, L., South, E.J., Peng, H., Varma, S.J., Herrera-Dominguez, L., Ralser, M. & Ledesma-Amaro, R. (2023) Spontaneously established syntrophic yeast communities improve bioproduction. *Nature Chemical Biology* 2023 19:8. 19 (8), 951–961. doi:10.1038/s41589-023-01341-2.

Bader, J., Mast-Gerlach, E., Popović, M.K., Bajpai, R. & Stahl, U. (2010) Relevance of microbial coculture fermentations in biotechnology. *Journal of Applied Microbiology*. 109 (2), 371–387. doi:10.1111/j.1365-2672.2009.04659.x.

Bange, G., Brodersen, D.E., Liuzzi, A. & Steinchen, W. (2021) Two P or Not Two P: Understanding Regulation by the Bacterial Second Messengers (p)ppGpp. *Annual review of microbiology*. 75, 383–406. doi:10.1146/ANNUREV-MICRO-042621-122343.

Bayer, E.A., Laura, A., Benatti, T., De, M., Teixeira, L. & Polizeli, M. (2023) Lignocellulolytic

Biocatalysts: The Main Players Involved in Multiple Biotechnological Processes for Biomass Valorization. *Microorganisms* 2023, Vol. 11, Page 162. 11 (1), 162. doi:10.3390/MICROORGANISMS11010162.

Becerra-Pérez, L.A., Rincón, L.E. & Posada-Duque, J.A. (2022) Logistics and Costs of Agricultural Residues for Cellulosic Ethanol Production. *Energies* 2022, Vol. 15, Page 4480. 15 (12), 4480. doi:10.3390/EN15124480.

Beig, B., Riaz, M., Raza Naqvi, S., Hassan, M., Zheng, Z., Karimi, K., Pugazhendhi, A., Atabani, A.E. & Thuy Lan Chi, N. (2021) Current challenges and innovative developments in pretreatment of lignocellulosic residues for biofuel production: A review. *Fuel*. 287, 119670. doi:10.1016/J.FUEL.2020.119670.

Ben-David, Y., Morais, S., Bayer, E.A. & Mizrahi, I. (2020) Rapid adaptation for fibre degradation by changes in plasmid stoichiometry within *Lactobacillus plantarum* at the synthetic community level. *Microbial Biotechnology*. 13 (6), 1748–1764. doi:10.1111/1751-7915.13584.

van den Berg, N.I., Machado, D., Santos, S., Rocha, I., Chacón, J., Harcombe, W., Mitri, S. & Patil, K.R. (2022) Ecological modelling approaches for predicting emergent properties in microbial communities. *Nature Ecology & Evolution* 2022 6:7. 6 (7), 855–865. doi:10.1038/s41559-022-01746-7.

Boehlke, K.W. & Friesen, J.D. (1975) Cellular content of ribonucleic acid and protein in *Saccharomyces cerevisiae* as a function of exponential growth rate: calculation of the apparent peptide chain elongation rate. *Journal of Bacteriology*. 121 (2), 429–433. doi:10.1128/jb.121.2.429-433.1975.

Boo, A., Ellis, T. & Stan, G.B. (2019) Host-aware synthetic biology. *Current Opinion in Systems Biology*. 14 pp.66–72. doi:10.1016/j.coisb.2019.03.001.

Boonvitthya, N., Bozonnet, S., Burapatana, V., O'Donohue, M.J. & Chulalaksananukul, W. (2013) Comparison of the heterologous expression of *Trichoderma reesei* endoglucanase II and cellobiohydrolase II in the yeasts *Pichia pastoris* and *Yarrowia lipolytica*. *Molecular biotechnology*. 54 (2), 158–169. doi:10.1007/S12033-012-9557-0.

Borkowski, O., Ceroni, F., Stan, G.-B. & Ellis, T. (2016) Overloaded and stressed: whole-cell considerations for bacterial synthetic biology. *Current Opinion in Microbiology*. 33, 123–130. doi:10.1016/J.MIB.2016.07.009.

Braga, A. & Belo, I. (2016) Biotechnological production of γ -decalactone, a peach like aroma, by *Yarrowia lipolytica*. *World Journal of Microbiology and Biotechnology*. 32 (10). doi:10.1007/s11274-016-2116-2.

Brocchieri, L. & Karlin, S. (2005) Protein length in eukaryotic and prokaryotic proteomes. *Nucleic Acids Research*. 33 (10), 3390–3400. doi:10.1093/nar/gki615.

Cao, L., Li, J., Yang, Z., Hu, X. & Wang, P. (2023) A review of synthetic biology tools in *Yarrowia lipolytica*. *World Journal of Microbiology and Biotechnology* 2023 39:5. 39 (5), 1–18. doi:10.1007/S11274-023-03557-9.

Cao, Z., Yan, W., Ding, M. & Yuan, Y. (2022) Construction of microbial consortia for microbial degradation of complex compounds. *Frontiers in Bioengineering and Biotechnology*. 10, 1051233. doi:10.3389/FBIOE.2022.1051233/BIBTEX.

Cardinale, S. & Arkin, A.P. (2012) Contextualizing context for synthetic biology--identifying causes of failure of synthetic biological systems. *Biotechnology journal*. 7 (7), 856–866. doi:10.1002/biot.201200085.

- Carlos, C., Fan, H. & Currie, C.R. (2018) Substrate Shift Reveals Roles for Members of Bacterial Consortia in Degradation of Plant Cell Wall Polymers. *Frontiers in Microbiology*. 0 (MAR), 364. doi:10.3389/FMICB.2018.00364.
- Cavallo, E., Charreau, H., Cerrutti, P. & Foresti, M.L. (2017) *Yarrowia lipolytica*: A model yeast for citric acid production. *FEMS Yeast Research*. 17 (8) p.84. doi:10.1093/femsyr/fox084.
- Celińska, E., Borkowska, M., Białas, W., Korpys, P. & Nicaud, J.-M. (2018) Robust signal peptides for protein secretion in *Yarrowia lipolytica*: identification and characterization of novel secretory tags. *Applied Microbiology and Biotechnology*. 102 (12), 5221–5233. doi:10.1007/s00253-018-8966-9.
- Celińska, E., Borkowska, M., Korpys-Woźniak, P., Kubiak, M., Nicaud, J.M., Kubiak, P., Gorczyca, M. & Białas, W. (2020) Optimization of *Yarrowia lipolytica*-based consolidated biocatalyst through synthetic biology approach: transcription units and signal peptides shuffling. *Applied Microbiology and Biotechnology*. 104 (13), 5845–5859. doi:10.1007/s00253-020-10644-6.
- Celińska, E. & Gorczyca, M. (2024) ‘Small volume—big problem’: culturing *Yarrowia lipolytica* in high-throughput micro-formats. *Microbial Cell Factories*. 23 (1), 1–17. doi:10.1186/S12934-024-02465-3/FIGURES/5.
- Celińska, E., Ledesma-Amaro, R., Larroude, M., Rossignol, T., Pauthenier, C. & Nicaud, J.-M. (2017) Golden Gate Assembly system dedicated to complex pathway manipulation in *Yarrowia lipolytica*. *Microbial biotechnology*. 10 (2), 450–455. doi:10.1111/1751-7915.12605.
- Celińska, E. & Nicaud, J.M. (2019) Filamentous fungi-like secretory pathway strayed in a yeast system: peculiarities of *Yarrowia lipolytica* secretory pathway underlying its extraordinary performance. *Applied Microbiology and Biotechnology*. 103 (1) pp.39–52. doi:10.1007/s00253-018-9450-2.
- Celińska, E., Nicaud, J.M. & Białas, W. (2021) Hydrolytic secretome engineering in *Yarrowia lipolytica* for consolidated bioprocessing on polysaccharide resources: review on starch, cellulose, xylan, and inulin. *Applied Microbiology and Biotechnology*. 105 (3) pp.975–989. doi:10.1007/s00253-021-11097-1.
- Ceroni, F., Algar, R., Stan, G.-B.B. & Ellis, T. (2015) Quantifying cellular capacity identifies gene expression designs with reduced burden. *Nature Methods*. 12 (5), 415–418. doi:10.1038/nmeth.3339.
- Ceroni, F., Boo, A., Furini, S., Gorochofski, T.E., Borkowski, O., Ladak, Y.N., Awan, A.R., Gilbert, C., Stan, G.B. & Ellis, T. (2018) Burden-driven feedback control of gene expression. *Nature Methods*. 15 (5), 387–393. doi:10.1038/nmeth.4635.
- Chandrasekaran, S. & Price, N.D. (2010) Probabilistic integrative modeling of genome-scale metabolic and regulatory networks in *Escherichia coli* and *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*. 107 (41), 17845–17850. doi:10.1073/PNAS.1005139107/SUPPL_FILE/SFIG08.TIF.
- Chappell, T.C. & Nair, N.U. (2017) Co-utilization of hexoses by a microconsortium of sugar-specific *E. coli* strains. *Biotechnology and Bioengineering*. 114 (10), 2309–2318. doi:10.1002/BIT.26351.
- Charubin, K. & Papoutsakis, E.T. (2019) Direct cell-to-cell exchange of matter in a synthetic *Clostridium* syntrophy enables CO₂ fixation, superior metabolite yields, and an expanded metabolic space. *Metabolic Engineering*. 52, 9–19. doi:10.1016/J.YMBEN.2018.10.006.
- Che, S. & Men, Y. (2019) Synthetic microbial consortia for biosynthesis and biodegradation:

promises and challenges. *Journal of Industrial Microbiology and Biotechnology*. 46 (9–10), 1343–1358. doi:10.1007/S10295-019-02211-4.

Chen, K.-J., Tang, J.-C., Xu, B.-H., Lan, S.-L. & Cao, Y. (2019) Degradation enhancement of rice straw by co-culture of *Phanerochaete chrysosporium* and *Trichoderma viride*. *Scientific Reports* 2019 9:1. 9 (1), 1–7. doi:10.1038/s41598-019-56123-5.

Chen, L., Du, J.L., Zhan, Y.J., Li, J.A., Zuo, R.R. & Tian, S. (2018) Consolidated bioprocessing for cellulosic ethanol conversion by cellulase–xylanase cell-surfaced yeast consortium. <https://doi.org/10.1080/10826068.2018.1487846>. 48 (7), 653–661. doi:10.1080/10826068.2018.1487846.

Chen, M.-T. & Weiss, R. (2005) Artificial cell-cell communication in yeast *Saccharomyces cerevisiae* using signaling elements from *Arabidopsis thaliana*. *Nature Biotechnology*. 23 (12), 1551–1555. doi:10.1038/nbt1162.

Chen, N., Koumpouras, G.C., Polizzi, K.M. & Kontoravdi, C. (2012) Genome-based kinetic modeling of cytosolic glucose metabolism in industrially relevant cell lines: *Saccharomyces cerevisiae* and Chinese hamster ovary cells. *Bioprocess and Biosystems Engineering*. 35 (6), 1023–1033. doi:10.1007/s00449-012-0687-3.

Chiu, H.C., Levy, R. & Borenstein, E. (2014) Emergent Biosynthetic Capacity in Simple Microbial Communities. *PLOS Computational Biology*. 10 (7), e1003695. doi:10.1371/JOURNAL.PCBI.1003695.

Colarusso, A. V., Goodchild-Michelman, I., Rayle, M. & Zomorodi, A.R. (2021) Computational modeling of metabolism in microbial communities on a genome-scale. *Current Opinion in Systems Biology*. 26, 46–57. doi:10.1016/J.COISB.2021.04.001.

Copete-Pertuz, L.S., Alandete-Novoa, F., Plácido, J., Correa-Londoño, G.A. & Mora-Martínez, A.L. (2019) Enhancement of ligninolytic enzymes production and decolourising activity in *Leptosphaerulina* sp. by co-cultivation with *Trichoderma viride* and *Aspergillus terreus*. *Science of The Total Environment*. 646, 1536–1545. doi:10.1016/J.SCITOTENV.2018.07.387.

Corrado, I., Petrillo, C., Isticato, R., Casillo, A., Corsaro, M.M., Sannia, G. & Pezzella, C. (2021) The power of two: An artificial microbial consortium for the conversion of inulin into Polyhydroxyalkanoates. *International Journal of Biological Macromolecules*. 189, 494–502. doi:10.1016/J.IJBIOMAC.2021.08.123.

Czajka, J.J., Nathenson, J.A., Benites, V.T., Baidoo, E.E.K., Cheng, Q., Wang, Y. & Tang, Y.J. (2018) Engineering the oleaginous yeast *Yarrowia lipolytica* to produce the aroma compound β -ionone. *Microbial Cell Factories*. 17 (1), 136. doi:10.1186/s12934-018-0984-x.

Dahal, S., Zhao, J. & Yang, L. (2020) Genome-scale Modeling of Metabolism and Macromolecular Expression and Their Applications. *Biotechnology and Bioprocess Engineering*. 25 (6) pp.931–943. doi:10.1007/s12257-020-0061-2.

Dahl, R.H., Zhang, F., Alonso-Gutierrez, J., Baidoo, E., Batth, T.S., Redding-Johanson, A.M., Petzold, C.J., Mukhopadhyay, A., Lee, T.S., Adams, P.D. & Keasling, J.D. (2013) Engineering dynamic pathway regulation using stress-response promoters. *Nature Biotechnology* 2013 31:11. 31 (11), 1039–1046. doi:10.1038/nbt.2689.

Darlington, A.P.S., Kim, J., Jiménez, J.I. & Bates, D.G. (2018) Dynamic allocation of orthogonal ribosomes facilitates uncoupling of co-expressed genes. *Nature Communications* 2018 9:1. 9 (1), 1–12. doi:10.1038/s41467-018-02898-6.

Darvishi, F., Fathi, Z., Ariana, M. & Moradi, H. (2017) *Yarrowia lipolytica* as a workhorse for biofuel

production. *Biochemical Engineering Journal*.127 pp.87–96.
<https://www.sciencedirect.com/science/article/pii/S1369703X17302164>.

Demiray, E., González-Fernández, C. & Tomás-Pejó, E. (2024) Kluyveromyces marxianus and Bacillus coagulans co-cultivation: Efficient co-production of bioethanol and lactic acid from pomegranate peels. *Bioresource Technology Reports*. 25, 101808. doi:10.1016/J.BITEB.2024.101808.

Dong, H., Nilsson, L. & Kurland, C.G. (1995) Gratuitous overexpression of genes in Escherichia coli leads to growth inhibition and ribosome destruction. *Journal of Bacteriology*. 177 (6), 1497–1504. doi:10.1128/JB.177.6.1497-1504.1995.

Du, C., Li, Y., Xiang, R. & Yuan, W. (2022) Formate Dehydrogenase Improves the Resistance to Formic Acid and Acetic Acid Simultaneously in Saccharomyces cerevisiae. *International Journal of Molecular Sciences*. 23 (6). doi:10.3390/IJMS23063406/S1.

Dujon, B., Sherman, D., Fischer, G., Durrens, P., Casaregola, S., et al. (2004) Genome evolution in yeasts. *Nature*. 430 (6995), 35–44. doi:10.1038/nature02579.

Dulermo, R., Brunel, F., Dulermo, T., Ledesma-Amaro, R., Vion, J., Trassaert, M., Thomas, S., Nicaud, J.-M. & Leplat, C. (2017) Using a vector pool containing variable-strength promoters to optimize protein production in Yarrowia lipolytica. *Microbial Cell Factories*. 16 (1), 31. doi:10.1186/s12934-017-0647-3.

Duncker, K.E., Holmes, Z.A. & You, L. (2021) Engineered microbial consortia: strategies and applications. *Microbial Cell Factories* 2021 20:1. 20 (1), 1–13. doi:10.1186/S12934-021-01699-9.

Duquesne, S., Bozonnet, S., Bordes, F., Dumon, C., Nicaud, J.-M. & Marty, A. (2014) Construction of a highly active xylanase displaying oleaginous yeast: comparison of anchoring systems. *PloS one*. 9 (4), e95128. doi:10.1371/journal.pone.0095128.

Eiteman, M.A., Lee, S.A. & Altman, E. (2008) A co-fermentation strategy to consume sugar mixtures effectively. *Journal of Biological Engineering*. 2 (1), 3. doi:10.1186/1754-1611-2-3.

El-Fallal, A., Abou, M., El-Sayed, A. & Omar, N. (2012) Starch and Microbial α -Amylases: From Concepts to Biotechnological Applications. In: *Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology*. InTech. p. doi:10.5772/51571.

Ellis, T. (2019) Predicting how evolution will beat us. *Microbial Biotechnology*.12 (1) pp.41–43. doi:10.1111/1751-7915.13327.

Ezeorba, T.P.C., Okeke, E.S., Mayel, M.H., Nwuche, C.O. & Ezike, T.C. (2024) Recent advances in biotechnological valorization of agro-food wastes (AFW): Optimizing integrated approaches for sustainable biorefinery and circular bioeconomy. *Bioresource Technology Reports*. 26, 101823. doi:10.1016/J.BITEB.2024.101823.

Fan, Z. (2014) Consolidated Bioprocessing for Ethanol Production. In: *Biorefineries: Integrated Biochemical Processes for Liquid Biofuels*. Elsevier Inc. pp. 141–160. doi:10.1016/B978-0-444-59498-3.00007-5.

Fedorec, A.J.H., Karkaria, B.D., Sulu, M. & Barnes, C.P. (2021) Single strain control of microbial consortia. *Nature Communications* 2021 12:1. 12 (1), 1–12. doi:10.1038/s41467-021-22240-x.

Fickers, P., Le Dall, M.T., Gaillardin, C., Thonart, P. & Nicaud, J.M. (2003) New disruption cassettes for rapid gene disruption and marker rescue in the yeast Yarrowia lipolytica. *Journal of Microbiological Methods*. 55 (3), 727–737. doi:10.1016/j.mimet.2003.07.003.

Firmenich (2023) CLEARWOOD®. 2023. <https://www.firmenich.com/product/clearwoodr-pe>

970953-0 [Accessed: 4 December 2023].

Flores, A.D., Ayla, E.Z., Nielsen, D.R. & Wang, X. (2019) Engineering a Synthetic, Catabolically Orthogonal Coculture System for Enhanced Conversion of Lignocellulose-Derived Sugars to Ethanol. *ACS Synthetic Biology*. 8 (5), 1089–1099. doi:10.1021/ACSSYNBIO.9B00007.

Freitag, M. & Morrell, J.J. (1992) Changes in selected enzyme activities during growth of pure and mixed cultures of white-rot decay fungus *Trametes versicolor* and the potential biocontrol fungus *Trichoderma harzianum*. *Canadian Journal of Microbiology*. 38 (4), 317–323. doi:10.1139/m92-053.

Fritts, R.K., McCully, A.L. & McKinlay, J.B. (2021) Extracellular Metabolism Sets the Table for Microbial Cross-Feeding. *Microbiology and Molecular Biology Reviews*. 85 (1). doi:10.1128/MMBR.00135-20.

Fujii, M. & Kawamura, Y. (1985) Synergistic action of α -amylase and glucoamylase on hydrolysis of starch. *Biotechnology and Bioengineering*. 27 (3), 260–265. doi:10.1002/bit.260270308.

Futcher, B., Latter, G.I., Monardo, P., McLaughlin, C.S. & Garrels, J.I. (1999) A Sampling of the Yeast Proteome. *Molecular and Cellular Biology*. 19 (11), 7357–7368. doi:10.1128/mcb.19.11.7357.

Ganesan, V., Spagnuolo, M., Agrawal, A., Smith, S., Gao, D. & Blenner, M. (2019) Advances and opportunities in gene editing and gene regulation technology for *Yarrowia lipolytica*. *Microbial cell factories*. 18 (1). doi:10.1186/S12934-019-1259-X.

Garner, K.L. (2021) Principles of synthetic biology. *Essays in Biochemistry*. 65 (5), 791. doi:10.1042/EBC20200059.

Gavrilescu, M. & Chisti, Y. (2005) Biotechnology—a sustainable alternative for chemical industry. *Biotechnology Advances*. 23 (7–8), 471–499. doi:10.1016/J.BIOTECHADV.2005.03.004.

Gladkov, G. V., Kimeklis, A.K., Afonin, A.M., Lisina, T.O., Orlova, O. V., Aksenova, T.S., Kichko, A.A., Pinaev, A.G. & Andronov, E.E. (2022) The Structure of Stable Cellulolytic Consortia Isolated from Natural Lignocellulosic Substrates. *International Journal of Molecular Sciences*. 23 (18), 10779. doi:10.3390/IJMS231810779/S1.

Glick, B.R. (1995) Metabolic load and heterologous gene expression. *Biotechnology Advances*. 13 (2) pp.247–261. doi:10.1016/0734-9750(95)00004-A.

Goelzer, A., Muntel, J., Chubukov, V., Jules, M., Prestel, E., Nölker, R., Mariadassou, M., Aymerich, S., Hecker, M., Noirot, P., Becher, D. & Fromion, V. (2015) Quantitative prediction of genome-wide resource allocation in bacteria. *Metabolic Engineering*. 32, 232–243. doi:10.1016/J.YMBEN.2015.10.003.

Gopal, C. V, Broad, D. & Lloyd, D. (1989) Bioenergetic consequences of protein overexpression in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*. 30 (2), 160–165. doi:10.1007/BF00264005.

Görgens, J.F., Van Zyl, W.H., Knoetze, J.H. & Hahn-Hägerdal, B. (2005) Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium. *Applied Microbiology and Biotechnology*. 67 (5), 684–691. doi:10.1007/s00253-004-1803-3.

Görgens, J.F., Van Zyl, W.H., Knoetze, J.H. & Hahn-Hägerdal, B. (2001) The metabolic burden of the PGK1 and ADH2 promoter systems for heterologous xylanase production by *saccharomyces cerevisiae* in defined medium. *Biotechnology and Bioengineering*. 73 (3), 238–245.

doi:10.1002/bit.1056.

Gorochowski, T.E., Avciilar-Kucukgoze, I., Bovenberg, R.A.L., Roubos, J.A. & Ignatova, Z. (2016) A Minimal Model of Ribosome Allocation Dynamics Captures Trade-offs in Expression between Endogenous and Synthetic Genes. *ACS Synthetic Biology*. 5 (7), 710–720. doi:10.1021/acssynbio.6b00040.

Guo, W., Sheng, J. & Feng, X. (2016) ¹³C-Metabolic Flux Analysis: An Accurate Approach to Demystify Microbial Metabolism for Biochemical Production. *Bioengineering*. 3 (1). doi:10.3390/BIOENGINEERING3010003.

Guo, X., Li, Z., Wang, X., Wang, J., Chala, J., Lu, Y. & Zhang, H. (2019) De novo phenol bioproduction from glucose using biosensor-assisted microbial coculture engineering. *Biotechnology and Bioengineering*. 116 (12), 3349–3359. doi:10.1002/BIT.27168.

Guo, X., Wang, X., Chen, T., Lu, Y. & Zhang, H. (2020) Comparing E. coli mono-cultures and co-cultures for biosynthesis of protocatechuic acid and hydroquinone. *Biochemical Engineering Journal*. 156. doi:10.1016/j.bej.2020.107518.

Guo, Z., Duquesne, S., Bozonnet, S., Cioci, G., Nicaud, J.M., Marty, A. & O'Donohue, M.J. (2015) Development of cellobiose-degrading ability in *Yarrowia lipolytica* strain by overexpression of endogenous genes. *Biotechnology for Biofuels*. 8 (1), 109. doi:10.1186/s13068-015-0289-9.

Guo, Z.P., Duquesne, S., Bozonnet, S., Cioci, G., Nicaud, J.M., Marty, A. & O'Donohue, M.J. (2017) Conferring cellulose-degrading ability to *Yarrowia lipolytica* to facilitate a consolidated bioprocessing approach. *Biotechnology for Biofuels*. 10 (1), 132. doi:10.1186/s13068-017-0819-8.

Guo, Z.P., Robin, J., Duquesne, S., O'Donohue, M.J., Marty, A. & Bordes, F. (2018) Developing cellulolytic *Yarrowia lipolytica* as a platform for the production of valuable products in consolidated bioprocessing of cellulose. *Biotechnology for Biofuels*. 11 (1), 141. doi:10.1186/s13068-018-1144-6.

Gupta, V., Sengupta, M., Prakash, J. & Tripathy, B.C. (2016) An Introduction to Biotechnology. *Basic and Applied Aspects of Biotechnology*. 1. doi:10.1007/978-981-10-0875-7_1.

Gutiérrez, M., Gregorio-Godoy, P., Pérez Del Pulgar, G., Munoz, L.E., Sáez, S. & Rodríguez-Patón, A. (2017) A New Improved and Extended Version of the Multicell Bacterial Simulator gro. *ACS Synthetic Biology*. 6 (8), 1496–1508. doi:10.1021/acssynbio.7b00003.

von der Haar, T. (2008) A quantitative estimation of the global translational activity in logarithmically growing yeast cells. *BMC Systems Biology*. 2, 87. doi:10.1186/1752-0509-2-87.

Han, Y.H., Kim, G. & Seo, S.W. (2023) Programmable synthetic biology tools for developing microbial cell factories. *Current Opinion in Biotechnology*. 79, 102874. doi:10.1016/J.COPBIO.2022.102874.

Hanly, T.J. (2013) Dynamic Modeling of Synthetic Microbial Consortia to Optimize the Co-fermentation of Glucose and Xylose. *University of Massachusetts AND#160;and#8211; Amherst*. http://scholarworks.umass.edu/open_access_dissertations/797/.

Hanly, T.J. & Henson, M.A. (2011) Dynamic flux balance modeling of microbial co-cultures for efficient batch fermentation of glucose and xylose mixtures. *Biotechnology and Bioengineering*. doi:10.1002/bit.22954.

Hanly, T.J. & Henson, M.A. (2013) Dynamic metabolic modeling of a microaerobic yeast co-culture: Predicting and optimizing ethanol production from glucose/xylose mixtures.

Biotechnology for Biofuels. doi:10.1186/1754-6834-6-44.

Hanly, T.J. & Henson, M.A. (2014) Dynamic model-based analysis of furfural and HMF detoxification by pure and mixed batch cultures of *S. cerevisiae* and *S. stipitis*. *Biotechnology and Bioengineering*. 111 (2), 272–284. doi:10.1002/BIT.25101.

Hanly, T.J., Urello, M. & Henson, M.A. (2012) Dynamic flux balance modeling of *S. cerevisiae* and *E. coli* co-cultures for efficient consumption of glucose/xylose mixtures. *Applied Microbiology and Biotechnology*. 93 (6), 2529–2541. doi:10.1007/s00253-011-3628-1.

Hapeta, P., Szczepańska, P., Neuvéglise, C. & Lazar, Z. (2021) A 37-amino acid loop in the *Yarrowia lipolytica* hexokinase impacts its activity and affinity and modulates gene expression. *Scientific Reports*. 11 (1), 6412. doi:10.1038/s41598-021-85837-8.

Harcombe, W.R., Chacón, J.M., Adamowicz, E.M., Chubiz, L.M. & Marx, C.J. (2018) Evolution of bidirectional costly mutualism from byproduct consumption. *Proceedings of the National Academy of Sciences*. 115 (47), 12000–12004. doi:10.1073/PNAS.1810949115.

Hartline, C.J., Schmitz, A.C., Han, Y. & Zhang, F. (2021) Dynamic control in metabolic engineering: Theories, tools, and applications. *Metabolic Engineering*. 63, 126–140. doi:10.1016/J.YMBEN.2020.08.015.

Harvey, E., Heys, J. & Gedeon, T. (2014) Quantifying the effects of the division of labor in metabolic pathways. *Journal of Theoretical Biology*. 360, 222–242. doi:10.1016/j.jtbi.2014.07.011.

Hays, S.G., Yan, L.L.W., Silver, P.A. & Ducat, D.C. (2017) Synthetic photosynthetic consortia define interactions leading to robustness and photoproduction. *Journal of Biological Engineering*. doi:10.1186/s13036-017-0048-5.

Henke, N.A., Wichmann, J., Baier, T., Frohwitter, J., Lauersen, K.J., Risse, J.M., Peters-Wendisch, P., Kruse, O. & Wendisch, V.F. (2018) Patchoulol Production with Metabolically Engineered *Corynebacterium glutamicum*. *Genes*. 9 (4). doi:10.3390/GENES9040219.

Henson, M.A. & Hanly, T.J. (2014) Dynamic flux balance analysis for synthetic microbial communities. *IET Systems Biology*. 8 (5), 214–229. doi:10.1049/iet-syb.2013.0021.

Hickert, L.R., Da Cunha-Pereira, F., De Souza-Cruz, P.B., Rosa, C.A. & Ayub, M.A.Z. (2013) Ethanogenic fermentation of co-cultures of *Candida shehatae* HM 52.2 and *Saccharomyces cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate. *Bioresource Technology*. 131, 508–514. doi:10.1016/J.BIORTECH.2012.12.135.

High Level Panel of Experts on Food Security and Nutrition (HLPE) (2014) *Food Losses and Waste in the Context of Sustainable Food Systems*. <https://www.fao.org/3/a-i3901e.pdf>.

Ho, J.C.H., Pawar, S. V., Hallam, S.J. & Yadav, V.G. (2018) An Improved Whole-Cell Biosensor for the Discovery of Lignin-Transforming Enzymes in Functional Metagenomic Screens. *ACS Synthetic Biology*. 7 (2), 392–398. doi:10.1021/ACSSYNBIO.7B00412/SUPPL_FILE/SB7B00412_SI_001.PDF.

Van Hoek, P., Flikweert, M.T., Van Der Aart, Q.J.M., Steensma, H.Y., Van Dijken, J.P. & Pronk, J.T. (1998) Effects of Pyruvate Decarboxylase Overproduction on Flux Distribution at the Pyruvate Branch Point in *Saccharomyces cerevisiae*. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*. 64 (6). <http://aem.asm.org/>.

Honjo, H., Iwasaki, K., Soma, Y., Tsuruno, K., Hamada, H. & Hanai, T. (2019) Synthetic microbial consortium with specific roles designated by genetic circuits for cooperative chemical

production. *Metabolic Engineering*. 55, 268–275. doi:10.1016/J.YMBEN.2019.08.007.

Ibrahim, M., Raajaraam, L. & Raman, K. (2021) Modelling microbial communities: Harnessing consortia for biotechnological applications. *Computational and Structural Biotechnology Journal*. 19, 3892. doi:10.1016/J.CSBJ.2021.06.048.

Idiris, A., Tohda, H., Kumagai, H. & Takegawa, K. (2010) Engineering of protein secretion in yeast: Strategies and impact on protein production. *Applied Microbiology and Biotechnology*. 86 (2), 403–417. doi:10.1007/S00253-010-2447-0/TABLES/1.

iGEM Parts Registry (n.d.) *iGEM Parts Registry*. (n.d.). *Anderson promoters*. <https://parts.igem.org/Promoters/Catalog/Anderson> [Accessed: 13 November 2021].

Ijoma, G.N. & Tekere, M. (2017) Potential microbial applications of co-cultures involving ligninolytic fungi in the bioremediation of recalcitrant xenobiotic compounds. *International Journal of Environmental Science and Technology*. 14 (8) pp.1787–1806. doi:10.1007/s13762-017-1269-3.

Intasit, R., Cheirsilp, B., Louhasakul, Y. & Boonsawang, P. (2020) Consolidated bioprocesses for efficient bioconversion of palm biomass wastes into biodiesel feedstocks by oleaginous fungi and yeasts. *Bioresource technology*. 315. doi:10.1016/J.BIORTECH.2020.123893.

Jawed, K., Yazdani, S.S. & Koffas, M.A. (2019) Advances in the development and application of microbial consortia for metabolic engineering. *Metabolic Engineering Communications*. 9. doi:10.1016/J.MEC.2019.E00095.

Jeanne, G., Goelzer, A., Tebbani, S., Dumur, D. & Fromion, V. (2018) Dynamical resource allocation models for bioreactor optimization. *IFAC-PapersOnLine*. 51 (19), 20–23. doi:10.1016/J.IFACOL.2018.09.020.

Jeswani, H.K., Figueroa-Torres, G. & Azapagic, A. (2021) The extent of food waste generation in the UK and its environmental impacts. *Sustainable Production and Consumption*. 26, 532–547. doi:10.1016/J.SPC.2020.12.021.

Jiang, T., Li, C., Teng, Y., Zhang, R. & Yan, Y. (2020) Recent advances in improving metabolic robustness of microbial cell factories. *Current Opinion in Biotechnology*. 66, 69–77. doi:10.1016/J.COPBIO.2020.06.006.

Jiang, Y., Guo, D., Lu, J., Dürre, P., Dong, W., Yan, W., Zhang, W., Ma, J., Jiang, M. & Xin, F. (2018) Consolidated bioprocessing of butanol production from xylan by a thermophilic and butanogenic *Thermoanaerobacterium* sp. M5. *Biotechnology for Biofuels*. 11 (1), 1–14. doi:10.1186/S13068-018-1092-1/FIGURES/5.

Jones, J.A., Vernacchio, V.R., Collins, S.M., Shirke, A.N., Xiu, Y., Englaender, J.A., Cress, B.F., McCutcheon, C.C., Linhardt, R.J., Gross, R.A. & Koffas, M.A.G. (2017) Complete Biosynthesis of Anthocyanins Using *E. coli* Polycultures. *mBio*. 8 (3). doi:10.1128/mBio.00621-17.

Jones, J.A., Vernacchio, V.R., Sinkoe, A.L., Collins, S.M., Ibrahim, M.H.A., Lachance, D.M., Hahn, J. & Koffas, M.A.G. (2016) Experimental and computational optimization of an *Escherichia coli* co-culture for the efficient production of flavonoids. *Metabolic Engineering*. 35, 55–63. doi:10.1016/j.ymben.2016.01.006.

Kafri, M., Metzl-Raz, E., Jona, G. & Barkai, N. (2016) The Cost of Protein Production. *Cell Reports*. 14 (1), 22–31. doi:10.1016/j.celrep.2015.12.015.

Karagöz, P. & Özkan, M. (2014) Ethanol production from wheat straw by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture in batch and continuous system. *Bioresource*

Technology. 158, 286–293. doi:10.1016/J.BIORTECH.2014.02.022.

Kavšček, M., Bhutada, G., Madl, T. & Natter, K. (2015) Optimization of lipid production with a genome-scale model of *Yarrowia lipolytica*. *BMC Systems Biology*. 9 (1), 72. doi:10.1186/s12918-015-0217-4.

Kazemi Seresht, A., Cruz, A.L., De Hulster, E., Hebly, M., Palmqvist, E.A., Van Gulik, W., Daran, J.M., Pronk, J. & Olsson, L. (2013) Long-term adaptation of *Saccharomyces cerevisiae* to the burden of recombinant insulin production. *Biotechnology and Bioengineering*. 110 (10), 2749–2763. doi:10.1002/bit.24927.

Ke, X., Hua, X., Sun, J.C., Zheng, R.C. & Zheng, Y.G. (2021a) Synergetic degradation of waste oil by constructed bacterial consortium for rapid in-situ reduction of kitchen waste. *Journal of Bioscience and Bioengineering*. 131 (4), 412–419. doi:10.1016/J.JBIOSEC.2020.12.005.

Ke, X., Sun, J.C., Liu, C., Ying, J.M., Zou, S.P., Xue, Y.P. & Zheng, Y.G. (2021b) Fed-in-situ biological reduction treatment of food waste via high-temperature-resistant oil degrading microbial consortium. *Bioresource Technology*. 340, 125635. doi:10.1016/J.BIORTECH.2021.125635.

Kerkhoven, E.J. (2022) Advances in constraint-based models: methods for improved predictive power based on resource allocation constraints. *Current Opinion in Microbiology*. 68, 102168. doi:10.1016/J.MIB.2022.102168.

Kerkhoven, E.J., Pomraning, K.R., Baker, S.E. & Nielsen, J. (2016) Regulation of amino-acid metabolism controls flux to lipid accumulation in *Yarrowia lipolytica*. *npj Systems Biology and Applications*. 2 (1), 16005. doi:10.1038/npjsba.2016.5.

Khandelwal, R.A., Olivier, B.G., Röling, W.F.M.M., Teusink, B. & Bruggeman, F.J. (2013) *Community Flux Balance Analysis for Microbial Consortia at Balanced Growth*. 8 (5), e64567. <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0064567>.

Klein, T., Lange, S., Wilhelm, N., Bureik, M., Yang, T.H., Heinzle, E. & Schneider, K. (2014) Overcoming the metabolic burden of protein secretion in *Schizosaccharomyces pombe* – A quantitative approach using ¹³C-based metabolic flux analysis. *Metabolic Engineering*. 21, 34–45. doi:10.1016/J.YMBEN.2013.11.001.

Klitgord, N. & Segrè, D. (2010) Environments that Induce Synthetic Microbial Ecosystems J.A. Papin (ed.). *PLoS Computational Biology*. 6 (11), e1001002. doi:10.1371/journal.pcbi.1001002.

Koch, S., Benndorf, D., Fronk, K., Reichl, U. & Klamt, S. (2016) Predicting compositions of microbial communities from stoichiometric models with applications for the biogas process. *Biotechnology for Biofuels*. 9 (1). doi:10.1186/s13068-016-0429-x.

Kogo, T., Yoshida, Y., Koganei, K., Matsumoto, H., Watanabe, T., Ogihara, J. & Kasumi, T. (2017) Production of rice straw hydrolysis enzymes by the fungi *Trichoderma reesei* and *Humicola insolens* using rice straw as a carbon source. *Bioresource Technology*. 233, 67–73. doi:10.1016/J.BIORTECH.2017.01.075.

Kong, W., Meldgin, D.R., Collins, J.J. & Lu, T. (2018) Designing microbial consortia with defined social interactions. *Nature Chemical Biology*. 14 (8), 821–829. doi:10.1038/s41589-018-0091-7.

Korpys-Woźniak, P. & Celińska, E. (2021) Global transcriptome profiling reveals genes responding to overproduction of a small secretory, a high cysteine- and a high glycosylation-bearing protein in *Yarrowia lipolytica*. *Biotechnology Reports*. 31, e00646. doi:10.1016/J.BTRE.2021.E00646.

Korpys-Woźniak, P., Kubiak, P., Biały, W. & Celińska, E. (2020) Impact of overproduced

heterologous protein characteristics on physiological response in *Yarrowia lipolytica* steady-state-maintained continuous cultures. *Applied Microbiology and Biotechnology*. 104 (22), 9785. doi:10.1007/S00253-020-10937-W.

Kruiswijk, T., Planta, R.J. & Mager, W.H. (1978) Quantitative Analysis of the Protein Composition of Yeast Ribosomes. *European Journal of Biochemistry*. 83 (1), 245–252. doi:10.1111/j.1432-1033.1978.tb12089.x.

Kumar, B., Bhardwaj, N., Agrawal, K., Chaturvedi, V. & Verma, P. (2020) Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept. *Fuel Processing Technology*. 199, 106244. doi:10.1016/J.FUPROC.2019.106244.

Kylilis, N., Tuza, Z.A., Stan, G.-B. & Polizzi, K.M. (2018) Tools for engineering coordinated system behaviour in synthetic microbial consortia. *Nature Communications*. 9 (1), 2677. doi:10.1038/s41467-018-05046-2.

de la Torre, D. & Chin, J.W. (2020) Reprogramming the genetic code. *Nature Reviews Genetics* 2020 22:3. 22 (3), 169–184. doi:10.1038/s41576-020-00307-7.

Lahtvee, P.-J.J., Sá Nchez, B.J., Smialowska, A., Kasvandik, S., Elsemman, I.E., Gatto, F., Nielsen, J., Sánchez, B.J., Smialowska, A., Kasvandik, S., Elsemman, I.E., Gatto, F. & Nielsen, J. (2017) Absolute Quantification of Protein and mRNA Abundances Demonstrate Variability in Gene-Specific Translation Efficiency in Yeast. *Cell Systems*. 4 (5), 495-504.e5. doi:10.1016/j.cels.2017.03.003.

Lal, R. (2005) World crop residues production and implications of its use as a biofuel. *Environment International*. 31 (4), 575–584. doi:10.1016/J.ENVINT.2004.09.005.

Lane, S., Xu, H., Oh, E.J., Kim, H., Lesmana, A., Jeong, D., Zhang, G., Tsai, C.S., Jin, Y.S. & Kim, S.R. (2018) Glucose repression can be alleviated by reducing glucose phosphorylation rate in *Saccharomyces cerevisiae*. *Scientific Reports* 2018 8:1. 8 (1), 1–12. doi:10.1038/s41598-018-20804-4.

Lang, G.I., Murray, A.W. & Botstein, D. (2009) The cost of gene expression underlies a fitness trade-off in yeast. *Proceedings of the National Academy of Sciences of the United States of America*. 106 (14), 5755–5760. doi:10.1073/pnas.0901620106.

Larroude, M., Celinska, E., Back, A., Thomas, S., Nicaud, J.-M.M. & Ledesma-Amaro, R. (2018a) A synthetic biology approach to transform *Yarrowia lipolytica* into a competitive biotechnological producer of β -carotene. *Biotechnology and Bioengineering*. 115 (2), 464–472. doi:10.1002/bit.26473.

Larroude, M., Celinska, E., Back, A., Thomas, S., Nicaud, J.M. & Ledesma-Amaro, R. (2018b) A synthetic biology approach to transform *Yarrowia lipolytica* into a competitive biotechnological producer of β -carotene. *Biotechnology and Bioengineering*. 115 (2), 464–472. doi:10.1002/BIT.26473.

Larroude, M., Rossignol, T., Nicaud, J.-M. & Ledesma-Amaro, R. (2018c) Synthetic biology tools for engineering *Yarrowia lipolytica*. *Biotechnology advances*. 36 (8), 2150–2164. doi:10.1016/j.biotechadv.2018.10.004.

Lawson, C.E., Harcombe, W.R., Hatzenpichler, R., Lindemann, S.R., Löffler, F.E., O'Malley, M.A., García Martín, H., Pfleger, B.F., Raskin, L., Venturelli, O.S., Weissbrodt, D.G., Noguera, D.R. & McMahon, K.D. (2019) Common principles and best practices for engineering microbiomes. *Nature reviews. Microbiology*. 17 (12), 725. doi:10.1038/S41579-019-0255-9.

Lazar, Z., Gamboa-Meléndez, H., Le Coq, A.M.C., Neuvéglise, C. & Nicaud, J.M. (2015) Awakening

- the endogenous Leloir pathway for efficient galactose utilization by *Yarrowia lipolytica*. *Biotechnology for Biofuels*. 8 (1), 185. doi:10.1186/s13068-015-0370-4.
- Ledesma-Amaro, R., Dulerio, T. & Nicaud, J.M. (2015) Engineering *Yarrowia lipolytica* to produce biodiesel from raw starch. *Biotechnology for Biofuels*. 8 (1), 148. doi:10.1186/s13068-015-0335-7.
- Ledesma-Amaro, R., Lazar, Z., Rakicka, M., Guo, Z., Fouchard, F., Coq, A.M.C. Le & Nicaud, J.M. (2016) Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose. *Metabolic Engineering*. 38, 115–124. doi:10.1016/j.ymben.2016.07.001.
- Ledesma-Amaro, R. & Nicaud, J.-M.M. (2016a) *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids. *Progress in Lipid Research*. 61 pp.40–50. <https://www.sciencedirect.com/science/article/pii/S0163782715300205?via%3Dihub>.
- Ledesma-Amaro, R. & Nicaud, J.M. (2016b) Metabolic Engineering for Expanding the Substrate Range of *Yarrowia lipolytica*. *Trends in Biotechnology*. 34 (10) pp.798–809. doi:10.1016/j.tibtech.2016.04.010.
- Lee, J.A., Baugh, A.C., Shevalier, N.J., Strand, B., Stolyar, S. & Marx, C.J. (2021) Cross-Feeding of a Toxic Metabolite in a Synthetic Lignocellulose-Degrading Microbial Community. *Microorganisms*. 9 (2), 1–20. doi:10.3390/MICROORGANISMS9020321.
- Lee, M.V., Topper, S.E., Hubler, S.L., Hose, J., Wenger, C.D., Coon, J.J. & Gasch, A.P. (2011) A dynamic model of proteome changes reveals new roles for transcript alteration in yeast. *Molecular Systems Biology*. 7 (1), 514. doi:10.1038/msb.2011.48.
- Levy, S. & Barkai, N. (2009) Coordination of gene expression with growth rate: A feedback or a feed-forward strategy? *FEBS Letters*. 583 (24) pp.3974–3978. doi:10.1016/j.febslet.2009.10.071.
- Li, D.D., Wang, J., Jiang, Y., Zhang, P., Liu, Y., Li, Y.Z. & Zhang, Z. (2024a) Quantifying functional redundancy in polysaccharide-degrading prokaryotic communities. *Microbiome*. 12 (1), 1–14. doi:10.1186/S40168-024-01838-5/FIGURES/8.
- Li, H. & Alper, H.S. (2016) Enabling xylose utilization in *Yarrowia lipolytica* for lipid production. *Biotechnology Journal*. 11 (9), 1230–1240. doi:10.1002/biot.201600210.
- Li, J., Tang, X., Chen, S., Zhao, J. & Shao, T. (2021) Ensiling pretreatment with two novel microbial consortia enhances bioethanol production in sterile rice straw. *Bioresource Technology*. 339, 125507. doi:10.1016/J.BIORTECH.2021.125507.
- Li, X., He, Y., Zhang, L., Xu, Z., Ben, H., Gaffrey, M.J., Yang, Y., Yang, S., Yuan, J.S., Qian, W.-J. & Yang, B. (2019) Discovery of potential pathways for biological conversion of poplar wood into lipids by co-fermentation of *Rhodococci* strains. *Biotechnology for Biofuels* 2019 12:1. 12 (1), 1–16. doi:10.1186/S13068-019-1395-X.
- Li, Z. & Rinas, U. (2020) Recombinant protein production associated growth inhibition results mainly from transcription and not from translation. *Microbial Cell Factories*. 19 (1), 1–11. doi:10.1186/S12934-020-01343-Y/TABLES/2.
- Li, Z., Waghmare, P.R., Dijkhuizen, L., Meng, X. & Liu, W. (2024b) Research advances on the consolidated bioprocessing of lignocellulosic biomass. *Engineering Microbiology*. 4 (2), 100139. doi:10.1016/J.ENGMIC.2024.100139.
- Li, Z., Wang, X. & Zhang, H. (2019) Balancing the non-linear rosmarinic acid biosynthetic pathway by modular co-culture engineering. *Metabolic Engineering*. 54, 1–11. doi:10.1016/j.ymben.2019.03.002.

- Liao, C., Blanchard, A.E. & Lu, T. (2017) An integrative circuit-host modelling framework for predicting synthetic gene network behaviours /631/553/552 /631/553/2695 article. *Nature Microbiology*. 2 (12), 1658–1666. doi:10.1038/s41564-017-0022-5.
- Lin, L. (2022) Bottom-up synthetic ecology study of microbial consortia to enhance lignocellulose bioconversion. *Biotechnology for Biofuels and Bioproducts*. 15 (1), 1–15. doi:10.1186/S13068-022-02113-1/FIGURES/4.
- Liu, F., Mao, J., Kong, W., Hua, Q., Feng, Y., Bashir, R. & Lu, T. (2020) Interaction variability shapes succession of synthetic microbial ecosystems. *Nature Communications*. 11 (1). doi:10.1038/s41467-019-13986-6.
- Liu, F., Mao, J., Lu, T. & Hua, Q. (2019a) Synthetic, Context-Dependent Microbial Consortium of Predator and Prey. *ACS Synthetic Biology*. 8 (8), 1713–1722. doi:10.1021/acssynbio.9b00110.
- Liu, G., Zhang, J. & Bao, J. (2016) Cost evaluation of cellulase enzyme for industrial-scale cellulosic ethanol production based on rigorous Aspen Plus modeling. *Bioprocess and Biosystems Engineering*. 39 (1), 133–140. doi:10.1007/S00449-015-1497-1/METRICS.
- Liu, L., Zhang, Z., Wang, J., Fan, Y., Shi, W., Liu, X. & Shun, Q. (2019b) Simultaneous saccharification and co-fermentation of corn stover pretreated by H₂O₂ oxidative degradation for ethanol production. *Energy*. 168, 946–952. doi:10.1016/J.ENERGY.2018.11.132.
- Liu, M., Lin, Y.C., Guo, J.J., Du, M.M., Tao, X., Gao, B., Zhao, M., Ma, Y., Wang, F.Q. & Wei, D.Z. (2021a) High-Level Production of Sesquiterpene Patchoulol in *Saccharomyces cerevisiae*. *ACS Synthetic Biology*. 10 (1), 158–172. doi:10.1021/ACSSYNBIO.0C00521/SUPPL_FILE/SB0C00521_SI_002.XLSX.
- Liu, Y., Tang, Y., Gao, H., Zhang, W., Jiang, Y., Xin, F. & Jiang, M. (2021b) Challenges and Future Perspectives of Promising Biotechnologies for Lignocellulosic Biorefinery. *Molecules*. 26 (17). doi:10.3390/MOLECULES26175411.
- Liu, Y., Yang, S. & Jia, X. (2020) Construction of a “nutrition supply–detoxification” coculture consortium for medium-chain-length polyhydroxyalkanoate production with a glucose–xylose mixture. *Journal of Industrial Microbiology and Biotechnology*. 47 (3), 343–354. doi:10.1007/S10295-020-02267-7.
- Loira, N., Dulermo, T., Nicaud, J.-M. & Sherman, D. (2012) A genome-scale metabolic model of the lipid-accumulating yeast *Yarrowia lipolytica*. *BMC Systems Biology*. 6 (1), 35. doi:10.1186/1752-0509-6-35.
- Losoi, P.S., Santala, V.P. & Santala, S.M. (2019) Enhanced Population Control in a Synthetic Bacterial Consortium by Interconnected Carbon Cross-Feeding. *Acs Synthetic Biology*. 8 (12), 2642–2650. doi:10.1021/acssynbio.9b00316.
- Lubbe, A., Bowen, B.P. & Northen, T. (2017) Exometabolomic Analysis of Cross-Feeding Metabolites. *Metabolites*. 7 (4), 50. doi:10.3390/METABO7040050.
- Lynd, L.R., Weimer, P.J., van Zyl, W.H. & Pretorius, I.S. (2002) Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews*. 66 (3), 506–577. doi:10.1128/MMBR.66.3.506-577.2002/ASSET/6F6A28CF-2C07-40BC-B7A7-BAF0F705A33E/ASSETS/GRAPHIC/MR032001411T.JPEG.
- Lyu, X., Nuhu, M., Candry, P., Wolfanger, J., Betenbaugh, M., Saldivar, A., Zuniga, C., Wang, Y. & Shrestha, S. (2024) Top-down and bottom-up microbiome engineering approaches to enable biomanufacturing from waste biomass. *Journal of Industrial Microbiology and Biotechnology*. 51 (2). doi:10.1093/JIMB/KUAE025.

- Ma, K. & Ruan, Z. (2015) Production of a lignocellulolytic enzyme system for simultaneous bio-delignification and saccharification of corn stover employing co-culture of fungi. *Bioresource Technology*. 175, 586–593. doi:10.1016/J.BIORTECH.2014.10.161.
- Madzak, C. (2015) *Yarrowia lipolytica*: recent achievements in heterologous protein expression and pathway engineering. *Applied Microbiology and Biotechnology*. 99 (11) pp.4559–4577. doi:10.1007/s00253-015-6624-z.
- Madzak, C., Gaillardin, C. & Beckerich, J.M. (2004) Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: A review. In: *Journal of Biotechnology*. 8 April 2004 J Biotechnol. pp. 63–81. doi:10.1016/j.jbiotec.2003.10.027.
- Maier, A., Volker, B., Boles, E. & Fuhrmann, G.F. (2002) Characterisation of glucose transport in *Saccharomyces cerevisiae* with plasma membrane vesicles (countertransport) and intact cells (initial uptake) with single Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, Hxt7 or Gal2 transporters. *FEMS Yeast Research*. 2 (4), 539–550. doi:10.1111/j.1567-1364.2002.tb00121.x.
- Maleki, N., Safari, M. & Eiteman, M.A. (2018) Conversion of glucose-xylose mixtures to pyruvate using a consortium of metabolically engineered *Escherichia coli*. *Engineering in Life Sciences*. 18 (1), 40–47. doi:10.1002/elsc.201700109.
- Malik, X., Smith, L., Stewart, I. & Burnett, N. (2024) *Food waste in the UK*. <https://researchbriefings.files.parliament.uk/documents/CBP-7552/CBP-7552.pdf>.
- Matyjaszkiewicz, A., Fiore, G., Annunziata, F., Grierson, C.S., Savery, N.J., Marucci, L. & Di Bernardo, M. (2017) BSim 2.0: An Advanced Agent-Based Cell Simulator. *ACS Synthetic Biology*. 6 (10), 1969–1972. doi:10.1021/acssynbio.7b00121.
- Mauri, M., Gouzé, J.L., de Jong, H. & Cinquemani, E. (2020) Enhanced production of heterologous proteins by a synthetic microbial community: Conditions and trade-offs. *PLoS Computational Biology*. 16 (4), e1007795. doi:10.1371/journal.pcbi.1007795.
- McCarty, N.S. & Ledesma-Amaro, R. (2019) Synthetic Biology Tools to Engineer Microbial Communities for Biotechnology. *Trends in biotechnology*. 37 (2), 181–197. doi:10.1016/j.tibtech.2018.11.002.
- Mee, M.T., Collins, J.J., Church, G.M. & Wang, H.H. (2014) Syntrophic exchange in synthetic microbial communities. *Proceedings of the National Academy of Sciences of the United States of America*. 111 (20), E2149–E2156. doi:10.1073/pnas.1405641111.
- Meng, X., Liu, L. & Chen, X. (2022) Bacterial photosynthesis: state-of-the-art in light-driven carbon fixation in engineered bacteria. *Current Opinion in Microbiology*. 69, 102174. doi:10.1016/J.MIB.2022.102174.
- Mewada, M., Albert, S. & Pandya, B. (2017) Enhancement of Ligninolytic & Xylanolytic Enzyme Activities in *Trichoderma reesei* co-Cultured with two White Rot Fungi. *International Journal of Biotechnology and Biochemistry*. 13 (4), 429–439. <http://www.ripublication.com>.
- Miano, A., Liao, M.J. & Hasty, J. (2020) Inducible cell-to-cell signaling for tunable dynamics in microbial communities. *Nature Communications* 2020 11:1. 11 (1), 1–8. doi:10.1038/s41467-020-15056-8.
- Michailidou, F. (2023) The Scent of Change: Sustainable Fragrances Through Industrial Biotechnology. *ChemBioChem*. 24 (19), e202300309. doi:10.1002/CBIC.202300309.
- Millán Acosta, A., Cosovanu, D., Cabañeros López, P., Thomsen, S.T., Gernaey, K. V. & Canela-Garayoa, R. (2021) Co-cultivation of a novel *Fusarium striatum* strain and a xylose consuming

Saccharomyces cerevisiae yields an efficient process for simultaneous detoxification and fermentation of lignocellulosic hydrolysates. *Chemical Engineering Journal*. 426, 131575. doi:10.1016/J.CEJ.2021.131575.

Milo, R. (2013) What is the total number of protein molecules per cell volume? A call to rethink some published values. *Bioessays*. 35 (12), 1050. doi:10.1002/BIES.201300066.

Minami, H., Kim, J.S., Ikezawa, N., Takemura, T., Katayama, T., Kumagai, H. & Sato, F. (2008) Microbial production of plant benzylisoquinoline alkaloids. *Proceedings of the National Academy of Sciences of the United States of America*. 105 (21), 7393–7398. doi:10.1073/PNAS.0802981105.

Minty, J.J., Singer, M.E., Scholz, S.A., Bae, C.H., Ahn, J.H., Foster, C.E., Liao, J.C. & Lin, X.N. (2013) Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. *Proceedings of the National Academy of Sciences of the United States of America*. 110 (36), 14592–14597. doi:10.1073/PNAS.1218447110/-/DCSUPPLEMENTAL/SAPP.PDF.

Mittal, P., Brindle, J., Stephen, J., Plotkin, J.B. & Kudla, G. (2018) Codon usage influences fitness through RNA toxicity. *Proceedings of the National Academy of Sciences of the United States of America*. 115 (34), 8639–8644. doi:10.1073/PNAS.1810022115/SUPPL_FILE/PNAS.1810022115.SD01.XLSX.

Moon, T.S., Lou, C., Tamsir, A., Stanton, B.C. & Voigt, C.A. (2012) Genetic programs constructed from layered logic gates in single cells. *Nature*. 491 (7423), 249–253. doi:10.1038/nature11516.

Moonsamy, T.A., Rajauria, G., Priyadarshini, A. & Jansen, M.A.K. (2024) Food waste: analysis of the complex and variable composition of a promising feedstock for valorisation. *Food and Bioproducts Processing*. 148, 31–42. doi:10.1016/J.FBP.2024.08.012.

Moraïs, S., Shterzer, N., Grinberg, I.R., Mathiesen, G., Eijsink, V.G.H., Axelsson, L., Lamed, R., Bayer, E.A. & Mizrahi, I. (2013) Establishment of a Simple *Lactobacillus plantarum* Cell Consortium for Cellulase-Xylanase Synergistic Interactions. *Applied and Environmental Microbiology*. 79 (17), 5242. doi:10.1128/AEM.01211-13.

Moraïs, S., Shterzer, N., Lamed, R., Bayer, E.A. & Mizrahi, I. (2014) A combined cell-consortium approach for lignocellulose degradation by specialized *Lactobacillus plantarum* cells. *Biotechnology for Biofuels*. 7 (1), 112. doi:10.1186/1754-6834-7-112.

Morris, B.E.L., Henneberger, R., Huber, H. & Moissl-Eichinger, C. (2013) Microbial syntrophy: interaction for the common good. *Fems Microbiology Reviews*. 37 (3), 384–406. doi:10.1111/1574-6976.12019.

Nair, A. & Sarma, S.J. (2021) The impact of carbon and nitrogen catabolite repression in microorganisms. *Microbiological Research*. 251, 126831. doi:10.1016/J.MICRES.2021.126831.

Naseeruddin, S., Desai, S. & Venkateswar Rao, L. (2021) Co-culture of *Saccharomyces cerevisiae* (VS3) and *Pichia stipitis* (NCIM 3498) enhances bioethanol yield from concentrated *Prosopis juliflora* hydrolysate. *3 Biotech*. 11 (1). doi:10.1007/s13205-020-02595-6.

Niehus, X., Crutz-Le Coq, A.M., Sandoval, G., Nicaud, J.M. & Ledesma-Amaro, R. (2018) Engineering *Yarrowia lipolytica* to enhance lipid production from lignocellulosic materials. *Biotechnology for Biofuels*. 11 (1). doi:10.1186/s13068-018-1010-6.

Nijland, J.G. & Driessen, A.J.M. (2020) Engineering of Pentose Transport in *Saccharomyces cerevisiae* for Biotechnological Applications. *Frontiers in Bioengineering and Biotechnology*. 7, 512089. doi:10.3389/FBIOE.2019.00464/BIBTEX.

Nikolados, E.-M., Weiße, A.Y., Oyarzún, D.A. & Oyarzún, O. (2020) *Prediction of cellular burden with host-circuit models*. <http://arxiv.org/abs/2004.00995>.

Nikolados, E.M., Weiße, A.Y. & Oyarzún, D.A. (2021) Prediction of Cellular Burden with Host-Circuit Models. *Methods in Molecular Biology*. 2229, 267–291. doi:10.1007/978-1-0716-1032-9_13.

Niu, F.X., He, X., Wu, Y.Q. & Liu, J.Z. (2018) Enhancing Production of Pinene in *Escherichia coli* by Using a Combination of Tolerance, Evolution, and Modular Co-culture Engineering. *Frontiers in Microbiology*. 9, 386469. doi:10.3389/FMICB.2018.01623/BIBTEX.

Nordahl, S.L., Devkota, J.P., Amirebrahimi, J., Smith, S.J., Breunig, H.M., Preble, C. V., Satchwell, A.J., Jin, L., Brown, N.J., Kirchstetter, T.W. & Scown, C.D. (2020) Life-Cycle Greenhouse Gas Emissions and Human Health Trade-Offs of Organic Waste Management Strategies. *Environmental Science and Technology*. 54 (15), 9200–9209. doi:10.1021/ACS.EST.0C00364/ASSET/IMAGES/LARGE/ES0C00364_0003.JPEG.

O'Brien, E.J., Lerman, J.A., Chang, R.L., Hyduke, D.R. & Palsson, B. (2013) Genome-scale models of metabolism and gene expression extend and refine growth phenotype prediction. *Molecular Systems Biology*. 9, 693. doi:10.1038/MSB.2013.52/SUPPL_FILE/MSB201352.REVIEWER_COMMENTS.PDF.

Olofsson, K., Bertilsson, M. & Lidén, G. (2008) A short review on SSF - An interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnology for Biofuels*. 1 (1), 1–14. doi:10.1186/1754-6834-1-7/FIGURES/4.

Pan, P. & Hua, Q. (2012) Reconstruction and In Silico Analysis of Metabolic Network for an Oleaginous Yeast, *Yarrowia lipolytica* S.S. Fong (ed.). *PLoS ONE*. 7 (12), e51535. doi:10.1371/journal.pone.0051535.

Pande, S., Merker, H., Bohl, K., Reichelt, M., Schuster, S., De Figueiredo, L.F., Kaleta, C. & Kost, C. (2014) Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria. *The ISME Journal*. 8 (5), 953–962. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3996690/pdf/ismej2013211a.pdf>.

Papanikolaou, S. & Aggelis, G. (2010) *Yarrowia lipolytica*: A model microorganism used for the production of tailor-made lipids. *European Journal of Lipid Science and Technology*. 112 (6), 639–654. doi:10.1002/ejlt.200900197.

Park, C.S., Chang, C.C. & Ryu, D.D.Y. (2000) Expression and high-level secretion of *Trichoderma reesei* endoglucanase I in *Yarrowia lipolytica*. *Applied biochemistry and biotechnology*. 87 (1), 1–15. doi:10.1385/ABAB:87:1:1.

Park, H., Patel, A., Hunt, K.A., Henson, M.A. & Carlson, R.P. (2020) Artificial consortium demonstrates emergent properties of enhanced cellulosic-sugar degradation and biofuel synthesis. *npj Biofilms and Microbiomes*. 6 (1). doi:10.1038/s41522-020-00170-8.

Park, Y.K., Peng, H., Hapeta, P., Sellés Vidal, L. & Ledesma-Amaro, R. (2024) Engineered cross-feeding creates inter- and intra-species synthetic yeast communities with enhanced bioproduction. *Nature Communications* 2024 15:1. 15 (1), 1–10. doi:10.1038/s41467-024-53117-4.

Pathania, S., Sharma, N. & Handa, S. (2017) Immobilization of co-culture of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in sodium alginate for bioethanol production using hydrolysate of apple pomace under separate hydrolysis and fermentation. <http://dx.doi.org/10.1080/10242422.2017.1368497>. 35 (6), 450–459.

doi:10.1080/10242422.2017.1368497.

Peng, H., Darlington, A.P.S., South, E.J., Chen, H.H., Jiang, W. & Ledesma-Amaro, R. (2024) A molecular toolkit of cross-feeding strains for engineering synthetic yeast communities. *Nature Microbiology* 2024 9:3. 9 (3), 848–863. doi:10.1038/s41564-023-01596-4.

Peng, Q.Q., Guo, Q., Chen, C., Song, P., Wang, Y.T., Ji, X.J., Ye, C. & Shi, T.Q. (2023) High-Level Production of Patchoulol in *Yarrowia lipolytica* via Systematic Engineering Strategies. *Journal of Agricultural and Food Chemistry*. 71 (11), 4638–4645. doi:10.1021/ACS.JAFC.3C00222/ASSET/IMAGES/LARGE/JF3C00222_0007.JPEG.

Perisin, M.A. & Sund, C.J. (2018) Human gut microbe co-cultures have greater potential than monocultures for food waste remediation to commodity chemicals. *Scientific Reports* 2018 8:1. 8 (1), 1–10. doi:10.1038/s41598-018-33733-z.

Perrino, G., Hadjimitsis, A., Ledesma-Amaro, R. & Stan, G.B. (2021) Control engineering and synthetic biology: working in synergy for the analysis and control of microbial systems. *Current Opinion in Microbiology*. 62, 68–75. doi:10.1016/J.MIB.2021.05.004.

Puentes-Téllez, P.E. & Falcao Salles, J. (2018) Construction of Effective Minimal Active Microbial Consortia for Lignocellulose Degradation. *Microbial Ecology*. 76 (2), 419. doi:10.1007/S00248-017-1141-5.

Qi-he, C., Krügener, S., Hirth, T., Rupp, S. & Zibek, S. (2011) Co-cultured Production of Lignin-Modifying Enzymes with White-Rot Fungi. *Applied Biochemistry and Biotechnology* 2011 165:2. 165 (2), 700–718. doi:10.1007/S12010-011-9289-9.

Qian, X., Chen, L., Sui, Y., Chen, C., Zhang, W., Zhou, J., Dong, W., Jiang, M., Xin, F. & Ochsenreither, K. (2020) Biotechnological potential and applications of microbial consortia. *Biotechnology Advances*. 40 p.107500. doi:10.1016/j.biotechadv.2019.107500.

Rafieenia, R., Atkinson, E. & Ledesma-Amaro, R. (2022) Division of labor for substrate utilization in natural and synthetic microbial communities. *Current opinion in biotechnology*. 75, 102706. doi:10.1016/J.COPBIO.2022.102706.

Rafieenia, R., Klemm, C., Hapeta, P., Fu, J., García, M.G. & Ledesma-Amaro, R. (2024) Designing synthetic microbial communities with the capacity to upcycle fermentation byproducts to increase production yields. *Trends in Biotechnology*. 0 (0). doi:10.1016/J.TIBTECH.2024.10.015/ATTACHMENT/1529700B-0E42-4B6A-8BD6-E2ACBE97FFB6/MMC3.PDF.

Rajpurohit, H. & Eiteman, M.A. (2020) Pretreatment and Detoxification of Acid-Treated Wood Hydrolysates for Pyruvate Production by an Engineered Consortium of *Escherichia coli*. *Applied Biochemistry and Biotechnology*. 192 (1), 243–256. doi:10.1007/s12010-020-03320-y.

Ramírez, D.M. & Bentley, W.E. (1993) Enhancement of recombinant protein synthesis and stability via coordinated amino acid addition. *Biotechnology and Bioengineering*. 41 (5), 557–565. doi:10.1002/bit.260410508.

Regot, S., Macia, J., Conde, N.N., Furukawa, K., Kjellén, J., Peeters, T., Hohmann, S., de Nadal, E.E., Posas, F. & Solé, R. (2011) Distributed biological computation with multicellular engineered networks. *Nature*. 469 (7329), 207–211. doi:10.1038/nature09679.

Regueira, A., Rombouts, J.L., Wahl, S.A., Mauricio-Iglesias, M., Lema, J.M. & Kleerebezem, R. (2021) Resource allocation explains lactic acid production in mixed-culture anaerobic fermentations. *Biotechnology and Bioengineering*. 118 (2), 745–758. doi:10.1002/BIT.27605.

- Van Rensburg, E., Den Haan, R., Smith, J., Van Zyl, W.H. & Görgens, J.F. (2012) The metabolic burden of cellulase expression by recombinant *Saccharomyces cerevisiae* Y294 in aerobic batch culture. *Applied Microbiology and Biotechnology*. 96 (1), 197–209. doi:10.1007/s00253-012-4037-9.
- Rollié, S., Mangold, M. & Sundmacher, K. (2012) Designing biological systems: Systems Engineering meets Synthetic Biology. *Chemical Engineering Science*. 69 (1) pp.1–29. doi:10.1016/j.ces.2011.10.068.
- Roth, R., Moodley, V. & Van Zyl, P. (2009) Heterologous expression and optimized production of an *aspergillus aculeatus* endo-1,4- β -mannanase in *Yarrowia lipolytica*. *Molecular Biotechnology*. 43 (2), 112–120. doi:10.1007/s12033-009-9187-3.
- Rouches, M. V., Xu, Y., Cortes, L.B.G. & Lambert, G. (2022) A plasmid system with tunable copy number. *Nature Communications* 2022 13:1. 13 (1), 1–12. doi:10.1038/s41467-022-31422-0.
- Rugbjerg, P., Myling-Petersen, N., Porse, A., Sarup-Lytzen, K. & Sommer, M.O.A. (2018) Diverse genetic error modes constrain large-scale bio-based production. *Nature Communications*. 9 (1), 787. doi:10.1038/S41467-018-03232-W.
- Ruhong, L., Liao, Q., Xia, A., Deng, Z., Huang, Y., Zhu, X. & Zhu, X. (2020) Synergistic Treatment of Alkali Lignin via Fungal Coculture for Biofuel Production: Comparison of Physicochemical Properties and Adsorption of Enzymes Used As Catalysts. *Frontiers in Energy Research*. 0, 231. doi:10.3389/FENRG.2020.575371.
- de Ruijter, J.C., Koskela, E. V., Nandania, J., Frey, A.D. & Velagapudi, V. (2018) Understanding the metabolic burden of recombinant antibody production in *Saccharomyces cerevisiae* using a quantitative metabolomics approach. *Yeast*. 35 (4), 331–341. doi:10.1002/yea.3298.
- Ryu, S., Hipp, J. & Trinha, C.T. (2016) Activating and Elucidating Metabolism of Complex Sugars in *Yarrowia lipolytica*. *Applied and Environmental Microbiology*. 82 (4), 1334. doi:10.1128/AEM.03582-15.
- Ryu, S. & Trinh, C.T. (2018) Understanding Functional Roles of Native Pentose-Specific Transporters for Activating Dormant Pentose Metabolism in *Yarrowia lipolytica*. *Applied and Environmental Microbiology*. 84 (3). doi:10.1128/AEM.02146-17.
- Saini, M., Lin, L.-J., Chiang, C.-J. & Chao, Y.-P. (2017) Synthetic Consortium of *Escherichia coli* for n-Butanol Production by Fermentation of the Glucose–Xylose Mixture. *Journal of Agricultural and Food Chemistry*. 65 (46), 10040–10047. doi:10.1021/ACS.JAFC.7B04275.
- Sanchez-Gorostiaga, A., Bajić, D., Osborne, M.L., Poyatos, J.F. & Sanchez, A. (2019) High-order interactions distort the functional landscape of microbial consortia W. Shou (ed.). *PLoS Biology*. 17 (12), e3000550. doi:10.1371/journal.pbio.3000550.
- Schlembach, I., Hosseinpour Tehrani, H., Blank, L.M., Büchs, J., Wierckx, N., Regestein, L. & Rosenbaum, M.A. (2020) Consolidated bioprocessing of cellulose to itaconic acid by a co-culture of *Trichoderma reesei* and *Ustilago maydis*. *Biotechnology for Biofuels*. 13 (1), 1–18. doi:10.1186/S13068-020-01835-4/FIGURES/7.
- Scholz, S.A., Graves, I., Minty, J.J. & Lin, X.N. (2018) Production of cellulosic organic acids via synthetic fungal consortia. *Biotechnology and Bioengineering*. 115 (4), 1096–1100. doi:10.1002/BIT.26509.
- Schwalm, N.D., Mojadedi, W., Gerlach, E.S., Benyamin, M., Perisin, M.A. & Akingbade, K.L. (2019) Developing a Microbial Consortium for Enhanced Metabolite Production from Simulated Food Waste. *Fermentation* 2019, Vol. 5, Page 98. 5 (4), 98. doi:10.3390/FERMENTATION5040098.

- Scott, M., Gunderson, C.W., Mateescu, E.M., Zhang, Z. & Hwa, T. (2010a) Interdependence of cell growth and gene expression: origins and consequences. *Science*. 330 (6007), 1099–1102. doi:10.1126/science.1192588.
- Scott, M., Gunderson, C.W., Mateescu, E.M., Zhang, Z. & Hwa, T. (2010b) Interdependence of cell growth and gene expression: Origins and consequences. *Science*. 330 (6007), 1099–1102. doi:10.1126/science.1192588.
- Scott, M. & Hwa, T. (2011) Bacterial growth laws and their applications. *Current Opinion in Biotechnology*. 22 (4) pp.559–565. doi:10.1016/j.copbio.2011.04.014.
- Shahab, R.L., Brethauer, S., Davey, M.P., Smith, A.G., Vignolini, S., Luterbacher, J.S. & Studer, M.H. (2020) A heterogeneous microbial consortium producing short-chain fatty acids from lignocellulose. *Science*. 369 (6507). doi:10.1126/science.abb1214.
- Shen, Y.P., Niu, F.X., Yan, Z.B., Fong, L.S., Huang, Y. Bin & Liu, J.Z. (2020) Recent Advances in Metabolically Engineered Microorganisms for the Production of Aromatic Chemicals Derived From Aromatic Amino Acids. *Frontiers in Bioengineering and Biotechnology*. 8, 407. doi:10.3389/FBIOE.2020.00407.
- Shong, J., Jimenez Diaz, M.R. & Collins, C.H. (2012) Towards synthetic microbial consortia for bioprocessing. *Current Opinion in Biotechnology*. 23 (5) pp.798–802. doi:10.1016/j.copbio.2012.02.001.
- Sickle, J.J., Ni, C., Shen, D., Wang, Z., Jin, M. & Lu, T. (2020) Integrative Circuit-Host Modeling of a Genetic Switch in Varying Environments. *Scientific Reports*. 10 (1), 1–9. doi:10.1038/s41598-020-64921-5.
- Silva, F., Queiroz, J.A. & Domingues, F.C. (2012) Evaluating metabolic stress and plasmid stability in plasmid DNA production by *Escherichia coli*. *Biotechnology Advances*. 30 (3) pp.691–708. doi:10.1016/j.biotechadv.2011.12.005.
- Singh, A., Bedore, S.R., Sharma, N.K., Lee, S.A., Eiteman, M.A. & Neidle, E.L. (2019) Removal of aromatic inhibitors produced from lignocellulosic hydrolysates by *Acinetobacter baylyi* ADP1 with formation of ethanol by *Kluyveromyces marxianus*. *Biotechnology for Biofuels*. 12 (1), 1–11. doi:10.1186/S13068-019-1434-7/TABLES/2.
- Singh, N., Verma, T. & Gaur, R. (2013) Detoxification of hexavalent chromium by an indigenous facultative anaerobic *Bacillus cereus* strain isolated from tannery effluent. *African Journal of Biotechnology*. 12 (10), 1091–1103. doi:10.5897/AJB12.1636.
- Sirohi, R., Lee, J.S., Yu, B.S., Roh, H. & Sim, S.J. (2021) Sustainable production of polyhydroxybutyrate from autotrophs using CO₂ as feedstock: Challenges and opportunities. *Bioresource Technology*. 341, 125751. doi:10.1016/J.BIORTECH.2021.125751.
- Siu, Y., Fenno, J., Lindle, J.M. & Dunlop, M.J. (2018) Design and Selection of a Synthetic Feedback Loop for Optimizing Biofuel Tolerance. *ACS Synthetic Biology*. 7 (1), 16–23. doi:10.1021/ACSSYNBIO.7B00260/SUPPL_FILE/SB7B00260_SI_001.PDF.
- Siwiak, M. & Zielenkiewicz, P. (2010) A comprehensive, quantitative, and genome-wide model of translation. *PLoS Computational Biology*. 6 (7), 4. doi:10.1371/journal.pcbi.1000865.
- Slopiecka, K., Liberti, F., Massoli, S., Bartocci, P. & Fantozzi, F. (2022) Chemical and physical characterization of food waste to improve its use in anaerobic digestion plants. *Energy Nexus*. 5, 100049. doi:10.1016/J.NEXUS.2022.100049.
- Snoeck, S., Guidi, C. & De Mey, M. (2024) “Metabolic burden” explained: stress symptoms and

its related responses induced by (over)expression of (heterologous) proteins in *Escherichia coli*. *Microbial Cell Factories*. 23 (1). doi:10.1186/S12934-024-02370-9.

Soufi, B., Krug, K., Harst, A. & Macek, B. (2015) Characterization of the *E. coli* proteome and its modifications during growth and ethanol stress. *Frontiers in Microbiology*. 6 (FEB), 103. doi:10.3389/FMICB.2015.00103/ABSTRACT.

Spagnuolo, M., Hussain, M.S., Gambill, L. & Blenner, M. (2018) Alternative substrate metabolism in *Yarrowia lipolytica*. *Frontiers in Microbiology*. 9 (MAY) p.1077. doi:10.3389/fmicb.2018.01077.

Srilekha Yadav, K., Naseeruddin, S., Sai Prashanthi, G., Sateesh, L. & Venkateswar Rao, L. (2011) Bioethanol fermentation of concentrated rice straw hydrolysate using co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis*. *Bioresource Technology*. 102 (11), 6473–6478. doi:10.1016/J.BIORTECH.2011.03.019.

Srivastava, S., Dafale, N.A., Tulsani, N., Jakhesara, S.J., Patil, N. V., Joshi, C.G. & Purohit, H.J. (2021) Evaluation of designed consortium SNH-1 for efficient hydrolysis of agriculture waste to benefit bioethanol production. *Journal of Cleaner Production*. 288, 125601. doi:10.1016/J.JCLEPRO.2020.125601.

Stern, J., Morais, S., Ben-David, Y., Salama, R., Shamsoum, M., Lamed, R., Shoham, Y., Bayer, E.A. & Mizrahi, I. (2018) Assembly of Synthetic Functional Cellulosomal Structures onto the Cell Surface of *Lactobacillus plantarum*, a Potent Member of the Gut Microbiome. *Applied and Environmental Microbiology*. 84 (8), e00282-18. doi:10.1128/AEM.00282-18.

Sufficiency, E., Qamar, S.A., Ferreira, L.F.R., Franco, M., Iqbal, H.M.N. & Bilal, M. (2022) Emerging biotechnological strategies for food waste management: A green leap towards achieving high-value products and environmental abatement. *Energy Nexus*. 6, 100077. doi:10.1016/J.NEXUS.2022.100077.

Sun, M.L., Shi, T.Q., Lin, L., Ledesma-Amaro, R. & Ji, X.J. (2022) Advancing *Yarrowia lipolytica* as a superior biomanufacturing platform by tuning gene expression using promoter engineering. *Bioresource Technology*. 347, 126717. doi:10.1016/J.BIORTECH.2022.126717.

Swietalski, P., Hetzel, F., Seitz, I. & Fischer, L. (2020) Secretion of a low and high molecular weight β -glycosidase by *Yarrowia lipolytica*. *Microbial Cell Factories*. 19 (1), 1–13. doi:10.1186/S12934-020-01358-5/FIGURES/5.

Tamsir, A., Tabor, J.J. & Voigt, C.A. (2011) Robust multicellular computing using genetically encoded NOR gates and chemical ‘wires’. *Nature*. 469 (7329), 212–215. doi:10.1038/nature09565.

Teague, B.P. & Weiss, R. (2015) Synthetic communities, the sum of parts: Complex behaviors are engineered from cooperating cell communities. *Science*. 349 (6251), 924–925. doi:10.1126/science.aad0876.

Theron, C.W., Vandermies, M., Telek, S., Steels, S. & Fickers, P. (2020) Comprehensive comparison of *Yarrowia lipolytica* and *Pichia pastoris* for production of *Candida antarctica* lipase B. *Scientific Reports*. 10 (1), 1–9. doi:10.1038/s41598-020-58683-3.

Thommes, M., Wang, T., Zhao, Q., Paschalidis, I.C. & Segrè, D. (2019) Designing Metabolic Division of Labor in Microbial Communities. *mSystems*. 4 (2). doi:10.1128/msystems.00263-18.

Tjørve, K.M.C. & Tjørve, E. (2017) The use of Gompertz models in growth analyses, and new Gompertz-model approach: An addition to the Unified-Richards family. *PLoS ONE*. 12 (6), e0178691. doi:10.1371/journal.pone.0178691.

Tomala, K. & Korona, R. (2013) Evaluating the fitness cost of protein expression in *saccharomyces cerevisiae*. *Genome Biology and Evolution*. 5 (11), 2051–2060. doi:10.1093/gbe/evt154.

Tran, H.T.M., Cheirsilp, B., Hodgson, B. & Umsakul, K. (2010) Potential use of *Bacillus subtilis* in a co-culture with *Clostridium butylicum* for acetone–butanol–ethanol production from cassava starch. *Biochemical Engineering Journal*. 48 (2), 260–267. doi:10.1016/J.BEJ.2009.11.001.

Tsai, S.L., Goyal, G. & Chen, W. (2010) Surface display of a functional minicellulosome by intracellular complementation using a synthetic yeast consortium and its application to cellulose hydrolysis and ethanol production. *Applied and Environmental Microbiology*. 76 (22), 7514–7520. doi:10.1128/AEM.01777-10.

Tsoi, R., Wu, F., Zhang, C., Bewick, S., Karig, D. & You, L. (2018a) Metabolic division of labor in microbial systems. *Proceedings of the National Academy of Sciences of the United States of America*. 115 (10), 2526–2531. doi:10.1073/pnas.1716888115.

Tsoi, R., Wu, F., Zhang, C., Bewick, S., Karig, D. & You, L. (2018b) Metabolic division of labor in microbial systems. *Proceedings of the National Academy of Sciences of the United States of America*. 115 (10), 2526–2531. doi:10.1073/pnas.1716888115.

UK Department for Business, I.& S. (2015) *Supporting growth of the UK bioeconomy: opportunities from waste* - GOV.UK. 2015. GOV.UK. <https://www.gov.uk/government/publications/supporting-growth-of-the-uk-bioeconomy-opportunities-from-waste> [Accessed: 21 October 2024].

Vemparala, G., Karumanchi, B., Begum, S. & Anupoju, G.R. (2023) Evaluating the potential of pectin de-esterifying bacterial cultures for the production of methanol from fruit waste: Optimization of critical operational parameters. *Renewable Energy*. 217, 119143. doi:10.1016/J.RENENE.2023.119143.

Verhoeven, M.D., de Valk, S.C., Daran, J.-M.G., van Maris, A.J.A. & Pronk, J.T. (2018) Fermentation of glucose-xylose-arabinose mixtures by a synthetic consortium of single-sugar-fermenting *Saccharomyces cerevisiae* strains. *FEMS Yeast Research*. 18 (8). doi:10.1093/femsyr/foy075.

Vidal, M.C., Wang, S.P., Rivers, D.M., Althoff, D.M. & Segraves, K.A. (2020) Species richness and redundancy promote persistence of exploited mutualisms in yeast. *Science*. 370 (6514), 346–350. doi:10.1126/SCIENCE.ABB6703/SUPPL_FILE/ABB6703_VIDAL_SM.PDF.

Vortmann, M., Stumpf, A.K., Sgobba, E., Dirks-Hofmeister, M.E., Krehenbrink, M., Wendisch, V.F., Philipp, B. & Moerschbacher, B.M. (2021) A bottom-up approach towards a bacterial consortium for the biotechnological conversion of chitin to l-lysine. *Applied Microbiology and Biotechnology*. 105 (4), 1547–1561. doi:10.1007/S00253-021-11112-5/FIGURES/10.

Waldron, C. & Lacroute, F. (1975) Effect of growth rate on the amounts of ribosomal and transfer ribonucleic acids in yeast. *Journal of Bacteriology*. 122 (3), 855–865. doi:10.1128/jb.122.3.855-865.1975.

Walsh, I.M., Bowman, M.A., Soto Santarriaga, I.F., Rodriguez, A. & Clark, P.L. (2020) Synonymous codon substitutions perturb cotranslational protein folding in vivo and impair cell fitness. *Proceedings of the National Academy of Sciences of the United States of America*. 117 (7), 3528–3534. doi:10.1073/PNAS.1907126117/SUPPL_FILE/PNAS.1907126117.SAPP.PDF.

Wang, S., Tang, H., Peng, F., Yu, X., Su, H., Xu, P. & Tan, T. (2019a) Metabolite-based mutualism enhances hydrogen production in a two-species microbial consortium. *Communications Biology* 2019 2:1. 2 (1), 1–11. doi:10.1038/s42003-019-0331-8.

- Wang, S., Xu, C., Song, L. & Zhang, J. (2022) Anaerobic Digestion of Food Waste and Its Microbial Consortia: A Historical Review and Future Perspectives. *International Journal of Environmental Research and Public Health*. 19 (15), 9519. doi:10.3390/IJERPH19159519.
- Wang, T. & Dunlop, M.J. (2019) Controlling and exploiting cell-to-cell variation in metabolic engineering. *Current Opinion in Biotechnology*. 57, 10–16. doi:10.1016/J.COPBIO.2018.08.013.
- Wang, W., Wei, H., Alahuhta, M., Chen, X., Hyman, D., Johnson, D.K., Zhang, M. & Himmel, M.E. (2014) Heterologous expression of xylanase enzymes in lipogenic yeast *Yarrowia lipolytica*. *PLoS ONE*. 9 (12). doi:10.1371/journal.pone.0111443.
- Wang, X., Cabales, A., Li, Z. & Zhang, H. (2019b) Biosensor-assisted high performing cell selection using an *E. coli* toxin/antitoxin system. *Biochemical Engineering Journal*. 144, 110–118. doi:10.1016/J.BEJ.2019.01.016.
- Wang, Y., Liu, C.L., Storey, J.D., Tibshirani, R.J., Herschlag, D. & Brown, P.O. (2002) Precision and functional specificity in mRNA decay. *Proceedings of the National Academy of Sciences of the United States of America*. 99 (9), 5860–5865. doi:10.1073/pnas.092538799.
- Warner, J.R. (1999) The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Sciences*. 24 (11) pp.437–440. doi:10.1016/S0968-0004(99)01460-7.
- Wei, H., Wang, W., Alper, H.S., Xu, Q., Knoshaug, E.P., Van Wychen, S., Lin, C.-Y., Luo, Y., Decker, S.R., Himmel, M.E. & Zhang, M. (2018) Ameliorating the Metabolic Burden of the Co-expression of Secreted Fungal Cellulases in a High Lipid-Accumulating *Yarrowia lipolytica* Strain by Medium C/N Ratio and a Chemical Chaperone. *Frontiers in microbiology*. 9, 3276. doi:10.3389/fmicb.2018.03276.
- Wei, H., Wang, W., Knoshaug, E.P., Chen, X., Van Wychen, S., Bomble, Y.J., Himmel, M.E. & Zhang, M. (2021) Disruption of the *Snf1* Gene Enhances Cell Growth and Reduces the Metabolic Burden in Cellulase-Expressing and Lipid-Accumulating *Yarrowia lipolytica*. *Frontiers in Microbiology*. 12, 757741. doi:10.3389/FMICB.2021.757741/BIBTEX.
- Weiße, A.Y., Oyarzún, D.A., Danos, V. & Swain, P.S. (2015) Mechanistic links between cellular trade-offs, gene expression, and growth. *Proceedings of the National Academy of Sciences of the United States of America*. 112 (9), E1038–E1047. doi:10.1073/pnas.1416533112.
- Williams, T.C., Nielsen, L.K. & Vickers, C.E. (2013) Engineered Quorum Sensing Using Pheromone-Mediated Cell-to-Cell Communication in *Saccharomyces cerevisiae*. *ACS Synthetic Biology*. 2 (3), 136–149. doi:10.1021/SB300110B.
- Woolford, J.L. & Baserga, S.J. (2013) Ribosome biogenesis in the yeast *Saccharomyces cerevisiae*. *Genetics*. 195 (3), 643–681. doi:10.1534/genetics.113.153197.
- Wu, G., Yan, Q., Jones, J.A., Tang, Y.J., Fong, S.S. & Koffas, M.A.G. (2016) Metabolic Burden: Cornerstones in Synthetic Biology and Metabolic Engineering Applications. *Trends in Biotechnology*. 34 (8) pp.652–664. doi:10.1016/j.tibtech.2016.02.010.
- Xia, T., Eiteman, M.A. & Altman, E. (2012) Simultaneous utilization of glucose, xylose and arabinose in the presence of acetate by a consortium of *Escherichia coli* strains. *Microbial Cell Factories*. 11 (1), 77. doi:10.1186/1475-2859-11-77.
- Xiong, X. & Chen, S. (2020) Expanding Toolbox for Genes Expression of *Yarrowia lipolytica* to Include Novel Inducible, Repressible, and Hybrid Promoters. *ACS synthetic biology*. 9 (8), 2208–2213. doi:10.1021/ACSSYNBIO.0C00243.
- Xu, Q., Alahuhta, M., Wei, H., Knoshaug, E.P., Wang, W., Baker, J.O., Vander Wall, T., Himmel,

- M.E. & Zhang, M. (2018) Expression of an endoglucanase-cellobiohydrolase fusion protein in *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Lipomyces starkeyi*. *Biotechnology for biofuels*. 11 (1). doi:10.1186/S13068-018-1301-Y.
- Xu, Q., Vandenkoornhuysse, P., Li, L., Guo, J., Zhu, C., Guo, S., Ling, N. & Shen, Q. (2022) Microbial generalists and specialists differently contribute to the community diversity in farmland soils. *Journal of Advanced Research*. 40, 17–27. doi:10.1016/J.JARE.2021.12.003.
- Xu, Z., Lei, P., Zhai, R., Wen, Z. & Jin, M. (2019) Recent advances in lignin valorization with bacterial cultures: microorganisms, metabolic pathways, and bio-products. *Biotechnology for Biofuels*. 12 (1). doi:10.1186/S13068-019-1376-0.
- YaPing, W., Ben, R., Ling, Z. & Lixin, M. (2017) High-level expression of two thermophilic β -mannanases in *Yarrowialipolytica*. *Protein Expression and Purification*. 133, 1–7. doi:10.1016/j.pep.2017.02.008.
- Yu, A., Zhao, Y., Li, J., Li, S., Pang, Y., Zhao, Y., Zhang, C. & Xiao, D. (2020) Sustainable production of FAEE biodiesel using the oleaginous yeast *Yarrowia lipolytica*. *MicrobiologyOpen*. 9 (7), e1051. doi:10.1002/mbo3.1051.
- Yuan, S.-F., Yi, X., Johnston, T.G. & Alper, H.S. (2020) De novo resveratrol production through modular engineering of an *Escherichia coli*–*Saccharomyces cerevisiae* co-culture. *Microbial Cell Factories*. 19 (1), 143. doi:10.1186/s12934-020-01401-5.
- Zahrl, R.J., Gasser, B., Mattanovich, D. & Ferrer, P. (2019) Detection and Elimination of Cellular Bottlenecks in Protein-Producing Yeasts. *Methods in Molecular Biology*. 1923, 75–95. doi:10.1007/978-1-4939-9024-5_2.
- Zeng, H. & Yang, A. (2020) Bridging substrate intake kinetics and bacterial growth phenotypes with flux balance analysis incorporating proteome allocation. *Scientific Reports 2020 10:1*. 10 (1), 1–10. doi:10.1038/s41598-020-61174-0.
- Zeng, L., Si, Z., Zhao, X., Feng, P., Huang, J., Long, X. & Yi, Y. (2022) Metabolome analysis of the response and tolerance mechanisms of *Saccharomyces cerevisiae* to formic acid stress. *The International Journal of Biochemistry & Cell Biology*. 148, 106236. doi:10.1016/J.BIOCEL.2022.106236.
- Zhang, H., Li, Z., Pereira, B. & Stephanopoulos, G. (2015) Engineering *E. coli*–*E. coli* cocultures for production of muconic acid from glycerol. *Microbial Cell Factories*. 14 (1), 1–10. doi:10.1186/S12934-015-0319-0/TABLES/3.
- Zhang, H. & Wang, X. (2016a) Modular co-culture engineering, a new approach for metabolic engineering. *Metabolic Engineering*. 37 pp.114–121. doi:10.1016/j.ymben.2016.05.007.
- Zhang, H. & Wang, X. (2016b) Modular co-culture engineering, a new approach for metabolic engineering. *Metabolic Engineering*. 37 pp.114–121. doi:10.1016/j.ymben.2016.05.007.
- Zhang, R., Goetz, H., Melendez-Alvarez, J., Li, J., Ding, T., Wang, X. & Tian, X.J. (2021) Winner-takes-all resource competition redirects cascading cell fate transitions. *Nature Communications 2021 12:1*. 12 (1), 1–9. doi:10.1038/s41467-021-21125-3.
- Zhang, W., Liu, H., Li, X., Liu, D., Dong, X.T., Li, F.F., Wang, E.X., Li, B.Z. & Yuan, Y.J. (2017a) Production of naringenin from D-xylose with co-culture of *E. coli* and *S. cerevisiae*. *Engineering in Life Sciences*. doi:10.1002/elsc.201700039.
- Zhang, X.L. & Reed, J.L. (2014) Adaptive Evolution of Synthetic Cooperating Communities Improves Growth Performance. *Plos One*. 9 (10). doi:10.1371/journal.pone.0108297.

- Zhang, Y., Wang, C., Wang, L., Yang, R., Hou, P. & Liu, J. (2017b) Direct bioethanol production from wheat straw using xylose/glucose co-fermentation by co-culture of two recombinant yeasts. *Journal of industrial microbiology & biotechnology*. 44 (3), 453–464. doi:10.1007/S10295-016-1893-9.
- Zhao, Y., Liu, S., Lu, Z., Zhao, B., Wang, S., Zhang, C., Xiao, D., Foo, J.L. & Yu, A. (2021) Hybrid promoter engineering strategies in *Yarrowia lipolytica*: isoamyl alcohol production as a test study. *Biotechnology for biofuels*. 14 (1). doi:10.1186/S13068-021-02002-Z.
- Zhou, K., Qiao, K., Edgar, S. & Stephanopoulos, G. (2015) Distributing a metabolic pathway among a microbial consortium enhances production of natural products. *Nature Biotechnology*. 33 (4), 377–383. doi:10.1038/nbt.3095.
- Zhou, L., Wang, Y., Han, L., Wang, Q., Liu, H., Cheng, P., Li, R., Guo, X. & Zhou, Z. (2021) Enhancement of Patchoulol Production in *Escherichia coli* via Multiple Engineering Strategies. *Journal of Agricultural and Food Chemistry*. 69 (27), 7572–7580. doi:10.1021/ACS.JAFC.1C02399/ASSET/IMAGES/LARGE/JF1C02399_0006.JPEG.
- Zhou, L., Wen, Z., Wang, Z., Zhang, Y., Ledesma-Amaro, R. & Jin, M. (2020) Evolutionary Engineering Improved d -Glucose/Xylose Cofermentation of *Yarrowia lipolytica*. *Industrial and Engineering Chemistry Research*. 59 (39), 17113–17123. doi:10.1021/ACS.IECR.0C00896/SUPPL_FILE/IE0C00896_SI_001.PDF.
- Zomorodi, A.R. & Maranas, C.D. (2012) OptCom: A Multi-Level Optimization Framework for the Metabolic Modeling and Analysis of Microbial Communities. *PLOS Computational Biology*. 8 (2), e1002363. doi:10.1371/JOURNAL.PCBI.1002363.
- Zomorodi, A.R. & Segrè, D. (2017) Genome-driven evolutionary game theory helps understand the rise of metabolic interdependencies in microbial communities. *Nature Communications*. 8 (1), 1563. doi:10.1038/s41467-017-01407-5.
- Zou, L., Ouyang, S., Hu, Y., Zheng, Z. & Ouyang, J. (2021) Efficient Lactic Acid Production from Dilute Acid Pretreated Lignocellulosic Biomass by a Synthetic Consortia of Engineered *Pseudomonas Putida* and *Bacillus Coagulans*. doi:10.21203/rs.3.rs-923947/v1.
- Zwietering, M.H., Jongenburger, I., Rombouts, F.M. & Van't Riet, K. (1990) Modeling of the Bacterial Growth Curve. *Applied and Environmental Microbiology*. 56 (6), 1875. doi:10.1128/AEM.56.6.1875-1881.1990.

9. Appendices

Appendix 9.1 Equations used in previous iteration of the model

$$\frac{ds_0}{dt} = s_{0in} - \frac{v_{ea} ea s_0}{s_0 + K_{M_{ea}}} - dg_{s_0} s_0$$

Equation W

$$\frac{ds_1}{dt} = n_{g0} \frac{v_{ea} ea s_0}{s_0 + K_{M_{ea}}} - \frac{v_{eb} eb s_1}{s_1 + K_{M_{eb}}} - dg_{s_1} s_1$$

Equation X

$$\frac{ds}{dt} = n_{g1} \frac{v_{eb} eb s_1}{s_1 + K_{M_{eb}}} - v(t, s) - dg_s s$$

Equation Y

$$v(t, s) = \frac{v_t t s}{s + K_{M_t}}$$

Equation Z

$$v(t, s) = \frac{v_t s (t_{cell_a} + t_{cell_b})}{s + K_{M_t}}$$

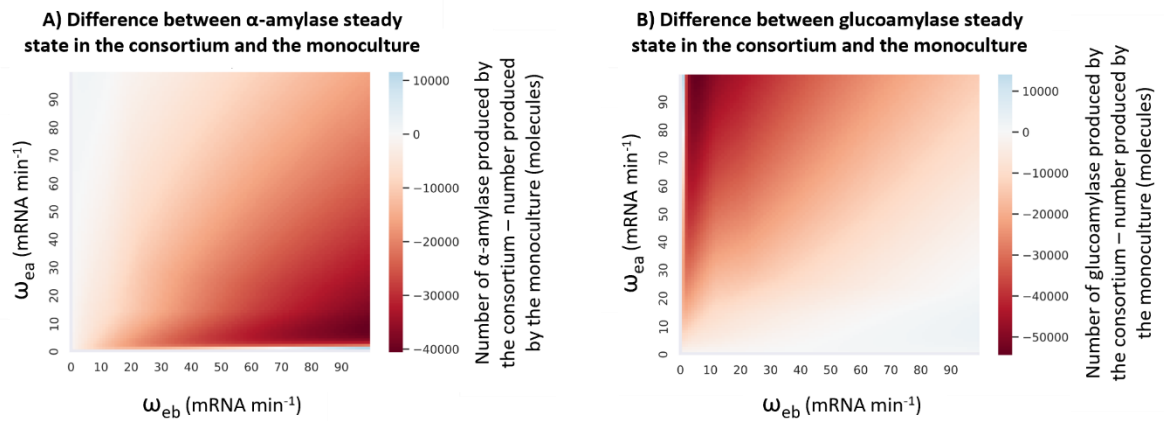
Equation Z'

Appendix 9.2 Steady states of all intracellular molecules for a single cell in the monoculture and for Cell A and Cell B in the consortium

Species	Description	Initial value	Molecules/cell		
			Monoculture single cell	Cell A	Cell B
m_ea	mRNA Enzyme A	0	86.89994	71.42561	
c_ea	mRNA:ribosome Enzyme A	0	177.796	252.7591	
ea	Enzyme A	0	16380.94	20130.16	
m_eb	mRNA Enzyme B	0	82.99183		68.70025
c_eb	mRNA:ribosome Enzyme B	0	206.6798		274.4006
eb	Enzyme B	0	13394.28		15660.84
m_q	mRNA housekeeping	0	922.3015	661.9858	697.3448
c_q	mRNA:ribosome housekeeping	0	1487.333	1836.807	1787.49
q	Housekeeping protein	0	210910.1	222435.3	220860.7
m_em	mRNA metabolic	0	18.85034	16.05385	16.52277
c_em	mRNA:ribosome metabolic	0	30.39866	44.54449	42.35249
em	Metabolic protein	0	4310.658	5394.288	5233.037
m_et	mRNA transport	0	18.85034	16.05385	16.52277
c_et	mRNA:ribosome transport	0	30.39866	44.54449	42.35249
et	Transport protein	0	4310.658	5394.288	5233.037
m_r	rRNA	0	88.46736	67.94208	71.05094
c_r	rRNA:ribosome	0	418.0531	573.9163	551.168
r	Ribosome	10	5.216145	9.408563	8.628292
si	Internal sugar	0	143.0823	143.0823	143.0823
a	Energy unit	1000	7.840284	9.076709	8.867173
Growth rate			0.015648	0.019581	0.018996
Doubling time			44.29714	35.39852	36.48929
Total ribosomes			2355.875	2761.98	2706.392
Total proteins			249311.8	253363.4	246996.3
Heterologous/total proteins (%)			11.94296	7.94517	6.34052

Appendix 9.3 Difference between the steady-state production of both heterologous proteins by 2 cells of a monoculture versus 2 cells in a consortium where only one cell produces each protein

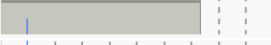
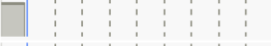
Here, the model predicts the difference in amylase produced by a 2-cell consortium and a 2-cell monoculture as the transcription rates of α -amylase and glucoamylase (ω_{ea} and ω_{eb} respectively) are varied. Difference in amylase is calculated as the consortium steady state minus the monoculture steady state. The monoculture will produce more than the consortium for almost all combinations of values of the maximum transcription rates (ω_{ea} and ω_{eb}).



Appendix 9.4 Fitting data for desirability model

The effect summaries indicate how each predictor variable affects the overall desirability of the outcomes. The LogWorth for each model effect, defined as $-\log_{10}(\text{p-value})$, adjusts p-values to provide an appropriate scale for graphing. A value that exceeds 2 (the blue dashed line) is significant at the 0.01 level. The summary of fit gives the adjusted R^2 values measuring the fit of the model to the data.

A) OsAA effect summaries

Response	Factor	LogWorth		PValue
Carrying Capacity	Promoter	14.638		0.00000
	Signal peptide*Promoter	1.817		0.01526
	Signal peptide	1.257		0.05536
Growth rate	Promoter	12.510		0.00000
	Signal peptide*Promoter	1.700		0.01995
	Signal peptide	0.828		0.14853
Inflection point	Promoter	12.321		0.00000
	Signal peptide*Promoter	3.501		0.00032
	Signal peptide	3.303		0.00050

B) TIGAMY effect summaries

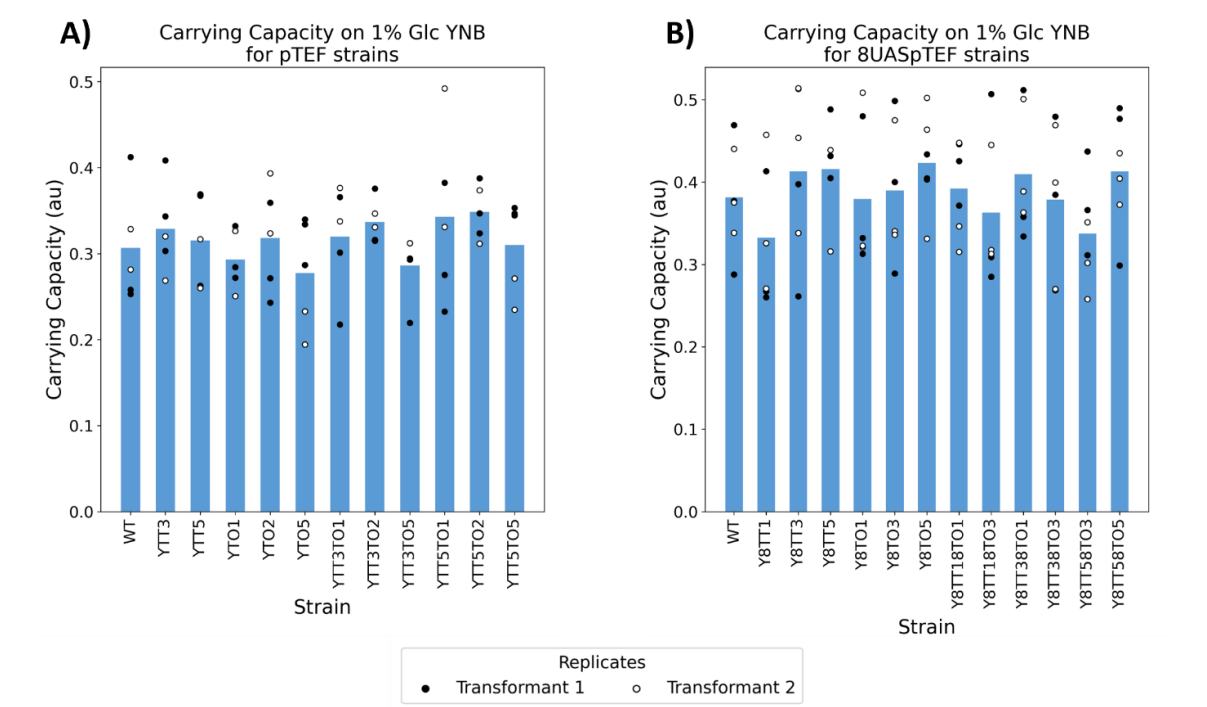
Response	Factor	LogWorth		PValue
Carrying capacity	Promoter	11.611		0.00000
	Signal peptide*Promoter	5.732		0.00000
	Signal peptide	1.966		0.01081
Growth rate	Promoter	6.135		0.00000
	Signal peptide*Promoter	2.279		0.00525
	Signal peptide	1.535		0.02919
Inflection point	Signal peptide*Promoter	9.231		0.00000
	Promoter	8.219		0.00000
	Signal peptide	3.312		0.00049

C) Summary of Fit

OsAA			TIGAMY		
Carrying capacity	RSquare	0.666008	Carrying capacity	RSquare	0.686249
	RSquare Adj	0.603663		RSquare Adj	0.627683
	Root Mean Square Error	0.063105		Root Mean Square Error	0.061246
Growth rate	RSquare	0.247514	Growth rate	RSquare	0.478752
	RSquare Adj	0.10705		RSquare Adj	0.381453
	Root Mean Square Error	0.035409		Root Mean Square Error	0.053603
Inflection point	RSquare	0.649821	Inflection point	RSquare	0.696287
	RSquare Adj	0.584454		RSquare Adj	0.639594
	Root Mean Square Error	2.573486		Root Mean Square Error	1.535139

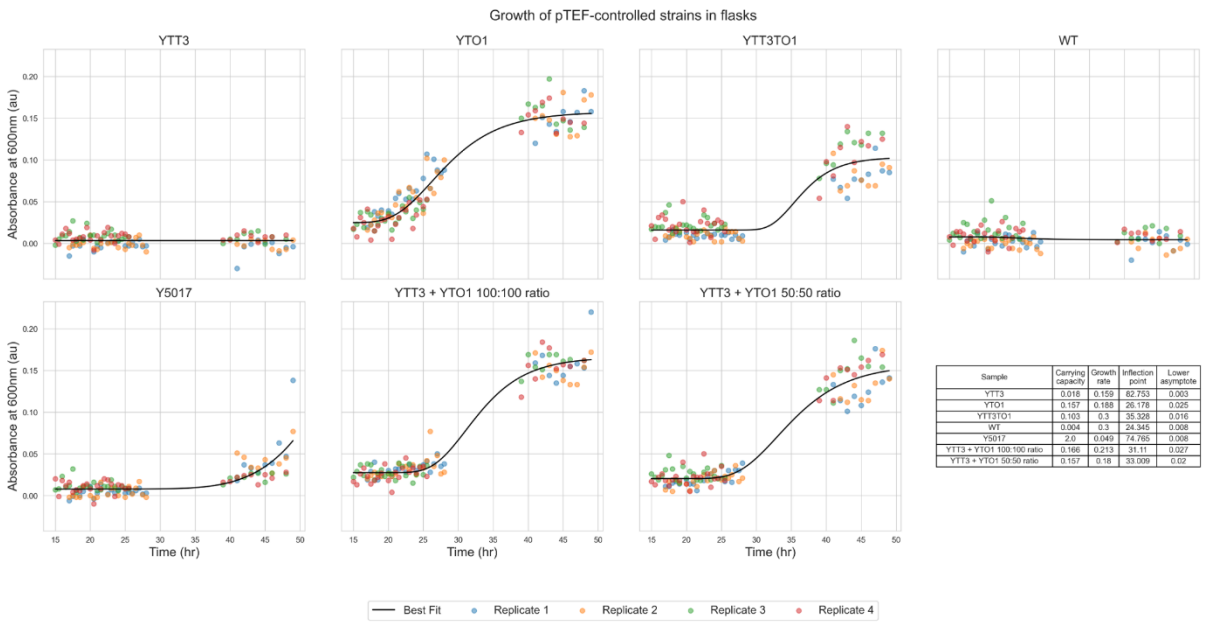
Appendix 9.5 Carrying capacity for single-amylase and double-amylase strains on 2% glucose YNB in 96-well plate format with A) pTEF promoter and B) 8UASpTEF promoter

No significant differences were observed for carrying capacity between the WT and the amylase-expressing strains.



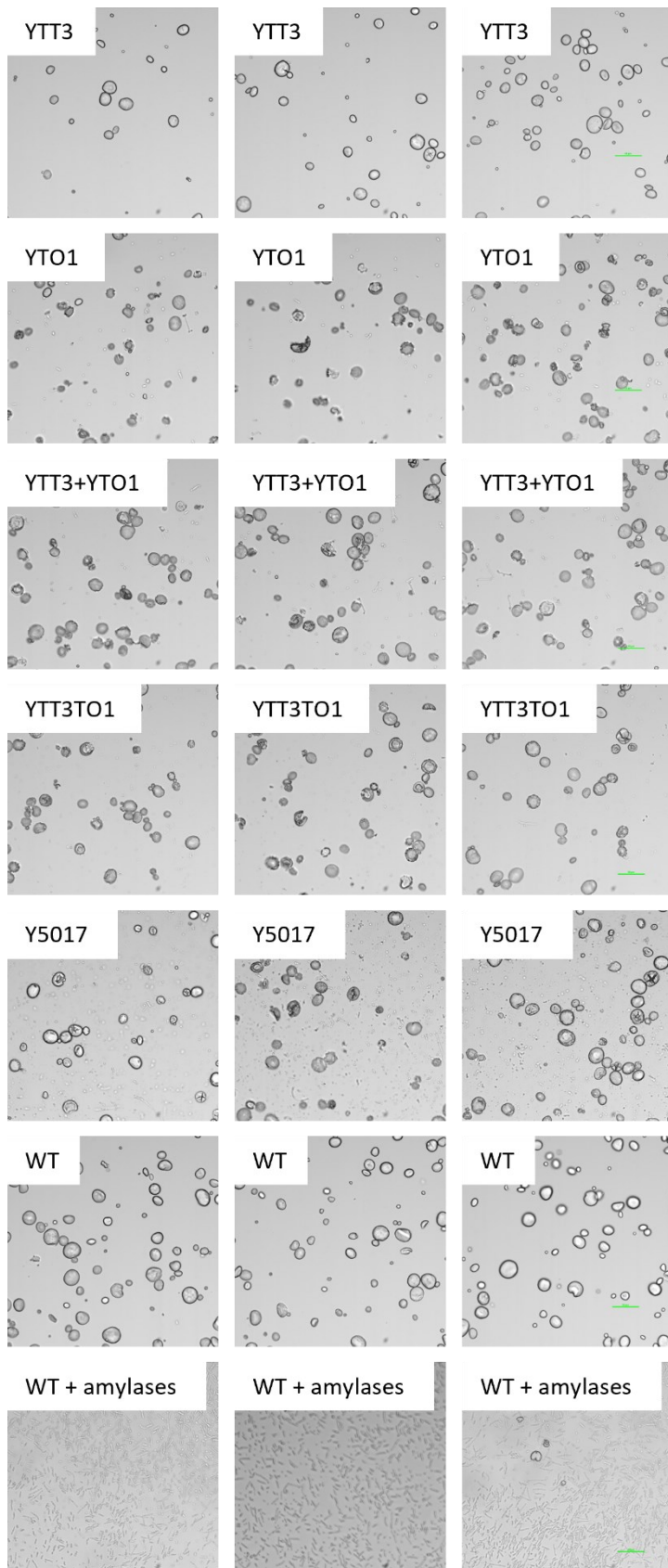
Appendix 9.6 Growth of YTT3TO1 monoculture and YTT3+YTO1 consortium on 1% potato starch YNB in flasks over 48 hours

Samples were taken during the day, meaning the data between 26 and 39 hours (overnight) is missing, making the fit to a Gompertz curve difficult, since it appears exponential phase happens overnight.



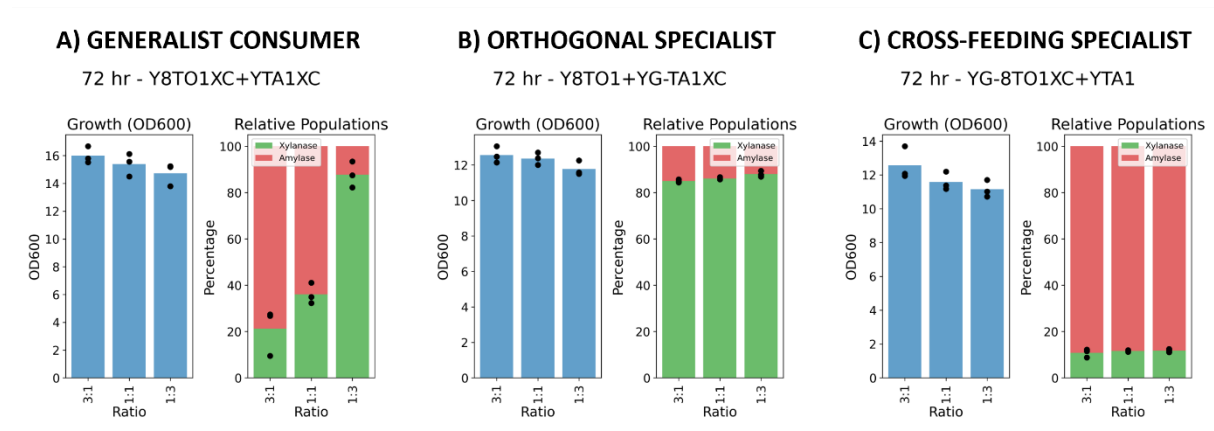
Appendix 9.7 Cultures under microscope

Cultures of *Y. lipolytica* strains grown on 1% starch YNB for 24 hours, imaged at x200 magnification.



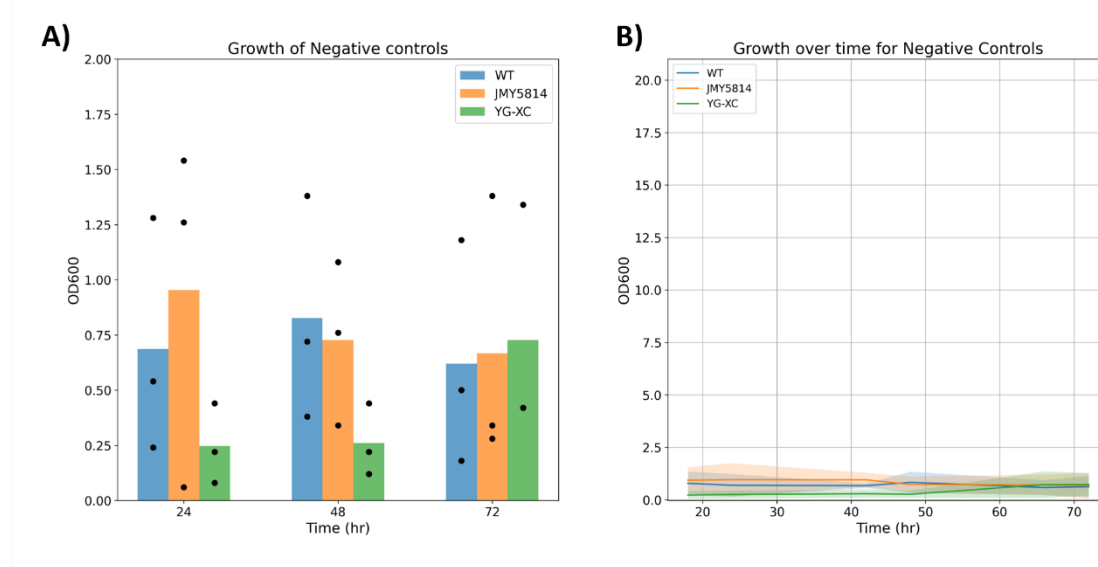
Appendix 9.8 Growth and population composition of DOL strategies with different inoculation ratios

Same experimental set-up as in Figure 4.4, except using different inoculation ratios. Here the OD600 for the three inoculation ratios was quite similar, hence I chose to use greater ratios in the next set of experiments.



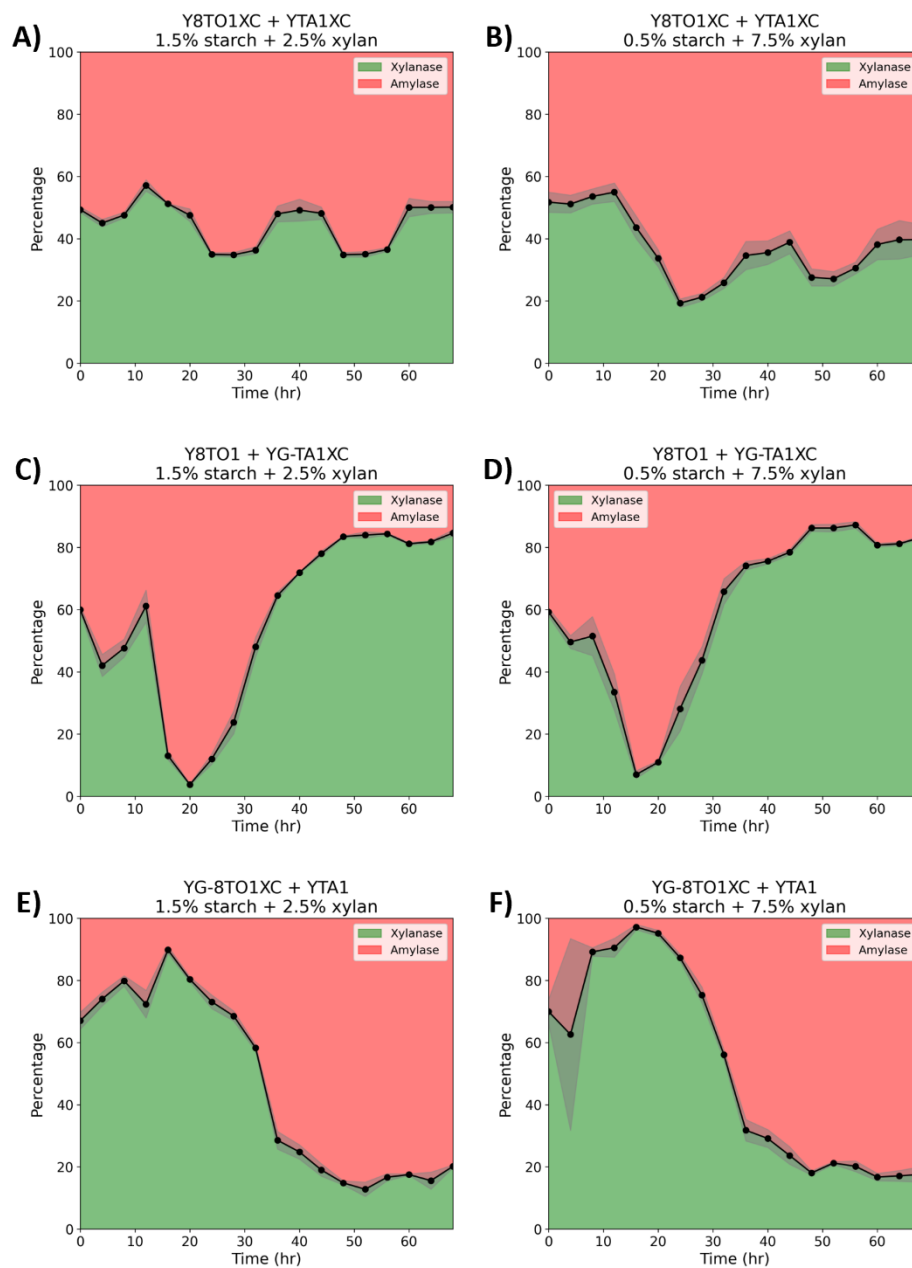
Appendix 9.9 Negative controls

The WT strain (Po1d), JMY5814 (Po1d with xylose cassette) and YG-XC (Po1d Δ HXT with xylose cassette) were grown on 1% starch + 1% xylan media.



Appendix 9.10 Population composition of DOL strategies on 1.5% starch + 2.5% xylan and 0.5% starch + 7.5% xylan YNB

This data shows the remaining two media compositions for Figure 4.8.



Appendix 9.11 Growth of different DOL strategies on starch and pre-treated xylan media – comparing media conditions for each DOL strategy

This is a reformatting of the data from Figure 4.9 to show the differences between the three media for the different DOL strategies. Here you can directly compare the pure starch and pure xylan medias to the mixed polysaccharide media.

