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REVIEW

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Division of labour enables *Yarrowia lipolytica* synthetic consortia to achieve higher growth rates from starch

Eliza Atkinson[1,2,3,4], Guy-Bart Stan[1,4] and Rodrigo Ledesma-Amaro[1,2,3,4*]

Abstract

Background *Yarrowia lipolytica* is a promising host for a range of biotechnological applications. However, the costs and sustainability of common feedstocks limit the commercial viability of current bioproduction. Consolidated bioprocessing using plant biomass offers a desirable alternative to reduce costs and improve sustainability, though such approaches are often constrained in single-organism systems due to metabolic burden. Here, we have investigated the use of division of labour (DOL) in *Y. lipolytica* consortia for the degradation of starch, a common component of plant biomass.

Results We engineered a panel of strains expressing α-amylase and/or glucoamylase, with varying promoter and signal peptide combinations. We found that stronger expression led to higher metabolic burden, and that expressing both amylases imposed a greater burden than expressing either enzyme alone. When combining both amylase strains, we found that some consortia could achieve faster growth rates than the equivalent monoculture. The fastest growing consortium identified from the panel was scaled-up into flasks, where the consortium achieved the highest final biomass, although the fastest growth rate was achieved by the α-amylase strain alone.

Conclusions These findings suggest that DOL is a promising strategy for degrading complex macromolecules in plant biomass, offering a potential route to more sustainable bioprocessing.

Keywords Microbial consortia, Division of labour, Sustainable biotechnology, Precision fermentation, Engineering biology, Metabolic engineering, Synthetic biology

Introduction

As global resource demands increase, the need for renewable approaches to replace conventional food, energy and material production has become a focus for national and global bodies for achieving their sustainability goals. One of the key bottlenecks to cost-efficiency and sustainability is the use of expensive purified sugar feedstocks. A better alternative is to use plant biomass from agricultural or food waste as a feedstock. The second most abundant constituent of plant biomass is starch, and high-starch foods, such as bread and potatoes, are common in food waste.

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Yarrowia lipolytica is a promising candidate host for this next generation of sustainable biotechnology. It is a non-conventional oleaginous yeast that has been used for the expression of several useful heterologous proteins [1] and the production of high-value compounds such as organic acids [2], aromas [3, 4], carotenoids [5], and lipid precursors for biofuels [6–8]. Of particular relevance, *Y. lipolytica* has a unique secretory pathway, giving it a higher secretory capacity than many alternative yeasts [9–11]. *Y. lipolytica* naturally produces high levels of proteases and lipases, allowing it to digest and utilise a range of substrates such as glycerol, crude waste of tallow and olive-mill wastewater [12]. However, *Y. lipolytica* cannot natively grow on lignocellulose substrates, or other complex polysaccharides such as 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 and a glucoamylase [13].

However, the expression of extracellular hydrolytic enzymes in yeast is limited by the metabolic burden of translation and secretion, as observed for both cellulases [14] and xylanases [15]. Overexpression of heterologous genes can lead to decreased growth rates and reduced yields of product due to intracellular resources being diverted away from essential endogenous bioprocesses [16–18]. Protein folding, post-translational modification and secretion are energy and resource-intensive processes, and inefficiency can lead to ER overload and stress responses, which in turn can reduce cell growth and lower yields in biotechnological applications [19–21].

To reduce metabolic burden, synthetic biology approaches can utilise microbial consortia that divide tasks among different populations, called division of labour (DOL). By distributing functions across specialised strains, DOL reduces the complexity within individual cells, as each expresses only a subset of the required proteins. This approach is particularly useful for tasks like plant biomass degradation, where the expression of multiple hydrolytic enzymes can be partitioned across different consortium members [22, 23]. For example, Tsai, Goyal & Chen (2010) engineered four different strains of *Saccharomyces cerevisiae*, each expressing a different subunit of the cellulosome. Altering the ratio of the four strains in the 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 [24–26].

In the current monoculture system for starch degradation in *Y. lipolytica*, α-amylase and glucoamylase are co-expressed [13]. Here, by dividing labour between two cells, each expressing a single amylase, we aim to study division of labour and metabolic burden as it relates to the degradation of starch in both monoculture and consortia (Fig. 1).

Results and discussion

Strain engineering of single-amylase strains and the effect of expression on metabolic burden

Y. lipolytica strain Po1d was engineered to express either *Oryza sativa* α-amylase (OsAA) or *Thermomyces*

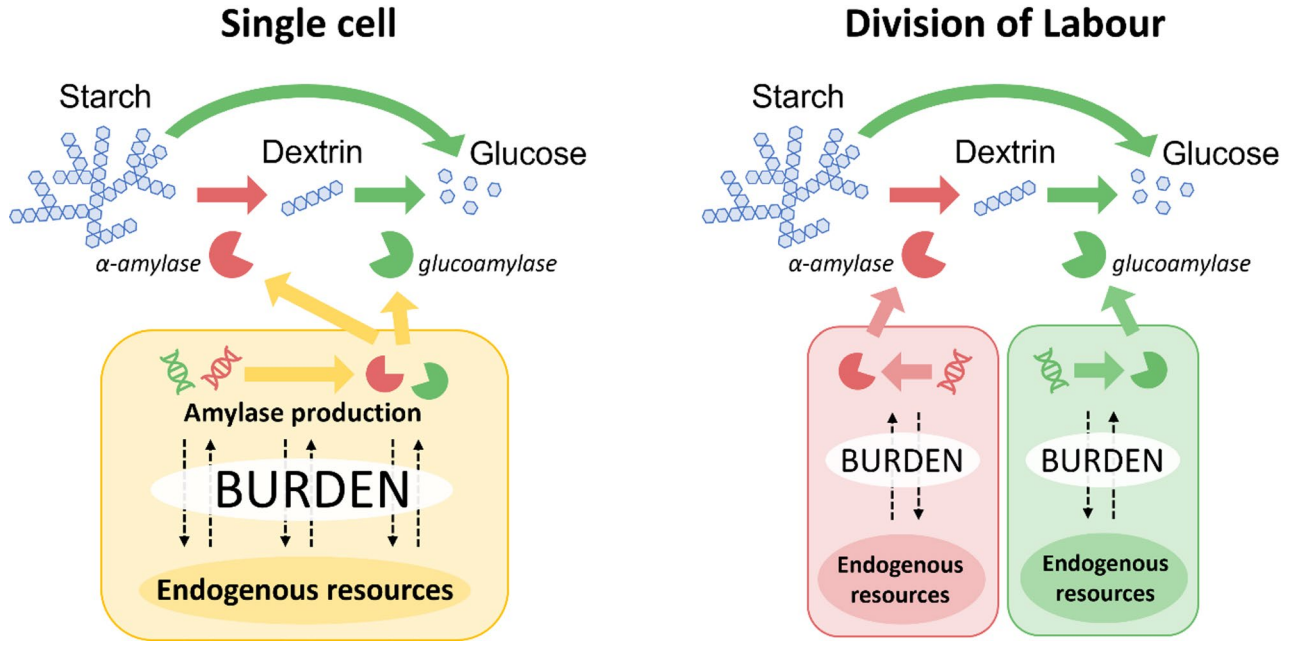


Fig. 1 Division of labour in a 2-strain consortium for starch degradation. A monoculture cell expresses both α-amylase and glucoamylase, and therefore has two heterologous genes interfering with endogenous bioprocesses. If this labour is divided into 2 cells, the burden will be reduced, and growth on starch should be improved

lanuginosus glucoamylase (TlGAMY), summarised in Table 1. Both these amylases have been previously used in *Y. lipolytica* to enable growth in combination with other amylases [13, 27]. To achieve a range of expression levels, each amylase was assembled with 3 different promoters: two native promoters, the weak pPGM and the widely used pTEF [28] and one strong synthetic promoter 8UASpTEF, made of pTEF and 8 upstream activating sequences [29]. A panel of 5 synthetic signal peptides (SPs) created by Celińska et al. were used to modulate the secretion level [27]. Two transformants were tested for growth to highlight any impacts of different integration sites or multicopy integration on growth.

Table 1) O. sativa α-amylase (OsAA) and T. lanuginosus glucoamylase (TlGAMY) strains

RLA No.	Strain	Insert/s	RLA No.	Strain	Insert/s
Single-amylase strains express either α-amylase or glucoamylase					
1806	YTO1	pTEF-SP1-OsAA	1811	YTT1	pTEF-SP1-TlGAMY
1807	YTO2	pTEF-SP2-OsAA	1812	YTT2	pTEF-SP2-TlGAMY
1808	YTO3	pTEF-SP3-OsAA	1813	YTT3	pTEF-SP3-TlGAMY
1809	YTO4	pTEF-SP4-OsAA	1814	YTT4	pTEF-SP4-TlGAMY
1810	YTO5	pTEF-SP5-OsAA	1815	YTT5	pTEF-SP5-TlGAMY
1826	YPO1	pPGM-SP1-OsAA	1831	YPT1	pPGM-SP1-TlGAMY
1827	YPO2	pPGM-SP2-OsAA	1832	YPT2	pPGM-SP2-TlGAMY
1828	YPO3	pPGM-SP3-OsAA	1833	YPT3	pPGM-SP3-TlGAMY
1829	YPO4	pPGM-SP4-OsAA	1834	YPT4	pPGM-SP4-TlGAMY
1830	YPO5	pPGM-SP5-OsAA	1835	YPT5	pPGM-SP5-TlGAMY
1846	Y8TO1	8UASpTEF-SP1-OsAA	1851	Y8TT1	8UASpTEF-SP1-TlGAMY
1847	Y8TO2	8UASpTEF-SP2-OsAA	1852	Y8TT2	8UASpTEF-SP2-TlGAMY
1848	Y8TO3	8UASpTEF-SP3-OsAA	1853	Y8TT3	8UASpTEF-SP3-TlGAMY
1849	Y8TO4	8UASpTEF-SP4-OsAA	1854	Y8TT4	8UASpTEF-SP4-TlGAMY
1850	Y8TO5	8UASpTEF-SP5-OsAA	1855	Y8TT5	8UASpTEF-SP5-TlGAMY
Double-amylase strains express both α-amylase and glucoamylase					
1867	YTT-3TO1	pTEF-SP3-TlGAMY-pTEF-SP1-OsAA			
1868	YTT-3TO2	pTEF-SP3-TlGAMY-pTEF-SP2-OsAA			
1869	YTT-3TO5	pTEF-SP3-TlGAMY-pTEF-SP5-OsAA			
1870	YTT-5TO1	pTEF-SP5-TlGAMY-pTEF-SP1-OsAA			
1871	YTT-5TO2	pTEF-SP5-TlGAMY-pTEF-SP2-OsAA			
1872	YTT-5TO5	pTEF-SP5-TlGAMY-pTEF-SP5-OsAA			
1887	Y8TT-18TO1	8UASpTEF-SP1-TlGAMY-8UASpTEF-SP1-OsAA			
1888	Y8TT-18TO3	8UASpTEF-SP1-TlGAMY-8UASpTEF-SP3-OsAA			
1889	Y8TT-38TO1	8UASpTEF-SP3-TlGAMY-8UASpTEF-SP1-OsAA			
1890	Y8TT-38TO3	8UASpTEF-SP3-TlGAMY-8UASpTEF-SP3-OsAA			
1891	Y8TT-58TO3	8UASpTEF-SP5-TlGAMY-8UASpTEF-SP3-OsAA			
1892	Y8TT-58TO5	8UASpTEF-SP5-TlGAMY-8UASpTEF-SP5-OsAA			

The following strains were transformed with corresponding assemblies containing the following constant elements integrated with the genome: NotI_InsUP-zeta_URA3_*variable insert*_InsDOWN_zeta_NotI.

These single-amylase strains were grown for 72 h on YNB 2% glucose in 96-well plates, measuring absorbance at 600 nm (Abs600) every 30 min. Growth parameters were obtained using curve-fitting to a four-parameter Gompertz curve [30]. Since amylases confer no survival advantage on glucose, any limitations to growth will reflect the burden of expressing the additional heterologous gene.

Figure 2A, B, and C show the growth parameters of each single-amylase strain. The most significant differences are seen with the strongest promoter, 8UASpTEF. Eight out of ten 8UASpTEF-SP variants exhibit a decreased growth rate and a later inflection point. Additionally, nine out of ten of the 8UASpTEF strains also show a slightly higher carrying capacity, the maximum population size, measured as the maximum Abs600 achieved by the culture when they plateau at stationary phase. Most of the other promoter-SP-amylase combinations have no significant difference in growth rate, with a few exceptions. Besides growth rate, many other promoter-SP-amylase combinations also show a significantly later inflection point and a non-significant decrease in carrying capacity— indicating a slower and lower overall growth when expressing these genes.

Overall, we do observe the effects of metabolic burden when expressing amylase genes, with the strong synthetic promoter causing the most significant burden.

Amylase activity shows the relative secretion strength of the amylase strains

There did not appear to be a strong correlation between the signal peptide and the burden to growth caused. However, secretion strength is important for the efficiency of starch degradation. The supernatants were tested for amylase activity to find the relative secretion strength of each strain. We used these results to select strains that exhibited a range of secretion levels for each amylase, which would be utilized in subsequent experiments comparing consortia to monocultures.

To test the activity of OsAA, which degrades large starch molecules into smaller maltodextrin chains, a micro-SIT assay was used [31], where iodine stains the large starch granules and provides a colorimetric output for OsAA removing starch. Supernatants of YTO1-5

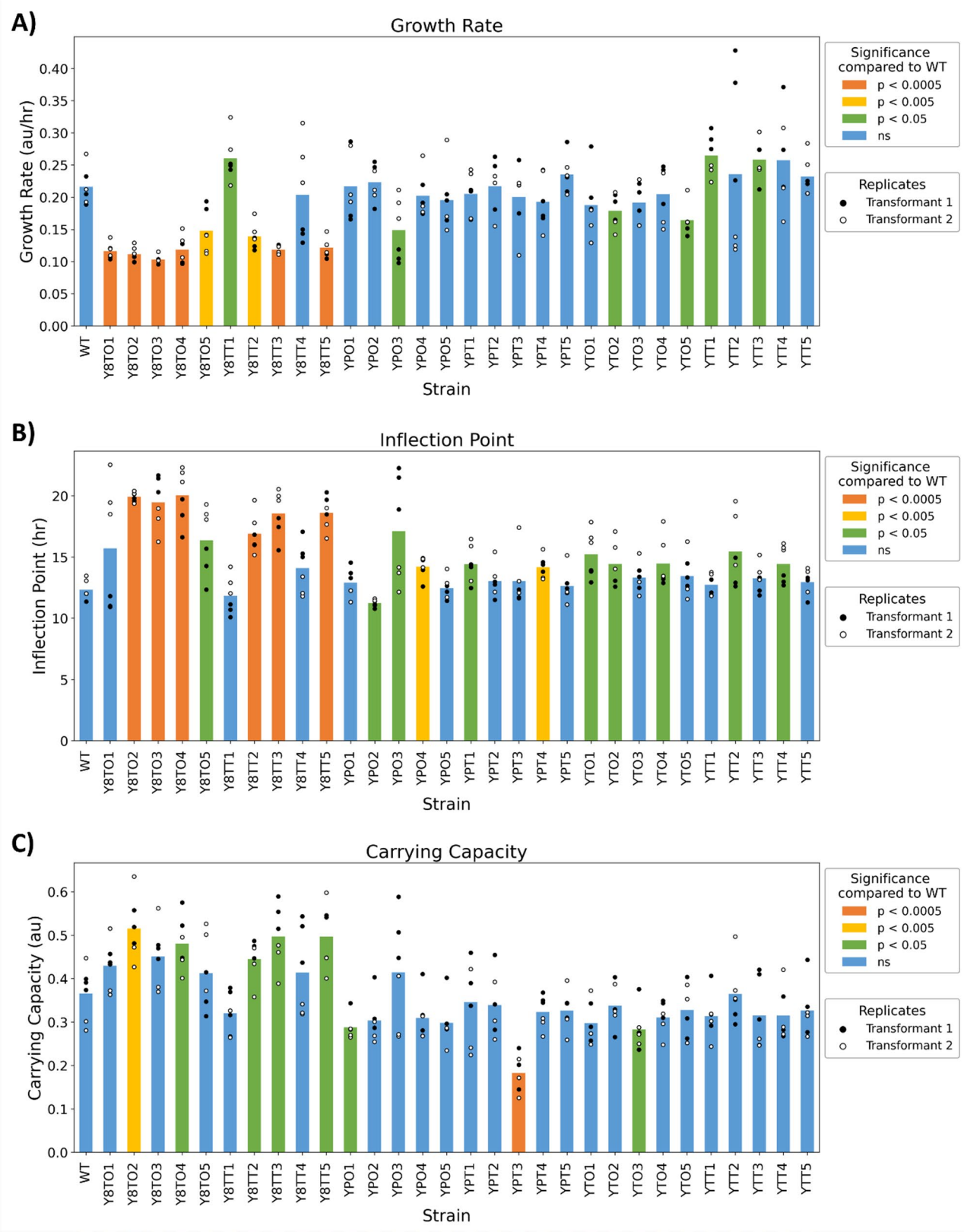


Fig. 2 (See legend on next page.)

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Fig. 2 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 (Materials and Methods 4.6) to derive the parameters of (**A**) growth rate (**B**) inflection point (which reflects lag time) and (**C**) carrying capacity (the maximum final absorbance value). Growth rate is decreased in most of the 8UASpTEF controlled strains, while the lag time is extended. This promoter also appeared to give a higher final carrying capacity. Data presented as the mean for n=6, where n=3 for each different transformant. Statistical analysis by Welch's T-test, pairwise analysis of each strain compared to the WT

transformant 1 were mixed with starch and micro-SIT measured the starch remaining after 15 min incubation. Figure 3A shows that YTO1 and YTO5 are the strongest strains for OsAA, with the least starch remaining; YTO3 and 4 are mid-level and YTO2 is the weakest. For our initial panel of strains, with the pTEF promoter, we chose to use YTO1, YTO2 and YTO5 for OsAA, to give both the strongest and weakest secretion.

To test the activity of TlGAMY, which produces single glucose units from starch, a micro-GOPOD (glucose oxidase peroxidase) assay was used, which provides a colorimetric output for glucose concentration. Supernatants of Y8TT1-5 transformant 1 were mixed with starch, and micro-GOPOD used to observe the concentration of glucose after 48 h. Figure 3B shows that YTT1 and 5 made the most glucose, and YTT2 the made the least. Specific enzyme activity was also calculated and shown in Supplementary Fig. 1. This contrasts with previously published results, which found SP3 to be the strongest and SP5 to be the weakest for TlGAMY [27], which can indicate a context-dependent effect. We chose to use the strains YTT3 and YTT5 for the pTEF-TlGAMY strains, since this would include a strong strain and a weak-mid strain, according to both results.

The WT strain, which was used as a negative control, also showed some glucose in the GOPOD assay. This glucose can be assumed to come from the starch solution, since the supernatants were produced using a culture grown on glycerol as the sole carbon source, therefore no glucose comes from the supernatant. In addition, while α-amylase primarily produces maltodextrins, it can also produce free glucose (Supplementary Fig. 2).

Double-amylase strains show more burden than single-amylase strains

Existing knowledge about burden suggests that the double-amylase strains, expressing both OsAA and TlGAMY, should have more burden than the single-amylase strains, expressing only one of the amylases [32]. Double-amylase strains were created with the same promoters and signal peptides, to be equivalent to a selection of the different single-amylase strains (Table 1). Single and double-amylase strains were grown for 48 h on 1% glucose YNB media in 96-well plates. 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.

Figure 4A shows that the growth rate is lower in many of the double-amylase strains than in the single-amylase strains. The double-amylase strains YTT3TO1, YTT3TO5, YTT5TO2 and YTT5TO5 all have significantly lower growth rates than the WT strain. In addition, YTT5TO2 has a significantly lower growth rate than both its equivalent single-amylase strains YTT5 and YTO2. Apart from YTT5TO1, the other double-amylase strains have a significantly reduced growth rate compared to the TlGAMY-expressing strains but not compared to the OsAA-expressing strains.

Figure 4B shows that the co-expression of both amylases also causes an increase in the time of the inflection point, indicating a longer lag time. The double-amylase YTT3TO2, YTT5TO2 and YTT5TO5 show a significant increase in their inflection point compared to WT. In addition to its lower growth rate, YTT5TO2 shows a significantly increased inflection point compared to both YTT5 and YTO2. YTT3TO2 and YTT5TO5 show a significant increase compared to YTT3 and YTT5, respectively, while there are no significant differences between the other double-amylase strains and their equivalent single-amylase strains. There are no significant differences in carrying capacity (Supplementary Fig. 3).

Considering both growth rate and inflection point, the double-amylase strains show a greater overall impact on growth compared to their single-amylase-expressing counterparts. Previous modelling of the effect of expression on burden [32] suggests that these growth impacts will be further exacerbated by increasing the expression and secretion of the genes. Therefore, we created consortia using the stronger 8UASpTEF promoter along with high- or mid-strength signal peptides (SPs) for both amylases. Figure 4C and D illustrate the growth rates and inflection points of strains controlled by the 8UASpTEF promoter. Compared to the weaker pTEF promoter strains, those with the 8UASpTEF promoter exhibit fewer significant differences between single and double-amylase strains. In Fig. 4C, all strains, except for Y8TT1, have a lower growth rate than the WT, although only 4 of these are significant. A significant decrease in growth rate is observed between Y8TT1 and both Y8TT18TO1 and Y8TT18TO3, with a similar trend for Y8TT5 compared to Y8TT58TO5. However, none of the double-amylase strains exhibit a significantly lower growth rate than both of their single-amylase counterparts. In Fig. 4D, and 8 out of 12 8UASpTEF strains have a significantly higher inflection point than WT. The significant decrease in growth

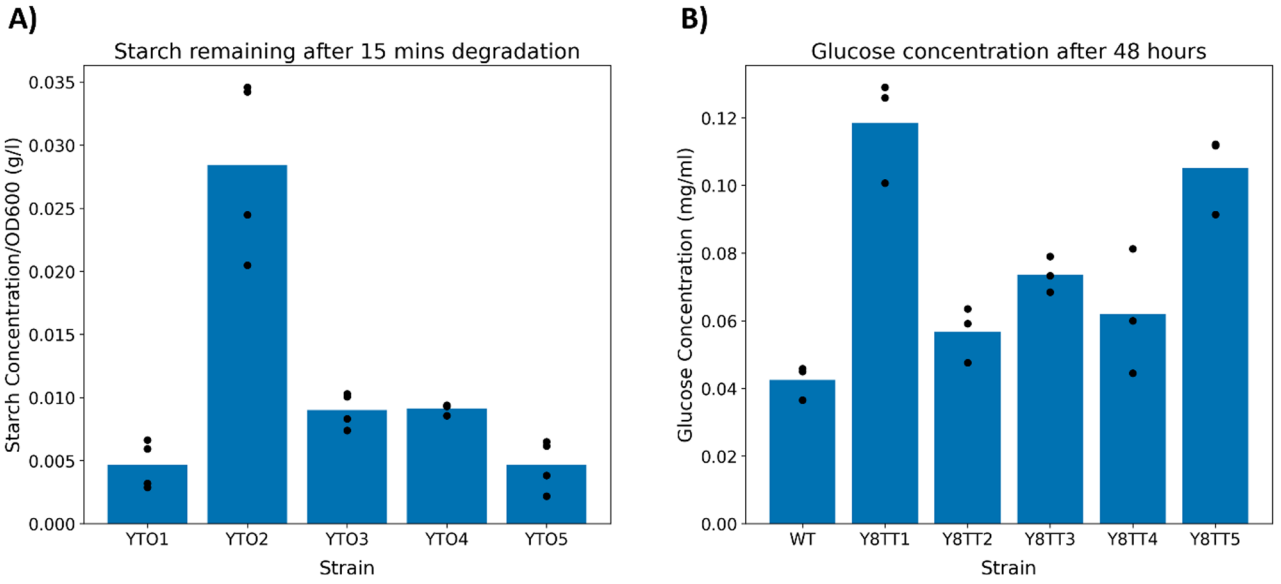


Fig. 3 **A** Micro-SIT and **B** Micro-GOPOD assays for the supernatants of OsAA and TlGAMY strains respectively. Supernatants from cultures of single-amylase strains were collected and tested for activity by micro-SIT assay, to observe how much starch is broken down, or micro-GOPOD, to test how much glucose is produced. Results were normalised by the final OD of the cultures. For both OsAA and TlGAMY, the strain containing SP1 (YTO1 and Y8TT1) and the strain containing SP5 (YTO5 and Y8TT5) are the strongest strains, while the SP2 strain (YTO2 and Y8TT2) is the weakest. Results of statistical analysis by Welch's T-test is shown in Supplementary Tables 1 and 2

rate for Y8TT18TO3 compared to Y8TT1 corresponds with a significant increase in the inflection point (Fig. 4D), but no other double-amylase strains showed a significant difference in inflection point compared to their single-amylase equivalent.

Two of the 8UASpTEF-OsAA strains (Y8TO3, Y8TO5) show a significantly decreased growth rate compared to WT, and all three 8UASpTEF-OsAA strains are significantly different for inflection point, despite only expressing one amylase. Since 8UASpTEF causes burden even with a single gene expression, demonstrated here and in Fig. 2, it may not be possible for the expression of a second gene to cause significantly higher burden than is caused by only one. This may mean that DOL will not confer a significant advantage for 8UASpTEF strains. However, the stronger promoter will increase the starch-degradation, which may lead to better growth despite additional burden.

Overall, we do observe that expressing two amylases in a single cell causes a lower growth rate and higher inflection point (longer lag time) than expressing a single amylase. Therefore, when combined in a 2-strain consortium, the two amylase strains should individually have lower metabolic burden than an equivalent double-amylase expressing monoculture. We hypothesised that this would allow DOL to achieve a higher growth rate and shorter lag time than the monoculture when grown on starch as the sole carbon source.

Comparing growth on starch of monoculture double-amylase strains and 2-strain consortia of single-amylase strains

Our previous modelling [32] predicted that a 2-strain consortium containing an α-amylase-expressing and a glucoamylase-expressing strain would achieve a higher average growth rate than an equivalent monoculture co-expressing both amylases when metabolic burden was limiting to growth. Since we have observed that metabolic burden is limiting to growth in glucose, we then studied whether DOL could achieve a higher growth rate on starch, as predicted. The double-amylase strains were grown on 1% starch YNB in 96-well plates and compared to 2-strain consortia, each containing a strain expressing OsAA and a strain expressing TlGAMY. The two single-amylase expressing strains were inoculated in a 1:1 ratio, with both strains at an initial OD of 0.01 OD, and the monoculture was also inoculated at an initial OD of 0.01, so that the consortia had an equal number of cells producing each amylase compared to the monoculture.

Figure 5A shows the growth rates of the pTEF-controlled consortia compared to their equivalent monoculture. The consortia of YTT3 + YTO1 and YTT5 + YTO1 exhibit increased growth rates compared to their equivalent double-amylase strains, although only the difference for the YTT3 + YTO1 was statistically significant. Figure 5B shows the growth rate of the 8UASpTEF-controlled consortia compared to their equivalent monoculture. Y8TT1 + Y8TO1, Y8TT3 + Y8TO1, and Y8TT5 + Y8TO5 consortia all have significantly increased

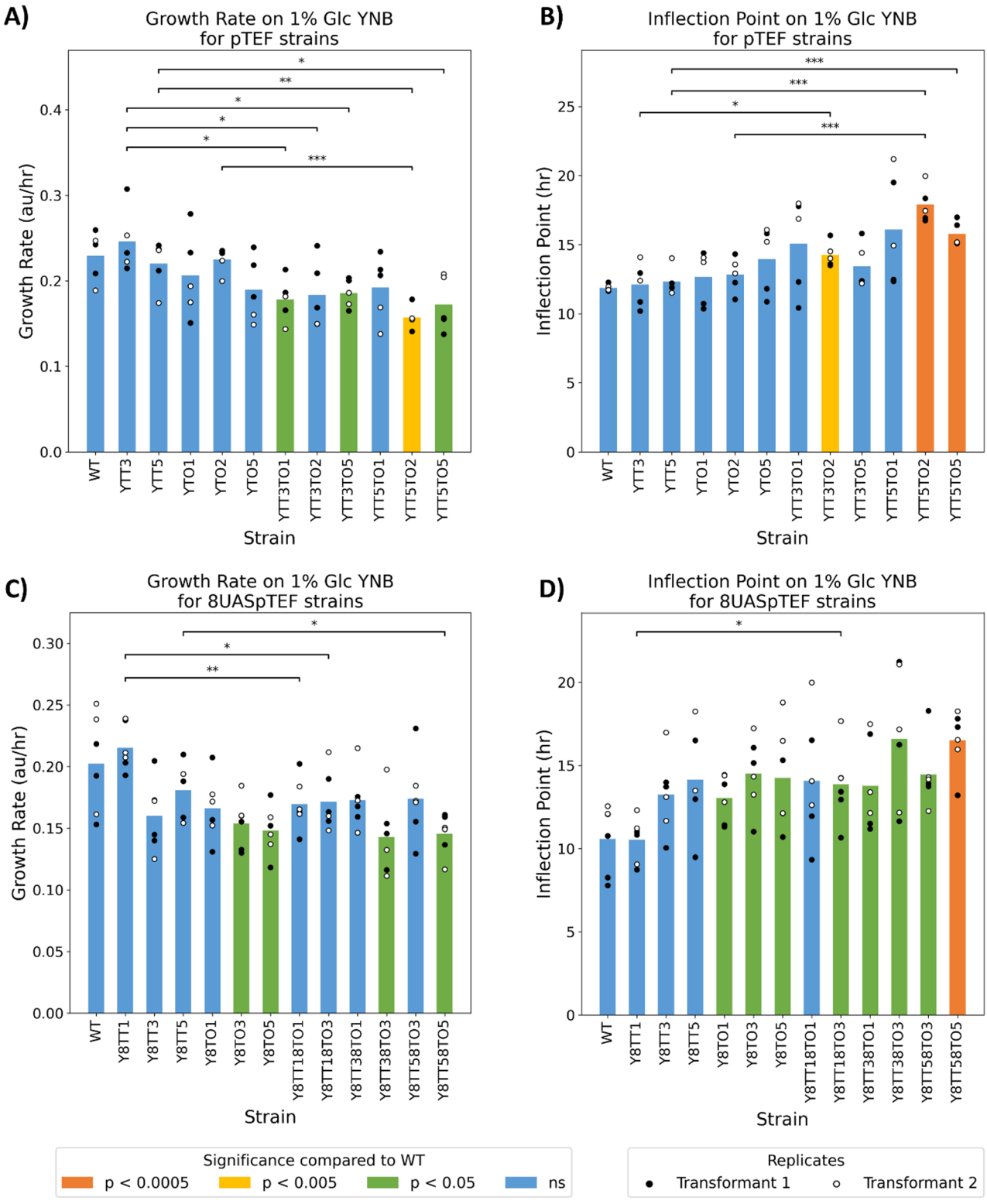


Fig. 4 (See legend on next page.)

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Fig. 4 Growth rate and inflection point of single and double-amylase-expression strains on glucose. Strains were grown for 48 hours on 1% glucose YNB media and growth rate was calculated by Gompertz 4-parameter curve fitting (Materials and Methods 4.6). **A** Double-amylase strains have lower mean growth rate than their single-amylase equivalents. However, only YTT5TO2 shows a significantly decreased growth rate compared to both its equivalent single-amylase strains; the other double-amylases show either non-significant differences, or only significant compared to the TlGAMY strain. **B** Double-amylase strains have higher inflection points than their single-amylase equivalents. Again, only YTT5TO2 shows a significantly higher inflection point compared to both its equivalent single-amylase strains. **C** All strains, except for Y8TT1, have a lower growth rate than the WT, although only 4 of these are significant. Y8TT18TO1, Y8TT18TO3 and Y8TT58TO5 show significantly slower growth rate than their equivalent TlGAMY-expressing strain. **D** 8 out of 12 8UASpTEF strains have a significantly higher inflection point than WT. But only one double-amylase strain is significantly higher than its TlGAMY-expressing equivalent. Data presented as the mean for n=5 (A & B) or n=6 (C & D, 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

growth rates compared to their equivalent double-amylase strains. In contrast, all other consortia have a lower growth rate than their equivalent monoculture, although only YTT5 + YTO5 and Y8TT5 + Y8TO3 have a significant decrease in growth rate. The consortia did also show a difference in inflection point and carrying capacity, although this may have been due to the higher initial OD (Supplementary Fig. 4).

The consortia that achieve higher growth rates than their monoculture counterparts do not align with the expression systems experiencing the highest burden. Among the monocultures, only YTT5TO2 shows a significantly different growth rate compared to both of its single-amylase strain counterparts. However, in starch media, the YTT5 + YTO2 consortium exhibited a lower growth rate than the YTT5TO2 monoculture. Strains YTT3TO1, YTT3TO5, YTT5TO5, Y8TT18TO1, Y8TT18TO3, and Y8TT58TO5 displayed significantly reduced growth rates compared to their TlGAMY-expressing equivalents. Among their consortium counterparts, only YTT3 + YTO1, Y8TT1 + Y8TO1, and Y8TT5 + Y8TO5 showed a significantly increased growth rate on starch compared to their respective monocultures. In contrast, consortia YTT3 + YTO5, YTT5 + YTO5, and Y8TT1 + Y8TO3 had lower growth rates than their monoculture equivalents. Therefore, measuring burden in glucose media does not reliably indicate that DOL will improve growth on starch. The two different media conditions, where starch media is likely providing a lower concentration of glucose than the pure glucose media, likely result in different metabolisms, meaning burden may not be exactly the same.

The Y8TT1 + Y8TO1 consortium, which had the highest average growth rate of any of the consortia, was carried forward for growth in flasks. The two single-amylase expressing strains were inoculated in a 1:1 ratio, with both strains at an initial OD of 0.05 OD, and the monocultures of single-amylase Y8TO1 and Y8TT1 and double-amylase Y8TT18TO1 were inoculated at an initial OD of 0.1.

Figure 5C shows that the Y8TT1 + Y8TO1 consortium achieves a statistically significant increase in OD600 at both 18 and 24 h. Fitting a Gompertz curve to the growth over time (Fig. 5D) allowed prediction of the growth rate and inflection point. For Y8TT18TO1 the carrying capacity was OD600 5.455, the growth rate was 0.230 h^{-1}, and the inflection point was 12.091 h. For Y8TT1 + Y8TO1 the carrying capacity was OD600 6.075, the growth rate was 0.155 h^{-1} and the inflection point was 13.064 h (Fitting for each replicate shown in Supplementary Fig. 5). So, the curve fitting predicted that the monoculture grew faster, but the consortium grew to a higher final OD.

Previous literature has shown that *Y. lipolytica* will only grow sufficiently on starch if OsAA is co-expressed with a glucoamylase [13]. However, not only do the OsAA-expressing strains grow on starch without the help of a TlGAMY-expressing strain, but Y8TO1 had a higher growth rate than either the Y8TT18TO1 monoculture or the Y8TT1 + Y8TO1 consortium, with a growth rate of 0.381 h^{-1} (Fig. 5D, Supplementary Fig. 5). The difference to previous literature can be explained by the high variability of the properties between different types of starch, or by the different secretion strength achieved by the signal peptides in this case. The high growth rate may be due to competition for glucose; when the OsAA strain is alone it can consume all the glucose available and therefore grow at its maximum rate. The carrying capacity of the OsAA strain alone, OD600 10.505, was lower than either the consortium or the double-amylase monoculture, indicating that while the growth rate may be slowed by competition for substrate, the consortium achieves the highest growth overall. When looking at the ratio of the two strains over time, we found that the inoculation ratio of 1:1 ratio ends at around 65% Y8TO1 and 35% Y8TT1, or a 13:7 ratio, favouring the OsAA strain (Supplementary Fig. 6).

Overall, we observe that DOL can achieve higher growth rates and higher final OD than double-amylase monoculture equivalents but have been unable to find a direct link with metabolic burden.

Conclusions

This work has investigated how division of labour (DOL) in *Yarrowia lipolytica* influences starch degradation. Stronger promoters and double-amylase expression both contributed to higher metabolic burden, but this effect plateaued beyond a certain threshold. When comparing growth on starch between a two-strain consortium and a

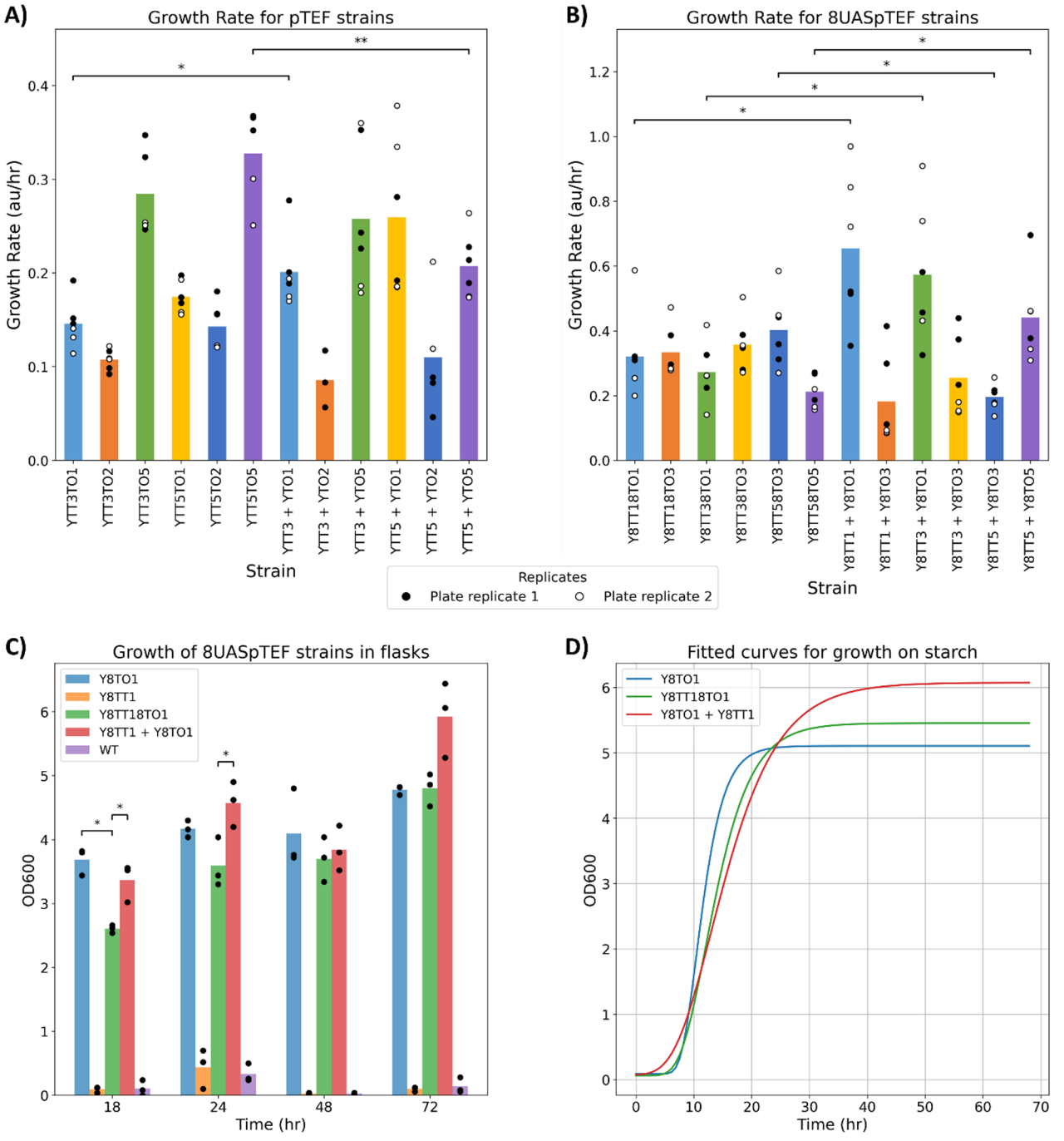


Fig. 5 Growth of double-amylase monoculture strains and 2-strain consortia on 1% soluble starch YNB. In the plate reader, strains were grown for 48 h on 1% starch YNB media and growth rate was calculated by Gompertz 4-parameter curve fitting. **A** The pTEF-controlled YTT3 + YTO1 and YTT5 + YTO1 consortia have a higher growth rate than their double-amylase equivalents. All other consortia grow slower than their equivalent monoculture, with YTT5 + YTO5 being significant. **B** For 8UASpTEF-controlled strains, the Y8TT1 + Y8TO1, Y8TT3 + Y8TO1 and Y8TT5 + Y8TO5 consortia have increased growth rate compared to their equivalent double-amylase strains. In contrast, Y8TT5 + Y8TO3 has a significant decrease in growth rate. The Y8TT1 + Y8TO1 consortia was scaled-up into flask cultures. **C** The consortium achieves a significantly higher OD600 than the monoculture at both 18 and 24 h and is higher by the end of the culture at 72 h, though not significantly (p-value = 0.064). **D** Growth curves were fitted to a Gompertz 4-parameter model. Here, the Y8TO1 strain has the steepest exponential slope, followed by the monoculture Y8TT18TO1 and then the consortium Y8TT1 + Y8TO1. But the consortium peaks at a higher carrying capacity, with the highest OD600 after 72 h. The full unfitted growth curves are shown in Supplementary Figure. Data presented as the mean for (A) $n = 6$ ($n = 3$ for YTT3 + YTO2 and $n = 5$ for YTT5 + YTO2) (B) $n = 6$ C) $n = 3$ D) $n = 3$. Shaded region on the line graph is the standard deviation about the mean. Statistical analysis by Welch's T-test; * represents a p-value < 0.05, ** p-value < 0.005 and *** p-value < 0.0005

monoculture expressing both amylases, certain consortia displayed superior growth rates, although metabolic burden in glucose was not a predictor for this increase.

A strain expressing only α-amylase exhibited a faster growth rate than either the consortium or monoculture. While the primary product of α-amylase activity are shorter oligosaccharides or maltose, it can also release some glucose units. In this case enough glucose must be produced from α-amylase alone to allow *Y. lipolytica* to grow. The reduced growth of the consortium compared to the α-amylase alone highlights the danger of competition within synthetic consortia. In the consortium the glucoamylase strain competed with the α-amylase strain for glucose, and therefore reduced the growth compared to α-amylase on its own. Nevertheless, the consortium of α-amylase and glucoamylase strains could achieve a higher final OD, or carrying capacity, than either of its component monoculture and higher than a monoculture co-expressing both amylases, demonstrating how DOL can assist in the degradation of starch.

Since metabolic burden alone did not determine the success of the consortium, future research should explore the factors influencing interactions within consortia. This could include investigating competition for sugars versus non-competitive sugar metabolism, such as when different strains consume distinct components of a polysaccharide. In natural consortia, behaviour is shaped by complex interactions and cross-feeding, which may facilitate competitive dynamics. This work establishes the first step, or the first module, of building a bottom-up synthetic consortium for polysaccharide degradation in *Y. lipolytica*. By adding new modules for degradation and metabolism, *Y. lipolytica* consortia may be capable of fully hydrolysing complex substrates more efficiently than a single-cell system.

Materials and methods

Golden gate

Inserts for transformation were combinatorially 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 µL PCR tubes as follows: 1.5 µL of type IIS restriction enzyme, 1.5 µL T7 ligase, 2 µL T4 ligase buffer, 1 µL of the vector backbone (25 fmol/µl), 1 µL of each biopart (50 fmol/µl), and made up to a total volume of 20 µL with nuclease-free H_2O. This mix was incubated in a thermocycler running the program: 30 cycles of restriction digestion at 42 °C for 2 min and ligation at 16 °C for 5 min, followed by a final digestion at 60 °C for 10 min and heat inactivation at 80 °C for 5 min. 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 marker gene. The full volume of the assembly mix was then used in an E. coli transformation.

Escherichia coli transformation for plasmid preparation

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 uL of LB and incubated at 37 °C for 1 h. The cells were spun down in the centrifuge and 800 uL of LB was removed. The cells were resuspended and plated on antibiotic LB selection plates (25 g/L of LB, 20 g/L, 34 ug/ml spectinomycin for level 1 assemblies or 100ug/ml ampicillin for level 2 assemblies).

Plasmids were extracted using the QIAGEN Miniprep Kit, following the manufacturers instructions. The size of the assembly was confirmed by NotI digest and gel electrophoresis. The correct parts were confirmed using either colony PCR, or sequencing.

Yarrowia lipolytica media

All media components were provided by Sigma, unless otherwise stated.

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 or glycerol to a final concentration of 40 g/L (for glucose-non-consuming strains).

YNB medium with ammonium sulfate was prepared with a final concentration of either 1.7 g/L (for plate-reader assays) or 6.7 g/L (for flask cultures) yeast nitrogen base (YNB) and 50 mM phosphate buffer (pH 6.8). The desired sugar or polysaccharide was added to the stated final concentration for each experiment. 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.

Yarrowia lipolytica transformation

Y. lipolytica Po1d (U-L-) was transformed using 100 µl of DTT transformation mixture (90 µl 50% PEG, 5 µl 2 M LiAc pH 6.0, 5 µl 2 M DTT), 5 µl of boiled DNA carrier (salmon sperm DNA) and 200 ng of the linearised DNA and incubated at 30 °C for 30 min, followed by 39 °C for 30 min. Transformed *Y. lipolytica* cells were washed with sterile water, plated onto 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.

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 min, followed by 28 cycles of 98 °C for 5 min, T_m for 5 min, 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.

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 was used, as outlined in [33].

Micro-SIT

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

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

Micro-GOPOD

To obtain cellular supernatants, cells were seeded to a starting OD600 of 0.1 in 10 ml media in a 15 ml culture flask and grown for 48 h. Final OD600 was measured, and the cells and supernatant separated by centrifugation.

The GOPOD assay was carried out by mixing 10 µl of 2% starch (Sigma) in solution with 10 µl of supernatant and incubating at 30 °C for 1 to 48 h. 10 µl of the resulting reaction mix was mixed with 10 µl of assay buffer and 150 µl of GOPOD reagent (Megazyme Glucose/Sucrose Assay kit), incubated at 50 °C for 20 min. The glucose produced was measured by absorbance at 510 nm and compared to a standard curve of known concentrations of glucose.

Growth assays in 96-well plates

Growth of single strains and consortia was determined primarily in 96-well plate format using a Biotek Synergy plate reader. Strains were grown to stationary phase and then seeded in 200 µl of the desired media to a initial absorbance at 600 nm (Abs_{600}) of 0.01. For consortia, both strains were seeded at 0.01, making the total starting inoculation 0.02, unless otherwise stated. Abs_{600} was measured every 30 min for 48–72 h with medium shaking.

Fitting growth parameters

Growth parameters were obtained using by fitting experimental Abs600 measurements to a four-parameter Gompertz curve [30]:

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

where $Y(t)$ is the Abs600 at time t, Y_0 is the initial Abs600, Y_s is the carrying capacity, r is the growth rate coefficient and t_i is time of the inflection point. The carrying capacity is the maximum Abs600 reached during stationary phase and the inflection point corresponds to the time at which growth rate is maximal (for Gompertz this is at 36.8% of the carrying capacity). All datapoints collected were fitted to this Gompertz curve using the scipy.optimize.curve_fit function. RMSE and R^2 were calculated for each replicate curve, and any fits with R^2 less than 0.5 for the plate reader, or 0.8 for the flask cultures, were considered anomalous and removed.

Growth assays in flasks

Strains were grown overnight and then seeded in 10 mL of media in 50 mL flasks. For consortia: the initial optical density (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. For the monocultures, they were seeded at an OD600 of 0.1. Cultures were incubated at 30 °C with shaking at 250 rpm. OD600 was measured using a spectrophotometer.

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. OD_{600} was measured every 4 h throughout the growth period.

Statistical analysis

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

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02899-3.

Supplementary Material 1.

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Author contributions
R.L-A. ideated the initial idea of the project. E.A. performed the experiments and analysed the results with support of R.L-A. E.A. and R.L-A. wrote the main manuscript text. E.A. prepared the first draft and the figures. R.L-A. and G.-B.S. supervised the work and secured funding.

Data availability
All data generated or analysed during this study are included in this published article and its supplementary information files. Any information about the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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