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Thermophilic mixed culture degradation of *Miscanthus x giganteus* as a guide to strategies for consolidated bioprocessing.

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Doctor of Philosophy

I declare that the work shown in this thesis is my own work unless stated otherwise. Any information taken from other sources has been appropriately referenced.

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Abstract.

The successful development of consolidated bioprocessing requires microorganisms capable of degrading lignocellulosic biomass and fermenting the resulting sugars. Commercial cellulases and hemicellulases are currently being used to access these sugars, adding to the cost of producing useful products from lignocellulose. This study reports the enrichment of thermophilic, miscanthus degrading bacterial cultures from a municipal composting facility. The detected and isolated bacteria were characterized by 16S rRNA gene sequence analysis and were mostly *Chitinophagaceae* family, *Meiothermus* spp. and *Geobacillus* spp. Other isolated species included *Cohnella* spp., *Brevibacillus* sp., *Chelatococcus* spp., *Thermobacillus* spp., *Thermoanaerobacterium* spp., *Thermobispora bispora, Bacillus* spp., *Staphylococcus* sp. *and Micrococcus* sp.

After enrichment, the mixed population was able to degrade greater than 50% of an ammonium hydroxide pre-treated *Miscanthus x giganteus* sample (1 g) over a six week incubation period at 55ºC, with a reduction in the amounts of all components, including acid soluble and acid insoluble lignin. The glycoside hydrolases and other enzymes identified in the culture supernatants included endo-1,4-β-glucanase A, glucoamylase, xylan 1,4-β-xylosidase, xylose isomerase, xylulokinase, superoxide dismutase, transaldolase, Mn-catalase, Δ-1-pyrroline-5-carboxylate dehydrogenase and endo-β-N-acetylglucoseaminidase H. The HPLC analysis showed that fermentation products formate and lactate were present in the culture supernatant.

Expression of an endoglycoside hydrolase (Csac_0137 from *Caldicellulosiruptor saccharolyticus*) gene in *Geobacillus thermoglucosidasius* strains, NCIMB 11955 and DL33, improved their β-glucosidase specific activity on cellobiose, and improved glycoside hydrolase activities of recombinant DL33 strain when grown on pre-treated *M. x giganteus*. Co-culturing of either transformed or wild-type NCIMB 11955 and DL33 with some of the isolated strains improved their glycoside hydrolase activity and growth on pretreated *M. x giganteus.*

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Table of Contents.

Abstract **3**

Acknowledgements. **4**

List of figures. **10**

List of tables **12**

Abbreviations. **13**

1. Introduction. **17**

1.1 Lignocellulosic biofuel as a promising alternative form of energy. 17

1.1.1 Fermentable biomass materials. 17

1.1.2 Pros and cons of lignocellulosic energy. 18

1.1.3 Worldwide biofuel production. 19

1.2 Lignocellulosic biomass. 22

1.2.1 Structure of lignocellulose. 22

1.2.1.1 Plant cell wall. 22

1.2.1.2 Cell wall structure and composition. 23

1.2.1.2.1 Cellulose. 24

1.2.1.2.2 Hemicellulose. 26

1.2.1.2.3 Pectin. 27

1.2.1.2.4 Lignin. 28

1.3 *Miscanthus x giganteus* 29

1.4 Pretreatment of biomass material. 30

1.4.1 Pretreatment technologies. 31

1.4.2 Consolidated bioprocessing (CBP). 33

1.5 (Hemi)cellulose degrading enzymes. 34

1.5.1 Cellulases. 34

1.5.2 Hemicellulases. 35

1.6.1 Classification of glycoside hydrolases. 36

1.6.2 Mechanisms of action of glycoside hydrolases. 37

1.6.3 Active-site topologies. 39

1.6.4 Carbohydrate binding molecules (CBMs) 41

1.7 Ligninases. 42

1.8 Improving microorganisms that are able to utilise the different types of sugars found in cellulosic biomass by genetic engineering. 44

1.8.1 Metabolic Engineering of microorganisms for lignocellulosic biofuel production. 44

1.8.2 Thermophiles and ethanol. 46

1.8.3 *Geobacillus* spp. as potential ethanologens. 47

1.9 Cocultures. 49

1.10 Molecular phylogeny. 51

1.11 Aim of project. 52

Specific objectives. 52

2. Materials and methods. **53**

2.1 Bacterial strains and Plasmids used in this study. 53

2.2 Sample Collection. 54

2.2.1 Preparation and pretreatment of *M. x giganteus*. 54

2.2.2 Composition analysis of untreated and pretreated *M. x giganteus* 55

2.2.2.1 Extractives content. 55
2.2.2.2 Determination of structural carbohydrates, acid-insoluble and acid-soluble lignin in pretreated and untreated *M. x giganteus* biomass. 56
2.2.3 Enzyme saccharification. 57
2.3 Phosphoric acid swollen cellulose. 58
2.4 Routine maintenance and growth of bacterial strains. 59
2.4.1 Sterilisation. 59
2.4.2 Antibiotics. 59
2.4.3 Luria-Bertani (LB) Medium. 59
2.4.4 2TY medium. 59
2.4.5 TGP aerobic growth medium. 59
2.4.6 Modified Ammonium salts medium (ASM). 60
2.4.7 Rastogi isolation medium (RIM). 60
2.4.8 Actinomycetes isolation medium. 60
2.5 Frozen glycerol stocks. 61
2.5.1 Preparation of 50% (v/v) glycerol. 61
2.5.2 Preparation and storage of stocks. 61
2.5.3 Reviving of thermophilic bacterial strains from stock cultures stored at -80°C. 61
2.5.4. Screening for possible factors that could be reviving cells from stock cultures after storing at -80°C. 61
2.6 Preparation of working stocks. 62
2.6.1 Preparation of overnight cultures. 62
2.7 Microbiological Methods. 62
2.7.1 Isolation of cellulolytic strains. 62
2.7.1.1 Isolation of microbial colonies from the TR1A subcultures. 63
2.7.1.2 Monitoring microbial growth for *M. x giganteus* cultures by total cell protein. 64
2.7.2 Determination of growth rates of WL strains on 2TY medium. 64
2.8 Centrifugation. 65
2.9 Optical density analysis. 65
2.10 HPLC analysis. 65
2.11 Protein methods and enzyme assay protocols. 66
2.11.1 Preparation of 0.1% Congo red. 66
2.11.2 Checking for cellulase activity on salts medium containing CMC, ... 66
2.12 Determination of enzyme specific activity. 66
2.12.1 Reagents. 66
2.12.1.1 Preparation of 3,5-dinitrosalicylic acid (DNS) solution. 66
2.12.1.2 Preparation of 2.5% w/v Carboxymethyl Cellulose (CMC). 67
2.12.1.3 Avicel or xylan. 67
2.12.1.4 Lactose solution. 67
2.12.2 Enzyme Assay. 67
2.13 Protein concentration by filtration. 68
2.14 Protein Assay. 68
2. 15 SDS PAGE. 69
2.15.1 Preparation of gels. 69
2.15.2 Preparation of coomassie blue stain. 69
2.15.3 Coomassie blue de-stainer. 69
2.15.4 Running buffer. 69
2.15.5 Running SDS PAGE gels. 69

2.16 Preparation of native PAGE gels. 70
2.16.1 Sample preparation and loading. 70
2.16.2 Staining of native PAGE gels. 71
2.17 Mass spectrometry analysis. 71
2.17.1 In gel digestion of proteins. 71
2.17.1.1 Excision of bands/spots. 71
2.17.1.2 Preparation for reduction carboxymethylation. 72
2.17.1.3 Gel Preparation/removal of gel stain. 72
2.17.1.4 Reduction/carboxymethylation. 72
2.17.1.5 Tryptic digestion. 73
2.17.1.6 Elution of peptides from the gel pieces. 73
2.17.1.7 Determination of the enzyme sequence. 74
2.18 Molecular biology methods. 74
2.18.1 Agarose gel preparation. 74
2.18.2 Isolation of Genomic DNA. 74
2.18.2.1 DNA extraction from compost sample cultures. 74
2.18.3 DNA fragment purification. 76
2.18.4 DNA Quantification. 76
2.18.5 Polymerase Chain Reaction (PCR). 76
2.18.6 DNA Ligation. 77
2.18.7 Preparation of chemically competent *E. coli* cells. 77
2.18.7.1 Transformation. 78
2.18.8 Preparation of electrocompetent *Geobacillus strains*. 79
2.18.8.1 Electroporation of electrocompetent *Geobacillus strains*. 80
2.18.9 Colony PCR. 80
2.18.10 Isolation of plasmid DNA. 80
2.18.11 Plasmid digestion. 81
2.18.12 Preparing DNA for sequencing. 81
3. Pretreatment of *M. x giganteus*. 82
3.1 Compositional analysis of pretreated and untreated *M. x giganteus*. 83
3.2 Enzymatic saccharification of the pretreated and untreated *M. x giganteus* biomass. 84
3.3 Discussion. 85
4. Preliminary studies of the WL isolates. 86
4.1 Introduction. 86
4.2. Standardisation of the revival procedure for WL strains from stock cultures stored at -80°C. 86
4.2.1 Screening for possible factors that could be reviving cells from stock cultures stored at -80°C. 87
4.2.2 Determination of the time at which cells of the WL14 strain start reviving from stock cultures stored at -80°C. 88
4.3 Growth profiles. 89
4.3.1 Determination of growth rates of strains WL3, WL6, WL14 and WL16 on 2TY medium. 89
4.3.2 Growth tests for the production of cellulases by WL strains. 91
4.4 Growth of WL3, WL6, WL14 and WL16 in liquid medium on CMC, avicel 92
4.5 Enzyme assays. 94
4.5.1 Determination of glycoside hydrolase activity. 94
4.5.2 SDS PAGE and native PAGE gel analysis. 96

4.6 Growth of WL3, WL6, WL14 and WL16 on pretreated and untreated *M. x giganteus* in unbuffered RIM. 98
4.6.1 Determination of enzyme activity of WL14 after growth on pretreated *M. x giganteus*. 100
4.7 Identifying the WL strains based on their 16S rRNA gene sequences. 100
4.7.1 PCR amplification of 16S rRNA gene sequences. 100
4.7.2 Phusion polymerase chain reaction. 102
4.7.3 Colony PCR. 103
4.7.4 Plasmid isolation and digestion. 104
4.7.5 Sequencing and analysis of the plasmid inserts. 104
4.8 Discussion. 106
5. New microbial isolations from domestic compost using pretreated *M. x giganteus* as carbon source. 110
5.1 Introduction. 110
5.2 Growth of compost cultures on pretreated *M. x giganteus*. 111
5.2.1 Monitoring growth of cultures. 111
5.2.2 Microbial growth on varying amounts of pretreated *M. x giganteus*. 114
5.2.3 Growth in buffered RIM. 115
5.2.4 The effect of increased aeration on growth of the TR1A subculture 6. 117
5.2.5 Microbial degradation of the pretreated *M. x giganteus* by the enriched culture. 117
5.2.6 Production of pigments during growth of TR1A subcultures. 119
5.2.7 Degradation of pre-treated miscanthus in TR1A subcultures. 120
5.3 Compositional analysis of lignocellulose residues from the TR1A subcultures. 121
5.4 HPLC analysis of the TR1A subculture supernatants. 124
5.5 Enzyme assay of the culture fluid. 128
5.5.1 Determination of glycoside hydrolase activity 128
5.5.2 PAGE gel analysis and zymograms. 130
5.5.2.1 Zymogram analysis of glycoside hydrolase activity in TR1A initial cultures and subcultures 1, 6, 7 and 8. 130
5.5.2.2 Identification of the enzymes detected in the supernatants of TR1A subcultures. 136
5.5.2.2.1 Identified enzymes and their importance in the degradation of lignocellulosic biomass. 140
5.5.2.2.1.1Glucoamylase. 140
5.5.2.2.1.2 Xylan 1,4-β-xylosidases/β-xylosidases. 141
5.5.2.2.1.3 Xylanase. 142
5.5.2.2.1.4 Endo-β -N-Acetylglucosaminidase H. 142
5.5.2.2.1.5 Endo-1,4-β-glucanase A. 142
5.5.2.2.1.6 Xylose isomerase. 143
5.5.2.2.1.7 Transaldolase. 144
5.5.2.2.1.8 Superoxide dismutase and catalase. 144
5.5.2.2.1.9 Acid α-amylase. 144
5.5.2.2.1.10 Other enzymes. 145
5.6 Discussion. 145
6. Identification of bacteria present in the TR1A culture by 16S rRNA gene analysis. 150
6.1 Introduction. 150
6.2 Isolation of bacteria present in the TR1A culture. 150
6.3 Identification of the bacteria present in the TR1A culture by 16S rRNA gene analysis. 151
6.3.1 Identification of microorganisms from 16S rRNA gene analysis of total DNA isolated from the TR1A subcultures. 155

6.3.1.1. Identification of the *Bacteroidetes* microorganisms. 155
6.3.1.2 Identification of the *Thermus* microorganisms. 155
6.3.1.3 Other microorganisms detected at less than 10% of the microbial population in TR1A subcultures 6 and 8. 157
6.3.2 Identification of microorganisms from 16Sr RNA gene analysis of the isolated microbial colonies from the TR1A subcultures. 158
6.3.2.1 The *Firmicutes* 158
6.3.2.1.1 *Geobacillus* spp. 158
6.3.2.1.2 The *Cohnella* spp. 160
6.3.2.1.3 The *Brevibacillus* spp. 161
6.3.2.1.4 *Bacillus aerius* or *Bacillus lichenformis* sp. 163
6.3.2.1.4.1 Growth and enzyme activity of *Bacillus lichenformis* TR1A_17 on 1% pretreated *M. x giganteus* in RIM. 166
6.3.2.2 *Staphylococcus warneri.* 169
6.3.3 The *Actinobacteria phylum*. 171
6.4 Discussion. 173
7. Expression of Csac_0137, an endoglucanase enzyme encoding gene from *Caldicellulosiruptor saccharolyticus*, in *Geobacillus thermoglucosidasius* strains, NCIMB 11955 and DL33. 175
7.1 Introduction. 175
7.2 Construction of plasmid pUCG4.81.1 and insertion of Csac_0137 into plasmid. 175
7.3 Growth of wild-type strains *G. thermoglucosidasius* strains NCIMB 11955 and *G. thermoglucosidasius* DL33 on xylan and other carbon sources. 176
7.4 Transformation of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 with Csac_0137. 178
7.5 Growth of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on 0.2% CMC and 0.1% cellobiose in modified ASM. 179
7.5.1 Assessment of the CMCase specific activity of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 after growth on 0.2% CMC and 0.1% cellobiose in modified ASM. 179
7.6 Growth of transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on 2% cellobiose. 181
7.6.1 Assessment of β-glucosidase activity of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 after growth on 2% cellobiose. 182
7.7 Growth of transformed and the wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on pretreated *M. x giganteus*. 184
7.7.1 Assessment of the avicelase and CMCase specific activity of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 after growth on 1% pretreated *M. x giganteus* in RIM. 185
7.7.2 Assessment of the xylanase specific activity of transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 and DL33 after growth on 1% pretreated *M. x giganteus* in RIM. 186
7.8 Discussion. 189
8. Cocultures of transformed and wild-type strains of *Geobacillus thermoglucosidasius* NCIMB 11955 and DL33, and strains isolated from the TR1A mixed culture, on pretreated *M. x giganteus*. 191
8.1 Introduction. 191

8.2 Cocultivation of *G. thermoglucosidasius* NCIMB 11955 and DL33 strains with *Brevibacillus thermoruber* sp. strain TR1A_13, on 1% pretreated *M. x giganteus*. 191
8.2.1 Assessment of the avicelase, β-glucosidase, CMCase and xylanase activity of cocultures of the transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 and DL33 with *B. thermoruber* TR1A_13, and the individual culture of *B. thermoruber* TR1A_13, after growth on 1% pretreated *M. x giganteus* in RIM. 193
8.2.1.1 Analysis of the CMCase and xylanase activity of the supernatant from the cocultures of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and DL33, with *B. thermoruber* TR1A_13 on native PAGE gel. 194
8.3. Assessment of growth and enzyme activity of the cocultures of transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 or DL33 with *B. lichenformis* TR1A_17. 196
8.4 Discussion. 197
9. General discussion and future work. 199
10. References. 210
Appendix. 235
Permissions. 258

List of figures.

Figure 1.1 Bioethanol and biodiesel production in the world. 20
Figure 1.2 World biofuel production trends. 21
Figure 1.3 Plant cell wall structure and composition. 24
Figure 1.4 Schematic illustration of the cellulose chain. 25
Figure 1.5 Schematic illustration of sugar units of hemicellulose. 26
Figure 1.6 Representative structures of specific regions of the pectic polysaccharide. 27
Figure 1.7 Lignin structure. 29
Figure 1.8 *Miscanthus x giganteus* field. 30
Figure 1.9 Pretreatment of cellulosic biomass and ethanol production. 31
Figure 1.10 Consolidated bioprocessing process. 33
Figure 1.11 Cellulose degradation. 35
Figure 1.12 The two major mechanisms for glycoside hydrolases. 39
Figure 1.13 The main fold of the catalytic domain in glycosyl hydrolases families 6 and 7. .40
Figure 1.14 The three types of active site topologies found in glycoside hydrolases. 41
Figure 1.15 Major metabolic pathways associated with ethanol production in *Geobacillus* spp. 49
Figure 2.1 Pretreatment of *M. x giganteus*. 55
Figure 2.2 A domestic compost heap at the sampling site. 63
Figure 3.1 Pretreated and untreated *M. x giganteus* 82
Figure 3.2 Chemical composition of untreated and pretreated *M. x giganteus* biomass. 83
Figure 3.3 Saccharification of pretreated and untreated *M. x giganteus* biomass. 84
Figure 4.1 Growth curves of WL strains on 2TY medium. 90
Figure 4.2 Congo red screen for cellulase activity in 24 h old WL strains cultures. 91
Figure 4.3 Congo red screen for cellulase activity in 8 d old WL strains cultures. 92
Figure 4.4 Growth profiles of WL strains on avicel, CMC and xylan. 94
Figure 4.5 Cellulase, xylanase and avicelase specific activity of WL strains. 95
Figure 4.6 Native CMC PAGE gels of protein from the cultures of WL strains. 97

Figure 4.7 Growth of WL strains on 1% pretreated and untreated *M. x giganteus* in unbuffered RIM. 99
Figure 4.8 Avicelase, CMCase and xylanase specific activity in WL14 culture broth after growth on pretreated *M. x giganteus* in unbuffered RIM. 100
Figure 4.9 PCR optimisation experiments using WL14 and WL16 strains. 101
Figure 4.10 Products from PCR amplification of the 16S rRNA gene from WL strains. 102
Figure 4.11 Products from colony PCR of JM109 pJET1.2 transformants believed to contain 16S rRNA genes. 103
Figure 4.12 Bgl II digestion of recombinant pJET1.2 believed to contain 16S rRNA gene inserts from WL strains. 104
Figure 4.13 Un-rooted phylogenetic tree based on 16S rRNA gene sequences amplified from the WL strains and their closest relatives. 106
Figure 5.1 Two week old initial subcultures started by using inoculum from the original TR1A and TR2A cultures. 112
Figure 5.2 Comparison of growth of the intial TR1A and TR2A subcultures on pretreated and untretreated *M. x giganteus*. 113
Figure 5.3 Comparing growth of TR1A mixed culture on varying amounts of pretreated *M. x giganteus*. 114
Figure 5.4 Growth of TR1A subcultures 6, 7 and 8. 116
Figure 5.5 The effect of increased aeration on the growth of TR1A subculture 6. 117
Figure 5.6 Growth of TR1A subculture using different sources of inoculum. 119
Figure 5.7 Pigmentation in TR1A cultures. 120
Figure 5.8 Colour of the pellets from TR1A subculture and control. 120
Figure 5.9 Weight of the remaining pellets from TR1A subculture 6, 7, 8, and their controls. 121
Figure 5.10 Relative compositional analysis of residues from the TR1A subculture 6 and control. 122
Figure 5.11 HPLC chromatograms with UV detection analysing supernatant from TR1A subculture 8. 126
Figure 5.12 HPLC chromatograms with RI detection analysing supernatant from TR1A subculture 8. 127
Figure 5.13 Specific enzyme activities in the TR1A subcultures 6, 7 and 8. 129
Figure 5.14 Native CMC PAGE gel (12%) of TR1A and TR2A cultures. 132
Figure 5.15 TR1A subculture 6 supernatant assayed for CMCase activity. 133
Figure 5.16 Native PAGE gel (12%) and zymogram for CMCase activity of supernatants of TR1A subculture 7 134
Figure 5.17 Native CMC and xylan PAGE gels (12%) of supernatant from TR1A subculture 8 135
Figure 6.1 Un-rooted phylogenetic trees of microorganisms detected in the TR1A subcultures. 153
Figure 6.2 Pie charts showing the approximate abundance (%) of the microbial species identified in the TR1A subcultures 6 and 8. 154
Figure 6.3 *Geobacillus* strains isolated from TR1A subcultures. 160
Figure 6.4 Colonies of TR1A_12 on 2TY. 161
Figure 6.5 *B. thermoruber* TR1A_13 grown on carbon sources. 162
Figure 6.6 *B. lichenformis* grown on carbon sources. 164
Figure 6.7 Enzyme activity of *B. lichenformis* after growth on 1% pretreated *M. x giganteus* in RIM. 168
Figure 6.8 The isolated *Staphylococcus* sp. strain TR1A_16. 170
Figure 6.9 The isolated *Micrococcus* sp. strain TR1A_14. 172

Figure 7.1 Plasmid pUCG4.81.1. 176
Figure 7.2 Growth of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on 2% xylan in modified ASM. 177
Figure 7.3 Digested plasmids of pUCG4.81.1Csac0137 and *G. thermoglucosidasius* strains, NCIMB 11955 and DL33. 178
Figure 7.4 Growth of transformed and wild strains of *G. thermoglucosidasius*, NCIMB 11955 and DL33 on 0.2% CMC and 0.1% cellobiose in modified ASM. 179
Figure 7.5 CMCase specific activity of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 after growth on 0.2% CMC and 0.1% cellobiose in modified ASM. 180
Figure 7.6 Growth of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 on 2% cellobiose in modified ASM. 182
Figure 7.7 β-glucosidase specific activity of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33. 183
Figure 7.8 Growth of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on 1% pretreated *M. x giganteus* in RIM. 184
Figure 7.9 Assessment of avicelase and CMCase specific activity of transformed and wild-type *G. thermoglucosidasius* strains of NCIMB 11955 and DL33 and the control. 186
Figure 7.10 Xylanase activity of transformed and wild-type *G. thermoglucosidasius* strains of NCIMB 11955 and DL33. 188
Figure 8.1 Growth of transformed or wild-type cocultures on pretreated *M. x giganteus*. 193
Figure 8.2 Assessment of the avicelase, β-glucosidase, CMCase and xylanase activity of the cocultures. 194
Figure 8.3 CMCase and xylanase native PAGE gels of the cocultures. 195
Figure 8.4 Xylanase activity of the cocultures. 197

List of tables.

Table 1.1 Cellulose, hemicellulose and lignin content of C_4 grasses and wood. 25
Table 1.2 Engineering of xylose metabolism genes into *S. cerevisiae*. 45
Table 2.1 Bacterial strains and plasmids used in this project. 53
Table 2.2 Sequences of primers used for PCR. 77
Table 4.1 Time course of cell revival from stock cultures stored at -80^{o}C. 87
Table4.2 Screening for possible soluble factors produced in liquid media which can revive cells previously stored at -80^{o}C. 88
Table 5.1 Identification of the enzymes detected in the supernatant of TR1A subcultures 6, 7 and 8. 137
Table 6.1 Glycoside Hydrolases and other important enzymes for lignocelluloses degradation coded for in the *Bacillus lichenformis* 9945A genome. 165
Table 6.2 Glycoside hydrolases coded for in the *Staphylococcus warner*i SG1 genome. 171

Abbreviations.

°C	degrees Centigrade
µg	micro-gram(s)
µl	micro-litre(s)
µM	micro-molar
16S rDNA	DNA encoding the small ribosomal RNA subunit
16S rRNA gene	DNA encoding the small ribosomal RNA subunit
AcDH	acetaldehyde dehydrogenase
Adh	alcohol dehydrogenase
AFEX	ammonia fiber expansion
AIL	acid insoluble lignin
EISA	Energy Independence and Security Act
Ambic	Ammonium hydrogen carbonate/Ammonium bicarbonate (NH_4HCO_3)
Amp	ampere(s)
ARP	ammonia recycle percolation
ASL	acid soluble lignin
ASM	ammonium salts medium
ATP	adenosine 5' triphosphate
Bp	base pairs
BSA	bovine serum albumin
CAZy	Carbohydrate-Active EnZymes
CBMs	Carbohydrate-binding modules
CBP	consolidated bioprocessing
CFU	colony forming units
CMC	carboxymethylcellulose
CMCase	carboxymethylcellulase (enzyme)
CoA	coenzyme A
CO_2	carbon dioxide

DMSO	dimethyl sulfoxide
DMC	Direct Microbial Conversion
DNA	deoxyribonucleic acid
DNS	3,5-dinitrosalisylic acid
dNTP	deoxy-nucleoside tri-phosphate
DTT	Dithiothreitol
EDTA	ethylenediaminotetra-acetate
EtOH	Ethanol
EERE	Energy Efficiency and Renewable Energy
g	gram(s)
GW	Gigawatts
GH	Glycoside hydrolase
GRFA	Global Renewable Fuels Association
h	hour(s)
ha	hectare
HPLC	high performance liquid chromatography
IU	International Unit
IUB	International Union of Biochemistry
kDa	kilodalton(s)
L	litre(s)
LB	Luria-Bertani (medium)
Ldh	L-lactate dehydrogenase (gene)
Ldh	L-lactate dehydrogenase (enzyme)
M	Molar
MeCN	Acetonitrile (CH_3CN)
min	minute(s)
mg	milligram(s)
ml	millilitre(s)

mm	millimeter
mM	milliMolar
Mol	mole(s)
MT	Metric tonnes
N	Normality
NaCl	Sodium chloride
NAD	nicotinamide-adenine dinucleotide (oxidised form)
NADH	nicotinamide-adenine dinucleotide (reduced form)
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometre(s)
NREL-LAP	National Renewable Energy Laboratory-Laboratory Analytical Procedure
OD	optical density
ODW	oven dry weight
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Pdh	pyruvate dehydrogenase (enzyme)
PEG	polyethylene glycol
Pfl	pyruvate formate lyase (enzyme)
*pfl*B	pyruvate formate lyase gene
Pmol	picomole(s)
rDNA	DNA encoding ribosomal RNA
RIM	Rastogi isolation medium
RNA	ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
Rpm	revolutions per minute

SAA	soaking in aqueous ammonia
s	second(s)
SDS	sodium dodecyl sulphate
sp.	species (singular)
spp.	species (plural)
SRS	sugar recovery standards
T	Tonnes
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TFA	Trifluoroacetic acid
TEMED	N,N,N',N'-tetramethyl ethylene diamine
TNTC	too numerous to count
TSS	Transformation and storage solution
U	International unity of enzyme activity
US	United States
UV	ultra violet
v	volt(s)
v/v	volume / volume
wt/wt	Weight/weight
w/v	weight / volume
YE	Bacto-yeast extract

1. Introduction.

1.1 Lignocellulosic biofuel as a promising alternative form of energy.

The world demand for fossil fuels is increasing and this has raised great concern over the effects of increased greenhouse gas emissions. This means that one of the most pressing issues for the 21st century is the need for renewable, carbon neutral and sustainable raw materials and energy for industry and society (Foster et al., 2010a). There is a worldwide search for renewable forms of energy that are environmentally clean and economically efficient (Kotchoni and Gachomo, 2008; Shao et al., 2010). Biofuel technology is now considered to be a promising interim (NSF. 2008), and possibly long term technology to replace fossil fuels with liquid fuels produced from renewable sources such as cellulosic biomass (Somerville et al., 2010).

Among various technical options, the fermentation of biomass hydrolysates is a favoured approach, but there has been slow progress in this area due to lack of microorganisms that are capable of metabolising all the sugars present in the hydrolysates and conversion into useful products (Cripps et al., 2009). Work has been done on engineering of microorganisms for production of biobutanol and biodiesel, but their productivity is low and the microorganisms being used have low tolerance to the solvent products (Sakuragi et al., 2011). Sakuragi et al. (2011) suggested that current scientific studies should be focused on engineering of microorganisms for the degradation of cellulosic biomass and the production of biofuels such as biobutanol, biodiesel and bioethanol at high efficiency and low cost.

1.1.1 Fermentable biomass materials.

The classes of fermentable biomass materials include sugar-based (sugarcane, sugar beet, etc), starch-based (sweet potato, potato, wheat, corn, etc) and wood/grass-based (waste wood, grass, rice straw, wastepaper, etc) feedstocks. Wood or grass based biomass raw materials can be stably secured because they have no utility as food and may, indeed, be available as wastes from food production (eg. straw, bagasse, corn-stover) (Limayem and Ricke, 2012). However, unlike sugar-based biomass, wood and grass-based biomass requires pretreatment to remove lignin which presents a barrier to enzyme access, and enzymatic saccharification before being fermented to produce biofuel, both of which increase the production costs (de Vos, 2006).

1.1.2 Pros and cons of lignocellulosic energy.

The price of biofuel must be competitive with that of gasoline if this alternative fuel is to become viable for use in transportation. Through the process of photosynthesis, plants have used solar energy to fix CO_2 into carbohydrates, making the combustion of biofuels derived from plant biomass a potentially carbon neutral process (Bassam, 1998). Sugar and starch-based biomass materials have been the most commonly used for the production of bioethanol until now. However, there are large amounts of lignocellulosic plant residues arising from either food grain production, such as corn stover and wheat straw, or forestry. Indeed, use of woody biomass for energy is considered a benefit in assisting the US Forest Service to manage excess wood residues which would otherwise increase fire risks (Somerville et al., 2010). It has been estimated that, in the USA, with efficient use of these residues there may be no need for expansion of land use for production of cellulosic biomass, which has recently been a controversial issue (Somerville et al., 2010).

However, removal of all of the crop residues can also cause problems due to loss of soil carbon and erosion, resulting in requirements for additional input of fertilizers to replace lost minerals, which can contribute to environmental contamination (Lal, 2005) and increase biofuel production costs arising from energy input and use of pesticides (Zhu and Zhuang, 2012). Recovery of stover after the grain harvest requires extra labour and transport to take the stover to the refineries (Biomass Conversion EPA 2007), with a beneficial effect on employment opportunities. However, this also pushes up lignocellulosic biofuel production costs (Sokhansanj et al., 2002).

Even with increased production capacity, the reliance of biofuel production on food crops is bound to cause conflicts in seasonal supply and demand, potentially causing unacceptable increases in food prices (Hill, 2007). Therefore, in the long term, the biofuel industry must move towards the use of crop residues and purpose grown non-food crops. It has also been thought that use of bioethanol may cause health problems. Research results have indicated that when compared to 100% gasoline, converting from gasoline to E85 (85% ethanol fuel, 15% gasoline), may cause health problems leading to a 9% and 4% increase in ozone-related mortality, hospitalisation and asthma, in Los Angeles and the US as a whole, respectively (Jacobson, 2007).

Use of bioethanol can lead to formation of aldehydes (formaldehyde and acetaldehyde). Aldehydes are partially oxygenated organic compounds containing a carbonyl group (Kumar et al., 2011). They are produced by combustion of hydrocarbons or due to partial oxidation of the un-burnt hydrocarbon fuels (Raine et al., 1997). A functional group of an aldehyde consists of a carbon atom bounded to a hydrogen and double-bonded to an oxygen atom (O=CH) (Kumar et al., 2011).

Aldehyde vapours can affect humans by causing irritation of eyes, throat, nose, asthma and can also affect pulmonary function (Kumar et al., 2011; Dahlgren et al., 2013). Formaldehyde is a known skin irritant, sensitizer and has been classified as a class 1 human carcinogen (Dahlgren et al., 2013). It is associated with development of nasopharyngeal cancers and has been reported to contribute to cancer related deaths (Winebrake et al., 2001).

Formaldehyde can form ozone by photochemical oxidation, contributing to environmental ozone formation (Degobert and Marshall, 1995). Kumar et al. (2011) indicated that when compared to neat petrol, a 10% v/v ethanol/petrol blend increased the emission of formaldehyde by approximately 25%, while emission of acetaldehyde increased by approximately 180% (ERDC, 1998). Another study carried out in Brazil reported that acetaldehyde was the main aldehyde emitted by light vehicles because they largely used ethanol (Pinto et al., 2014). It was also observed during the same study that the burning of B3-diesel (3% biodiesel and 97% diesel) increased the emissions of carbonyl compounds, such as acetaldehyde, and also contributed to ozone formation.

1.1.3 Worldwide biofuel production.

By 2010, worldwide biofuel production reached approximately 105 billion litres, up 17% from 2009 (http://www.worldwatch.org/biofuels-make-comeback-despite-tough-economy). The biofuels, mostly ethanol and biodiesel, provided 2.7% of the world's fuels for road transport (Fig. 1.1). Brazil and the United States of America (USA) produced 90% of the 86 billion litres of the worldwide ethanol produced in 2010, while 53% of biodiesel was produced by the European Union (http://www.worldwatch.org/biofuels-make-comeback-despite-tough-economy). By 2011, biofuels were blended at the national level by 31 countries and 29 states or provinces (http://www.worldwatch.org/biofuels-make-comeback-despite-tough-economy). In 2012, the USA was reported to be using 114 million metric tonnes of corn to produce 50.41 million litres of ethanol (http://ethanolrfa.org/page/-

/PDFs/RFA%202013%20Ethanol%20Industry%20Outlook.pdf?nocdn=1). However, corn is also a food crop and competition for the same resource for food and fuel can cause economic tensions. Starch–based ethanol is also reported to give a low reduction in greenhouse gases compared to fossil fuels (Maki et al., 2009). The aim of the National Biofuel Action Plan, based on the Energy Independence and Security Act 2007 (EISA) and Food and conservation Act of 2008 (FCEA), is to reduce or replace fossil fuel by production of 136.3 million litres of renewable fuels by 2022 (Affuso and Duzy, 2013).

The increase in biofuel production would raise the production of corn by 122.89%, and could result into nitrogen loss of 20% (Affuso and Duzy, 2013). Land use to grow corn for ethanol production will raise nitrogen losses associated with fertilization. Less productive lands would require large amounts of fertilizer especially nitrogen fertilization, to produce corn (Affuso and Duzy, 2013). During rainfall the nitrogen and other nutrients are mobilised and contribute to eutrophication in larger water bodies (Affuso nd Duzy, 2013). This could bring about direct or indirect changes in land use, with a potential negative impact on the environment, possibly increasing carbon emissions due to release of carbon stored in soils (Dumortier et al., 2011).

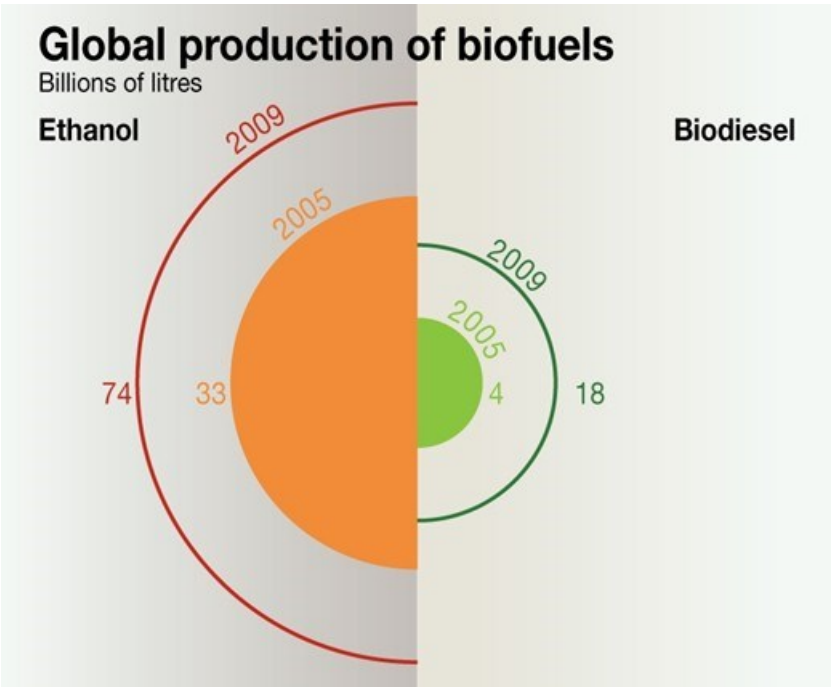


Figure 1.1 Bioethanol and biodiesel production in the world.
Biofuels Platform, Geographic distribution of bioethanol and biodiesel production in the world in 2008. Source: Ren 21, Renewables status report, 2006 and 2010 by Nieves Lopez Izquierdo.
http://www.grida.no/graphicslib/detail/global-production-of-biofuels_4d36. Accessed on 05/01/2015.

Global annual production of biofuel shows continued growth and has been forecasted to exceed 90 billion litres in 2014, with global ethanol output 2.7% higher than that of 2013 (http://www.biofuelsjournal.com/info/bf_articles.html?ID=139867). Both ethanol and biodiesel production show steadly increasing trends (Fig. 1.2), with the demand for ethanol being higher than that for diesel. Brazil and the United States of America are the two largest producers of ethanol, with the European Union gradually increasing its production (Fig. 1.2), while Africa is emerging as another contributor to ethanol production, according to Global Renewable Fuels Alliance (GRFA) and F.O. Licht reports (http://www.biofuelsjournal.com/info/bf_articles.html?ID=139867).

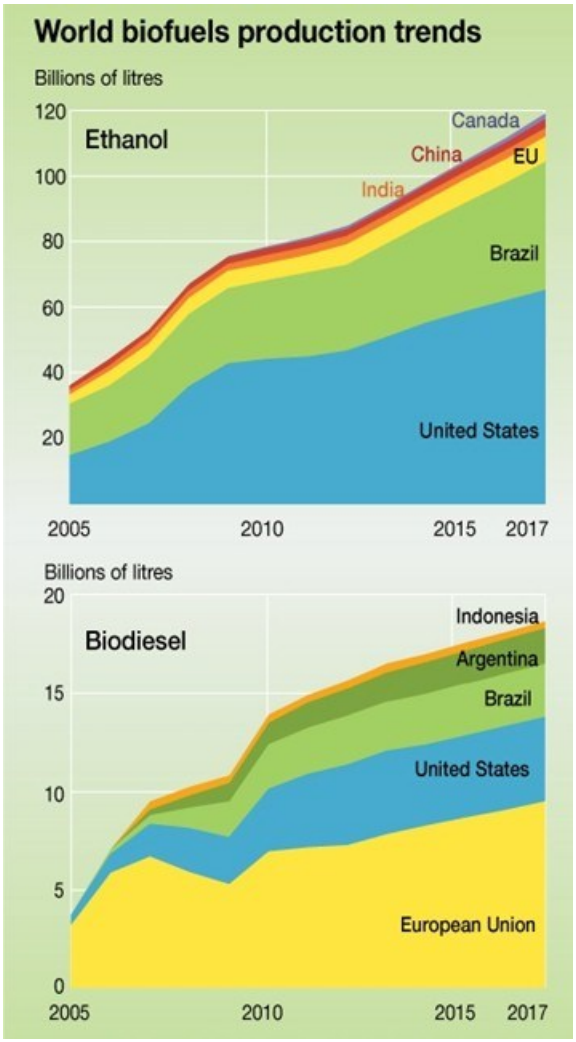


Figure 1.2 World biofuel production trends.
Trends in the production of biofuel in the world. Source: FAPRI, U.S. and world Agricultural Oultlook, 2008. (http://www.grida.no/graphicslib/detail/world-biofuels-production-trends_d3ec) by Riccardo Pravettoni, UNEP/GRID-Arendal. Accessed on 05/01/2015.

1.2 Lignocellulosic biomass.

Green energy from second generation bioenergy crops offers an alternative solution to first generation, starch or sugar-based biofuels. Second generation bioenergy involves the use of fast growing plants, such as trees and energy grasses, that are produced and used in CO_2 neutral conditions (Bassam, 1998). Lignocellulose is the most abundant raw material on earth, comprising about 50% of the world biomass (Claassen et al., 1999). The annual production of lignocellulosic biomass is estimated at $1x10^{10}$ MT worldwide and is considered to be the only feasible and sustainable resource for renewable energy and other important products (Sanchez and Cardona, 2008).

Agriculture and forestry can serve as sources of lignocellulose biomass. The lignocellulose can be broken down by enzymes to produce sugars that can be fermented to produce ethanol. Lignocellulose biomass has been documented as capable of producing between 110 to 300 liters of ethanol from 1 ton of raw biomass, depending on the source, according to Oak Ridge National Laboratory (2006) and other researchers (Mabee et al., 2006; Sims et al., 2010). The quantity of potential European environmentally-compatible biomass has recently been assessed by the European Environment Agency, showing the possibility to increase by nearly 35% by the end of 2030 without harming biodiversity, soil and water resources (Zuber et al., 2013).

1.2.1 Structure of lignocellulose.

1.2.1.1 Plant cell wall.

Plant biomass is mostly composed of cell walls, which are polysaccharide and protein rich macromolecular structures that present at the plant cell surface (Caffall and Mohnen, 2009). Photosynthetically fixed carbon is stored in the plant cell walls, which are a resource in carbon recycling (Lee et al., 2011).

Plant cell walls contribute extensively to the strength and structural integrity of the plant, as well as other cellular activities such as warding off pathogens, allowing water to be transported throughout the plant by allowing it to adhere to the walls of the cells that form the xylem vessels as the water moves along, cell-to-cell adhesion, growth and differentiation because of their ability to stretch and allow cell expansion to a certain extent during cell growth (Foster et al., 2010a; Foster et al., 2010b; Lee et al., 2011). Components of plant cell walls are also

exploited in many human activities and are used as food because they are the major component of plant derived foods (e.g. green vegetables, fruits, carrots, sweet potatoes, etc.), food additives, industrial enzymology, fibres, textiles, paper, pulp products, lumber, fine chemicals, adhesives, polymers for implantation devices, drug delivery, neutraceuticals, pharmaceuticals and biofuels (Lee et al., 2011).

1.2.1.2 Cell wall structure and composition.

The primary cell wall is the first to be laid down and is composed predominantly of the energy rich polymers, cellulose, various hemicelluloses, and pectin (Lacayo et al., 2013). Some cells differentiate and form secondary walls, which have reduced levels of pectin, increased amounts of cellulose and hemicellulose, and are often rigidified by impregnation of heterogenous polyphenolic lignins (Foster et al., 2010a; Gilbert, 2010; Lee et al., 2011) (Figures. 1.3A and B). Extractives, mostly phenols and sterols containing aromatic compounds (Villaverde et al., 2009), are also present in the lignocellulosic materials, but are of lower molecular weight than cellulose, hemicelluloses and lignin, and are only present in small quantities. Softwoods and hardwoods contain 1-5% and 2-8% of extractives, respectively, while wheat has been reported to have a high percentage of extractives in the range 21-31%, (Thomas, 1977).

The two types of wood, softwoods and hardwoods, can be distinguished by the wavenumber values of the carbonyl stretching vibration and one of the ring-breathing modes of lignin, and the fibrous structure of the cut pieces (Barker and Owen, 1999). Softwoods contain glucomannan (copolymer of glucose and mannose), as the main hemicellulose, with galactoglucomannan and glucomannan (depending on the galactose content) as the two major types (Vărnai et al., 2010). The main hemicellulose in hardwoods is xylan whose main components are xylose and 4-o-methylglucuronic acid (Pinto et al., 2005).

Gymnosperms or conifers are plants that bear seeds which are not enclosed in the ovary of the flower and they belong to the softwoods group of plants. Members of the hardwoods are plants that bear enclosed seeds and they are called angiosperms. The lignin of the softwoods is composed mainly of coniferyl alcohol derivatives [Fig. 1.7A (ii)] with a small amount of coumaryl alcohol [Fig. 1.7A (i)], while the hardwoods have lignin composed of coniferyl and sinapyl alcohols [Fig. 1.7A (iii)], and low amounts of coumaryl alcohols (Barker and Owen, 1999).

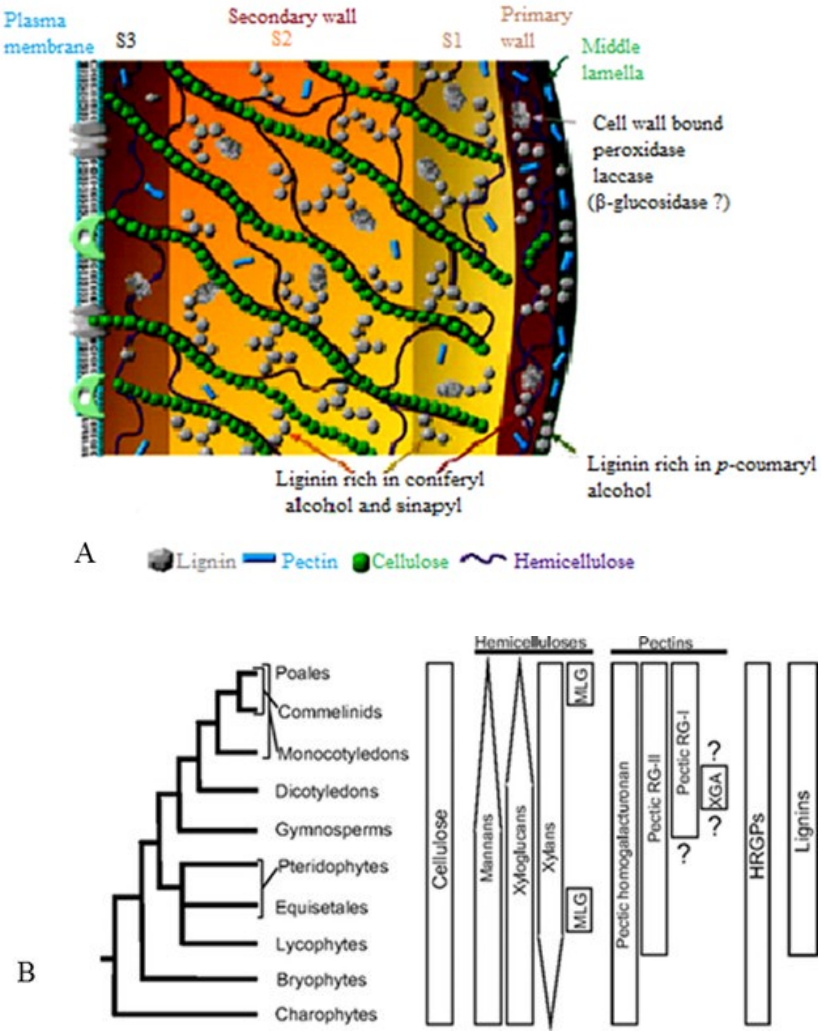


Figure 1.3 Plant cell wall structure and composition.
(A) Plant cell wall structure, reproduced from (Achyuthan et al., 2010) (B) Schematic outline of the major cell wall polymers in relation to Charophycean Green Algae and the major extant groups of land plants. Tapering boxes indicate that polymers are present at relatively reduced levels. Question marks indicate where little is known outside the groups that have been examined. MLG, mixed-linkage glucan; XGA, xylogalacturonan; HRGPs, hydroxyproline-rich glycoproteins. Reproduced from (Lee et al., 2011).

1.2.1.2.1 Cellulose.

Cellulose is made up of glucose molecules linked by β-1,4 glycosidic bonds, forming long polymeric chains (Fig. 1.4). The cellulose chains are joined together by hydrogen bonds, and form bundles of cellulose which are substantially crystalline (Gilbert, 2010). The bundles of linear chains of cellulose are longitudinally arranged in the cell wall. The cellulose content varies between grass species; miscanthus has the highest content among the grass groups presented in Table 1.1.

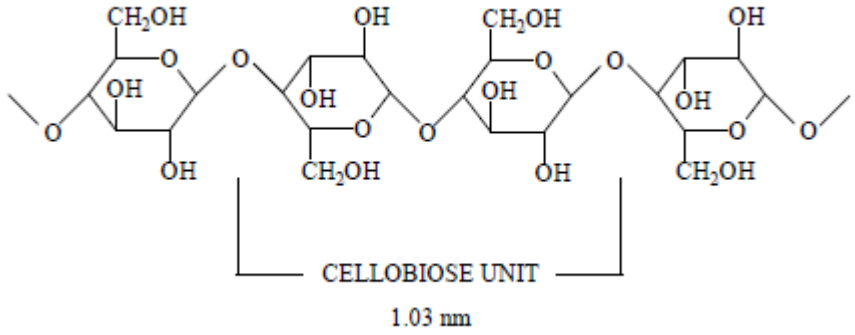


Figure 1.4 Schematic illustration of the cellulose chain.
Source: Fengel and Wegner, (1989).

Table 1.1 Cellulose, hemicellulose and lignin content of C_4 grasses and wood.
Cellulose, hemicellulose and lignin content of various C_4 energy grasses and wood presented as percentage of dry weight biomass. Modified from (van der Weijde et al., 2013).

Lignocellulosic feedstock	**Cellulose**	**Hemicellulose**	**Lignin**	**References**
Maize (stover)	~27–40%	~25–34%	~9–15%	(Jung and Bernardo, 2012; Lorenz et al., 2009; Lorenzana et al., 2010; Templeton et al., 2009; Wolfrum et al., 2009)
Switchgrass	~28–37%	~25–34%	~9–13%	(Sladden et al., 1991; Vogel et al., 2011)
Sorghum (stover)	~21–45%	~11–28%	~9–20%	(Murray et al., 2008; Rooney et al., 2007; Shiringani et al., 2010; Stefaniak et al., 2012)
Sugarcane (bagasse)	~35–45%	~25–32%	~16–25%	(Canilha et al., 2011; Masarin et al., 2011)
Miscanthus	~28–49%	~24–32%	~15–28%	(Hodgson et al., 2010; Zhang et al., 2012a; Zhang et al., 2012b)
Wheat straw	~31-41%	~24-30%	~8-14%	(Thomas, 1977)
Softwoods	~40-44%	~25-29%	~25-31%	
Hardwoods	~43-47%	~25-35%	~16-24%	

1.2.1.2.2 Hemicellulose.

Hemicelluloses differ from cellulose in having different monosaccharide units including pentoses, branched polymer chains and lacking crystallinity. Sugar monomers in hemicellulose include xylose, mannose, galactose, rhamnose and arabinose (Fig. 1.5). Xylan represents the most abundant hemicellulosic polysaccharide (Fig.1.3B) and is primarily composed of xylose, arabinose, and glucuronic acid (Fengel and Wegner, 1989). The backbone sugars of all hemicellulosic polysaccharides are β-linked, and are decorated with a variety of sugars and acetyl groups, giving the hemicellulosic polysaccharides a non-crystalline form (Gilbert, 2010).

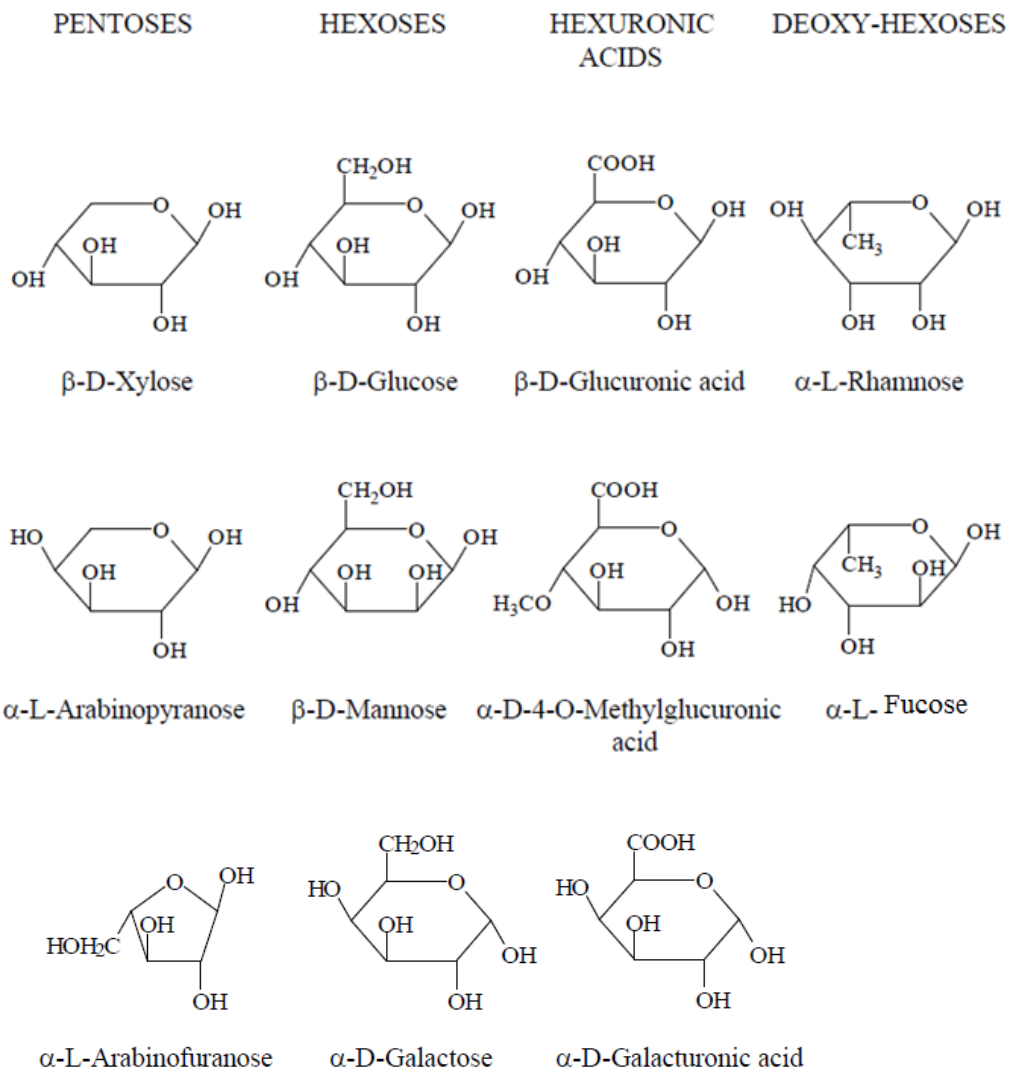


Figure 1.5 Schematic illustration of sugar units of hemicellulose.
Adapted from Fengel and Wegner, (1989).

1.2.1.2.3 Pectin.

Pectin is composed of three major forms of polysaccharide: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (Mohnen, 2008). The backbone of homogalacturonan is made up of polygalacturonic acid molecules. The backbone of rhamnogalacturonan I is composed of an alternating disaccharide, $[(\alpha\text{-}1,4)\text{-D-GalA}\rightarrow(\alpha\text{-}1,2)\text{-L-Rha}]_n$, with extensive decorations at the O-4 of the rhamnose (Rha) residues (Mohnen, 2008). Rhamnogalacturonan II consists of 13 different sugars and over 20 different linkages and is more complex than the other two pectic polysaccharides (O'Neill et al., 2004), (Fig. 1.6).

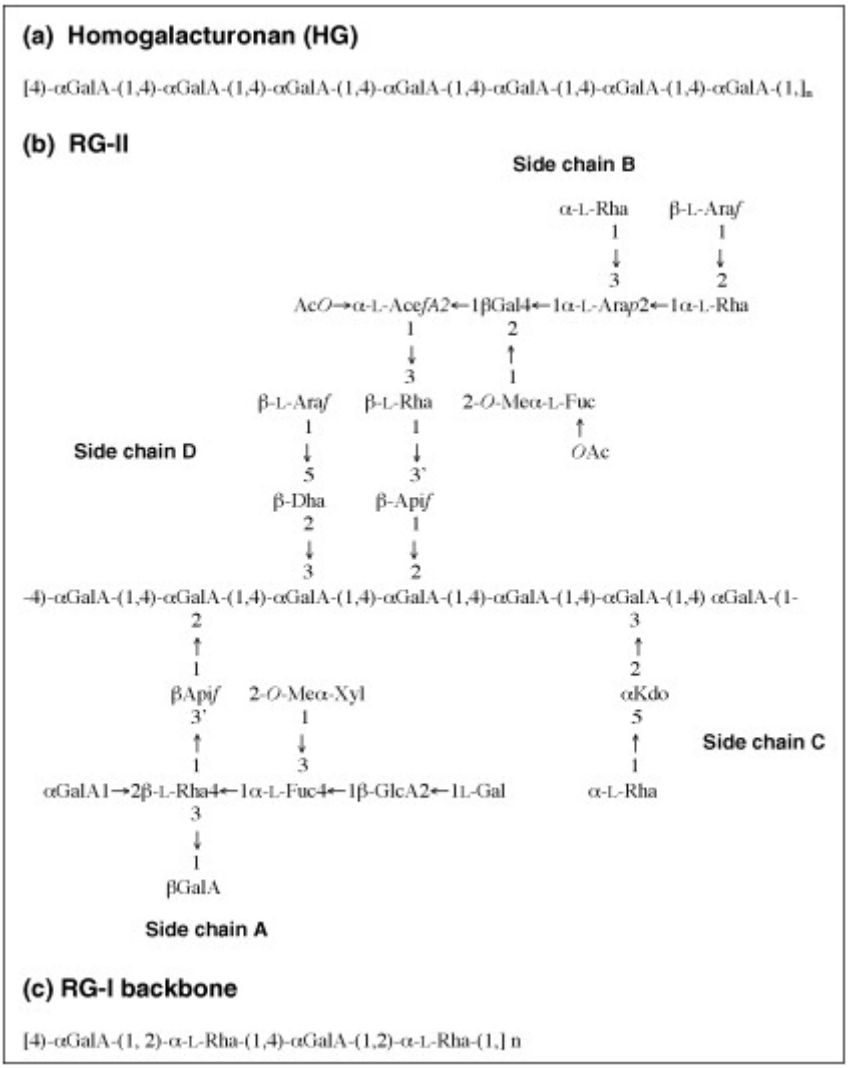


Figure 1.6 Representative structures of specific regions of the pectic polysaccharide.
(a) homogalacturonan (b) rhamnogalacturonan II, and (c) rhamnogalacturonan I. Abreviations: GalA-galacturonic Acid; Rha-Rhamnose; Ara*f,* Arabinofuranoside; A*c;* aceric acid; Ace-Acetyl ester; Api-Apiose; Me-Methyl; Fuc-Fucose; Gal-galactose; Glc-glucuronic acid; Kdo-3-deoxy-*D*-manno-octulosonic acid; Xyl-Xylose; Dha-3-deoxy-D-lyxoheptulosaric acid. Source: (O'Neill et al., 2004).

1.2.1.2.4 Lignin.

Lignin is a complex racemic, three-dimensional aromatic heteropolymer mainly formed from three hydroxycinnamyl alcohol monomers, *p*-coumaryl**M1H**, coniferyl**M1G**, and sinapyl**M1S** alcohols (Fig. 1.7A), which, respectively, produce *p*-hydroxyphenyl H, guaiacyl G, and syringyl S phenylpropanoid units in the lignin (Barker and Owen, 1999; Martinez et al., 2005; Masai et al., 2007). These monolignols differ in their degree of methoxylation, consisting of dimethoxylated, monoxylated or non-methoxylated phenylpropanoid units (Martinez et al., 2005), and are joined by different types of linkage, some of which are more resistant to chemical degradation (Boerjan et al., 2003). Lignin is the second most abundant terrestrial biopolymer, after cellulose, accounting for close to 30% of the organic carbon in the biosphere (Boerjan et al., 2003). It is especially high in the secondary cell walls of plants where it fills the spaces between the cellulose, hemicellulose and pectin components, making the cell wall rigid and hydrophobic, resulting in lignocellulose resistance to degradation (Bandounas et al., 2011; Perez et al., 2002).

It is a crosslinked large molecular structure (Fig. 1.7B) that encrusts the sugar based polymers and provides strengthening (Jones et al., 2001; Kumar et al., 2008; Rubin, 2008) and waterproofing (Myerly et al., 1981). It protects plants against pathogens (Boerjan et al., 2003; Kumar et al., 2008; Rubin, 2008). It also serves as a disposal mechanism for hydrogen peroxide (H_2O_2), a metabolic by-product generated during aerobic respiration (Giorgio et al., 2007). In the presence of H_2O_2 the lignin side chains of non-phenolic lignin units undergo single electron oxidation, forming free-radicals (Coelho-Moreira et al., 2013) with the asssociated degradation of H_2O_2.

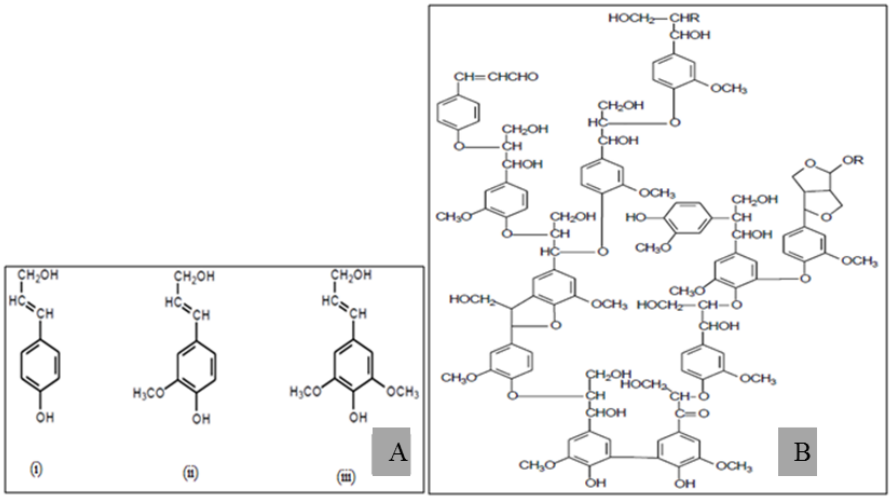


Figure 1.7 Lignin structure.
(A) The three monomeric precursors of lignin (i) p-coumaryl M1H, (ii) coniferyl M1G, and (iii) sinapyl M1S alcohols which respectively produce *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in lignin, (B) Structure of lignin, typical of a soft wood. Source: (Barker and Owen, 1999).

1.3 *Miscanthus x giganteus*.

Miscanthus x giganteus, also known as elephant grass or Giant Chinese Silver Grass is a C_4 perennial rhizomatous grass (Murnen et al., 2007). Like any other C_4 plant, *M. x giganteus* has high light, water, and nitrogen use efficiency. C_4 plants do not require a lot of tillage; in addition their perennial root system adds carbon to the soil and prevents soil erosion. Perennial grasses, such as miscanthus, mobilize mineral nutrients from the stem and leaves to the roots at the end of the growing season (Somerville et al., 2010). Winter is the best time for harvest because there would be low rates of removal of minerals from the field (Dohleman et al., 2009).

Miscanthus grows rapidly and can grow to a height of over 12 feet (Murnen et al., 2007) in favourable regions, (Fig. 1.8). It can easily be grown in poor-quality soil and does not require a lot of inputs such as herbicides, nitrogen and water for cultivation (Murnen et al., 2007; Vrije et al., 2009); it also retains high growth efficiency even in cooler climates (Murnen et al., 2007). It has been reported to have relatively high yields in England at 52^{o}N, where a peak biomass of 30 dry tons/hectare/year (T/ha/year) and harvestable biomass of 20 T/ha/year was recorded as the highest for a cool temperate climate (Beale and Long, 1995). Yields of 8 to 15 ton dry weight per ha have also been reported in Western European regions (Vrije et al., 2009). Its yields tend to be higher than that of switch grass (*Panicum vergatum),* though it is more expensive to establish than switch grass (Murnen et al., 2007).

M. x giganteus attracted attention and has been studied widely because of its potential for future energy supply either as fuel for electricity generation or for conversion to ethanol. It is considered as a good source of lignocellulosic biomass in Northern America and Europe. Like hardwoods, *M. x giganteus* has higher cellulose content than most crop residues, making it a suitable source of glucose which can be converted into ethanol (Murnen et al., 2007; Ververis et al., 2004). *M. x giganteus* lacks seeding and can therefore not cause problems as an invasive species.

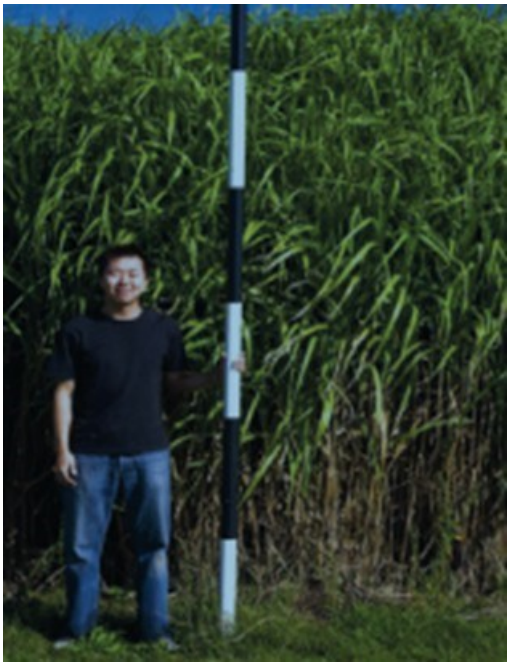


Figure 1.8 *Miscanthus x giganteus* field.
M. x giganteus in Illinois. Source: (Somerville et al., 2010).

1.4 Pretreatment of biomass material.

The efficient conversion of plant biomass to biofuels has a major limitation due to the recalcitrant nature of the plant cell wall. The first step in the methods currently used for processing lignocellulosic biomass into biofuel is preatment (Fig. 1.9). The preatment step involves some form of physico-chemical preatment and brings about the liberation of the cellulose/hemicellulose molecules from the lignin so as to enable subsequent enzymatic break down into simple sugars which can easily be fermented to ethanol by yeasts or bacteria (Lynd et al., 2008).

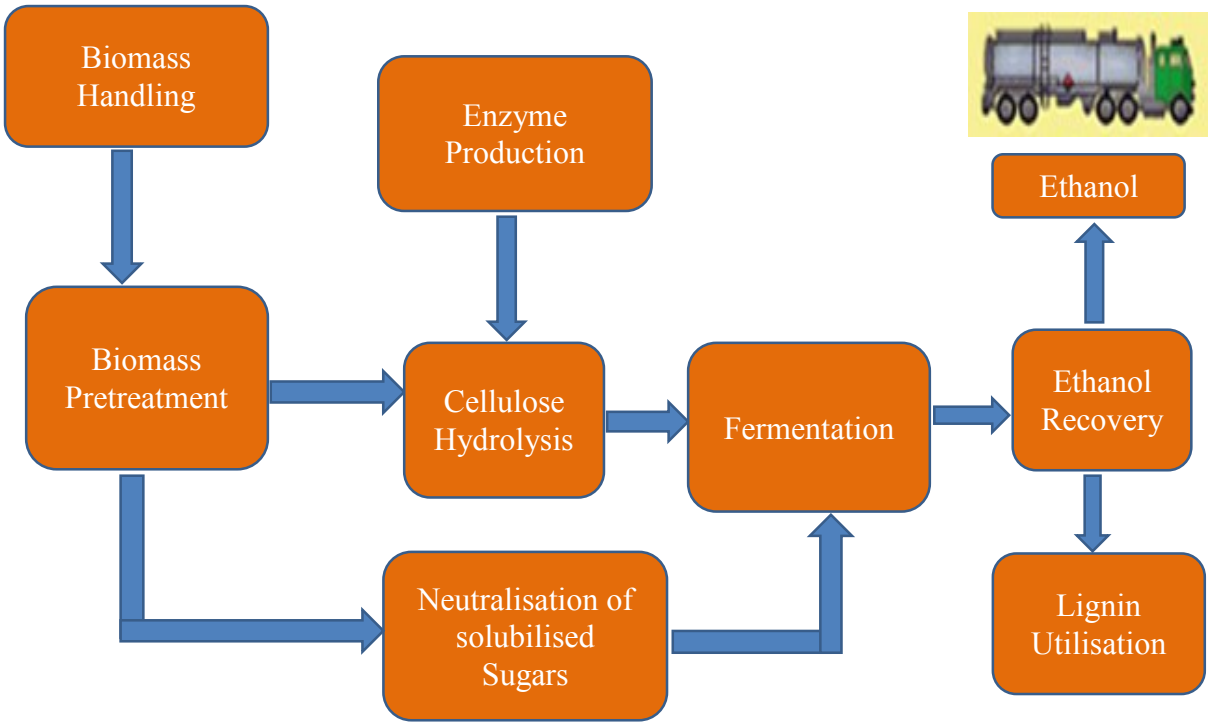


Figure 1.9 Pretreatment of cellulosic biomass and ethanol production.
Process currently being used for producing ethanol from cellulosic biomass. Modified from Energy Efficiency and Renewable Energy (EERE). (Courtesy of the U.S. Department of Energy- http://www1.eere.energy.gov/bioenergy/pdfs/Archive/abcs_biofuels.html).

1.4.1 Pretreatment technologies.

Pretreatment technologies include grinding, acid hydrolysis, steam explosion, ammonia fiber expansion (AFEX), alkaline wet oxidation, ammonia recycle percolation (ARP), and ozone preatment (ozonolysis). No single method is best for all types of feedstock.

- **Grinding-** leads to degradation of the crystalline structure of lignocellulose
- **Steam explosion-** a physico-chemical preatment in which biomass is subjected to high-pressure saturated steam, followed by rapid depressurisation (Sawada and Nakamura, 2001).
- **Alkaline wet oxidation-** water, sodium carbonate, and pressure interact with biomass by breaking ester bonds that cross-link hemicellulose with other components (Taherzadeh and Karimi, 2008).
- **Ozone**- acts primarily by degrading lignin, via attack and cleavage of aromatic ring structures.
- **Acid hydrolysis**- concentrated or dilute mineral acids penetrate biomass by breaking down hemicellulose into monomeric sugars (at high acid concentration) and removing part of the lignin (Taherzadeh and Karimi, 2008). However, the monomers further react

under acidic conditions producing furfurals which can inhibit the subsequent fermentation process. To be economic, the use of strong acids requires acid recovery so most studies focus on using weak acids that hydrolyse hemicellulose but leave the cellulose intact.

- **AFEX-** one of the advanced pretreatment processes currently being developed for biomass pretreatment. It is a physico-chemical preatment in which pre-wetted lignocellulosic material is treated with liquid anhydrous ammonia at moderate to high temperature and pressure, then pressure is rapidly released, explosively disrupting the lignocellulose fibres and the linkages between lignin and hemicelluloses, resulting in release of sugar polymers (Balan et al., 2009). AFEX brings about lignin depolymerisation during fibre expansion and allows much greater enzyme access to the carbohydrates (Zheng et al., 2009), but does not extensively solubilise the lignin.

- **ARP**- brings about greater delignification, does not require expensive facilities and works at high temperatures (Kumar et al., 2009). The left over ammonia is recyclable due to its volatility (Dale and Moreira, 1982), and residual ammonia can be used by the microorganisms that are involved in fermentation (Balan et al., 2009).

AFEX is carried out with liquid ammonia, while ARP is carried out with aqueous ammonia (10-15%), (Kumar et al., 2009). The resulting lignin can be recovered and burnt as a fuel in the refinery, leading to a net reduction in greenhouse gas emissions (Balan et al., 2009). Solubilisation of lignin during ARP is advantageous for consolidated bioprocessing (CBP), also known as direct microbial conversion (DMC), in which cellulase production, enzymatic hydrolysis and ethanol fermentation are done in a single process stage (Fig. 1.10). Lignin solubilisation will reduce the amount of material passing through the fermentation process resulting in reduced enzyme sequestration, because there will be less lignin to prevent the enzymes from binding with their substrates, though there may be a reduction in its fuel value. AFEX and ARP preatment methods have been found to be effective for pre-treating grasses such as miscanthus (Yang and Wyman, 2008). As well as modifying the lignin, they can reduce the crystallinity in the cellulose with limited hydrolysis of hemicellulose and cellulose (Balan et al., 2009) .

1.4.2 Consolidated bioprocessing (CBP).

In the current methods for utilization of lignocellulosic biomass, preatment is followed by enzymatic hydrolysis with commercial cellulases (and other enzymes if required). This may involve separate or simultaneous saccharification and fermentation, the latter combining both hydrolysis and fermentation processes in a single-step process (Fig. 1.10) (Ado et al., 2009). However, the need to purchase cellulase adds cost to the process and it might be more cost effective if the fermenting organism also produced the cellulase and any other enzymes that might be required for complete carbohydrate fermentation. This underlies the concept of CBP, which is a simplified process of converting feedstock to product (Taherzadeh and Karimi, 2007).

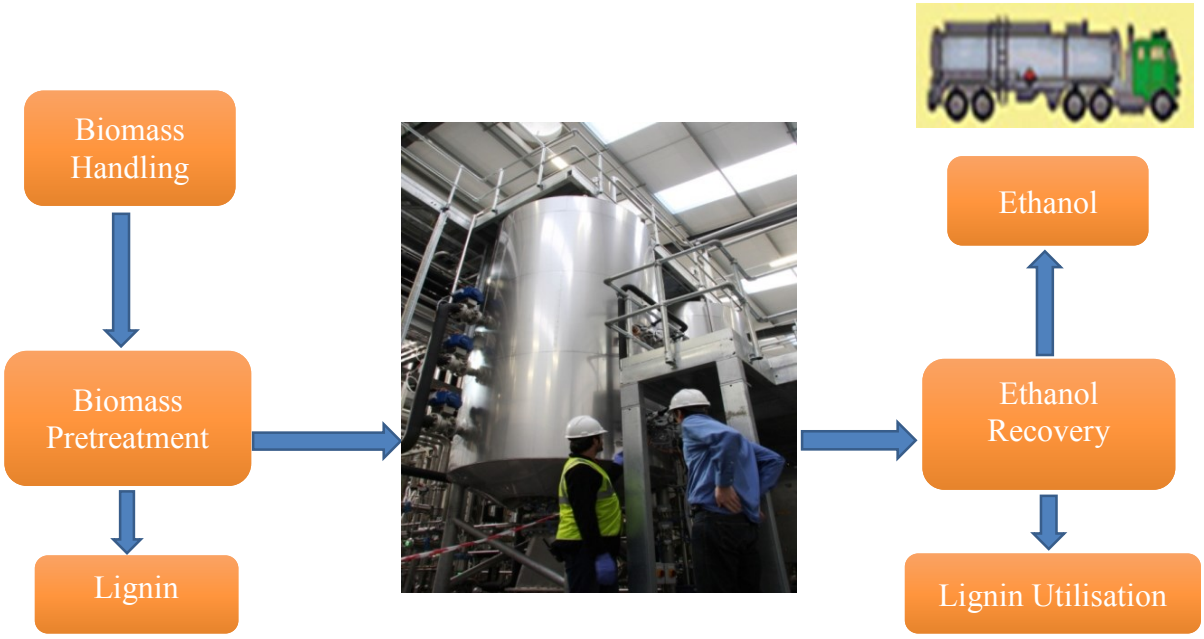


Figure 1.10 Consolidated bioprocessing process.
The (hemi)cellulase production, enzymatic hydrolysis and fermentation are combined in one process stage. Picture provided by Courtesy of TMO Renewables. Modified from EERE. (Courtesy of the U.S. Department of Energy-http://www1.eere.energy.gov/bioenergy/pdfs/Archive/abcs_biofuels.html).

The CBP process involves engineering of a single microorganism or a consortium of mixed-culture microbes (Taherzadeh and Karimi, 2007). These produce enzymes capable of degrading the carbohydrates in lignocellulose and ferment the resulting sugars into ethanol and other useful products. This process is expected to cut down on the costly addition of enzymes as used in separate or simultaneous saccharification and fermentation processes (Balan et al., 2009). Researchers have strongly promoted this approach, but there is no evidence in the

literature to show that it can be effective using a real feedstock (Lynd et al., 2005; Schuster and Chinn, 2013; Zhang et al., 2007). It is also not clear whether the increased energy demand of the implied high solids fermentation process outweighs the benefits.

Various organisms already combine multiple functions, but no organism or compatible combinations of microorganisms are available that produce cellulase and other enzymes at the required high yields and concentrations (Hamelinck et al., 2005; Olson et al., 2012; Taherzadeh and Karimi, 2007; van Zyl et al., 2007; Xu et al., 2009).

1.5 (Hemi)cellulose degrading enzymes.

1.5.1 Cellulases.

Cellulosic biomass is the most abundant substrate for the economical and environmentally sustainable production of fuels, solvents and other building blocks (Mazzoli, 2012). Cellulose is highly recalcitrant to biodegradation, and requires a mixture of enzymes known collectively as cellulase for complete hydrolysis (Mazzoli, 2012; Olson et al., 2012). The costs of pretreatment and production of cellulases and ancillary enzymes are still the most difficult hurdles to overcome for commercial cellulosic biofuel production (Mohanram and Gode, 2013).

A classic cellulase enzyme system is made up of three types of enzymes, and these work synergistically during the hydrolysis of cellulose to glucose (Deswal et al., 2011; Himmel et al., 1999; Kuhad et al., 2011; Kuhad et al., 1997). The three enzymes are endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Kuhad et al., 2011). Endoglucanases are involved in the random hydrolysis of the intramolecular β-1,4-glucosidic bonds of amorphous regions of cellulose chains resulting in production of new chain ends (Zhang et al., 2006). The exoglucanases (cellobiohydrolases) attack the cellulose chain ends to produce cellobiose, the repeating unit of cellulose, or glucose, while β-glucosidase hydrolyses cellobiose to glucose (Zhang et al., 2006) (Fig. 1.11). Crystalline cellulose is degraded from the chain ends by a combination of exoglucanases and β-glucosidase.

Use of commercial cellulases in biofuel production adds significant cost (Brunecky et al., 2011) so a lot of effort is going into efforts to reduce their cost, both in terms of cellulase production and also in improved catalytic turnover.

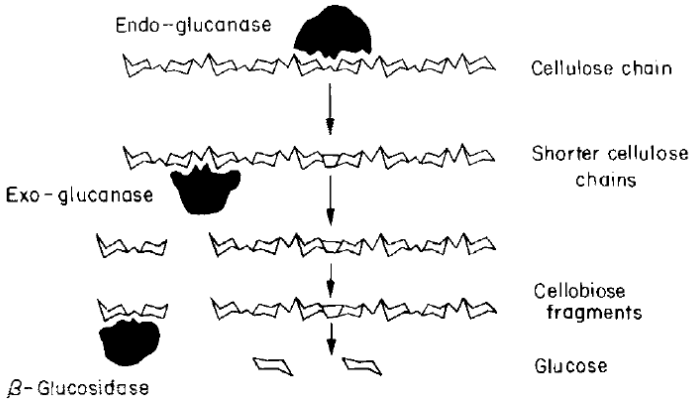


Figure 1.11 Cellulose degradation.
The action of endoglucanse, exoglucanase and β-glucosidase results in deconstruction of cellulose. Source: (Wright et al., 1988).

1.5.2 Hemicellulases.

The successful deconstruction and modification of hemicellulose requires a complex mixture of enzymes collectively called hemicellulases (Karboune et al., 2009). Enzyme degradation of hemicellulases involves mild conditions and does not result in the formation of toxic products such as those formed when acid hydrolysis is used (Juturu and Wu, 2013).

The post translational modifications of hemicellulases result into production of many types of enzymes, enabling them to deconstruct the heterogeneous hemicellulose structure (Juturu and Wu, 2013). Because of the structural complexity of hemicellulose these enzymes act synergistically to achieve full hemicellulose hydrolysis (Juturu and Wu, 2013; Karboune et al., 2009). They include endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), arabinase (endo α-L-arabinase, E.C.3.2.1.99), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55), feruloyl xylan esterase (E.C.3.2.1.73), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139) and acetyl xylan esterase (E.C.3.2.1.1.72) (Juturu and Wu, 2013). Endoglucanases can also degrade hemicellulose (Karboune et al., 2009). Cellulases and hemicellulases act synergistically to achieve complete hydrolysis of the cellulose and hemicellulose components of the lignocellulosic biomass (Gao et al., 2011).

1.6 Glycoside hydrolases and carbohydrate binding modules.

Glycoside hydrolases are the most diverse group of enzymes used by microbes to degrade biomass (Murphy et al., 2011; Rigden, 2005). They are produced by a range of bacteria and fungi (McCartney et al., 2004), and they often contain multiple structurally and functionally

diverse modules on the same polypeptide chain (Bayer et al., 1998; Davies and Henrissat, 1995; Gilkes et al., 1991; Henrissat, 1997). Glycoside hydrolases typically hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Henrissat and Davies, 2000). The cellulases have two independent globular modules (Arai et al., 2003): a catalytic domain, responsible for the hydrolysis reaction itself, and a cellulose-binding module, without catalytic activity but promoting the adsorption of the enzyme on to the substrate (Davies and Henrissat, 1995; Henrissat and Davies, 2000).

There is a wide stereochemical variation among carbohydrates, which is paralleled by a large multiplicity of enzymes involved in their metabolism (Henrissat, 1991; Henrissat and Bairoch, 1996). The process of hydrolysis is related to energy metabolism and storage, selective hydrolysis of the glycosidic bonds is therefore very important for energy uptake (Davies and Henrissat, 1995).

1.6.1 Classification of glycoside hydrolases.

Glycoside hydrolases are found in the three major kingdoms of life (archaea, eubacteria and eukaryotes) (Henrissat, 1991). These enzymes, together with their non-catalytic carbohydrate-binding modules (CBMs), have been grouped into sequence-based families based on the amino acid sequences of their catalytic domains, which directly determines structural, functional and mechanistic features of these enzymes, on the continuously updated Carbohydrate-Active EnZymes (CAZy) database (Cantarel et al., 2009; Lombard et al., 2014; http://www.cazy.org/Glycoside-Hydrolases.html).

The International Union of Biochemistry (IUB) Enzyme Nomenclature (1984) is based on the type of reaction that the enzymes catalyse and on their substrate specificity and has been used to classify glycoside hydrolases by a numerical classification (Henrissat, 1991). Under this classification glycoside hydrolases are denoted as “EC 3.2.1.x”, the first three digits indicate enzymes that hydrolyse *o*-glycosyl linkages whereas the last number indicates the substrate and sometimes indicates the molecular mechanism (Henrissat, 1991). EC represents Enzyme Commission number.

The CAZy classification is based on similarities in amino acid sequence and structure (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996). This classification differs significantly from that of the IUB nomenclature and was designed to integrate both structural and mechanistic features of the enzymes (Henrissat and Davies, 2000).

There is a direct relationship between sequence and folding similarities (Chothia and Lesk, 1986). Members of one GH family most likely share the same folding characteristics, thereby enabling homology modeling, if the three-dimensional structure of one is known (Henrissat, 1991).

However, enzymes with different substrate specificities are sometimes found in the same family, an indication for an evolutionary divergence to acquire new specificities, as observed in families 1, 13 and 16 (Davies and Henrissat, 1995). On the other hand enzymes that hydrolyse the same substrate are sometimes found in different families. Cellulases are a good example, they are found in 11 different families (Davies and Henrissat, 1995). Current reports indicate that there are 133 glycoside hydrolase families that have been classified (http://www.cazy.org/Glycoside-Hydrolases.html, 08/09/2014).

The glycoside hydrolases thought to have a common ancestry are classified into a clan. Members of a clan are recognized by significant similarities in tertiary structure together with the conservation of catalytic residues and catalytic mechanism (Henrissat and Bairoch, 1996). The relationship between some glycoside hydrolases families which can be grouped into clans have been revealed by the growing number of three dimensional structures solved for glycoside hydrolases and/or improved sequence comparison strategies (Henrissat and Bairoch, 1996).

1.6.2 Mechanisms of action of glycoside hydrolases.

Enzymatic hydrolysis of glycosidic bonds follows general acid catalysis that needs two critical residues: a proton donor and a nucleophile/base (Sinnott, 1990). There are two major mechanisms for glycoside hydrolases, leading to overall retention or inversion of the stereochemistry at the cleavage point (Sinnott, 1990), as shown in Fig.1.12. The position of the proton donor is within hydrogen bonding distance of the glycosidic oxygen (Davies and Henrissat, 1995). The type of mechanism followed appears to be conserved within each family (Gebler et al., 1992). However, the glycoside hydrolase family 97 (GH97) includes both retaining and inverting glycoside hydrolases, suspected to have resulted from the hopping of the functional group during evolution (Kitamura et al., 2008; Okuyama et al., 2009).

A detailed primary-structure analysis of glycoside hydrolases gives information about the location of the potential active-site residues on the basis of identification of appropriate invariant amino acids (Henrissat, 1990; Henrissat et al., 1989; Zvelebil and Sternberg, 1988).

The first glycoside hydrolases to have their three dimensional structures (3D) located were the lysozymes in which the two catalytic amino acids were identified as aspartate and glutamate residues (Blake et al., 1965; Matthews and Remingto, 1974). In a subset of the α-mannosidase family GH92 the usual general acid glutamic acid is replaced by glutamine (Tiels et al., 2012).

One of the mechanisms has been found to use NAD as a cofactor. The glycoside hydrolases of family 4 (Rajan et al., 2004) and 109 use a mechanism that requires a NAD cofactor (Sulzenbacher et al., 2010). The NAD remains tightly bound throughout catalysis and the mechanism involves anionic transition states with elimination and redox steps (Rajan et al., 2004). It has been shown that other residues may sometimes be involved in glycosidic bond cleavage (Davies and Henrissat, 1995). The sialidases and trans-sialidases, which are members of glycoside hydrolase families 33 and 34, utilise a tyrosine as a catalytic nucleophile; a neutral nucleophile, which gets activated by an adjacent base residue (Amaya et al., 2004; Watson et al., 2003; Watts et al., 2003). Typical examples include viral neuraminidase and bacterial sialidase, in which the transition state is thought to be stabilised with the help of a tyrosine (Burmeister et al., 1993; Crennell et al., 1993).

Glycoside hydrolase families 18, 20, 25, 56, 84, and 85 hydrolyse substrates containing an *N*-acetyl (acetamido) or *N*-glycolyl group at the carbon 2-position. This group of enzymes has no catalytic nucleophile, their catalytic mechanism uses the 2-acetamido group as an intramolecular nucleophile (Davies and Henrissat, 1995). Through neighbouring group participation, the 2-acetamido group can lead to formation of an oxazoline (oxazolinium ion) intermediate (Knapp et al., 1996; Terwisscha Van Scheltinga et al., 1995; Vocadlo and Withers, 2005). Some GH families are also able to employ novel mechanisms other than the typical carboxylate base/nucleophile. These include substrate assisted mechanisms, proton transferring networks, utilization of non-carboxylate residues and utilization of an exogenous base/nucleophile (Vuong and Wilson, 2010).

Glycoside hydrolases have also developed mechanisms to lower the energy barrier of the hydrolysis reaction by distorting their substrate into a sofa or half-chair conformation (Kuroki et al., 1993; Strynadka and James, 1991). Protonation of the glycosidic bond is accompanied by a substantial lengthening of this bond (Tanaka et al., 1994).

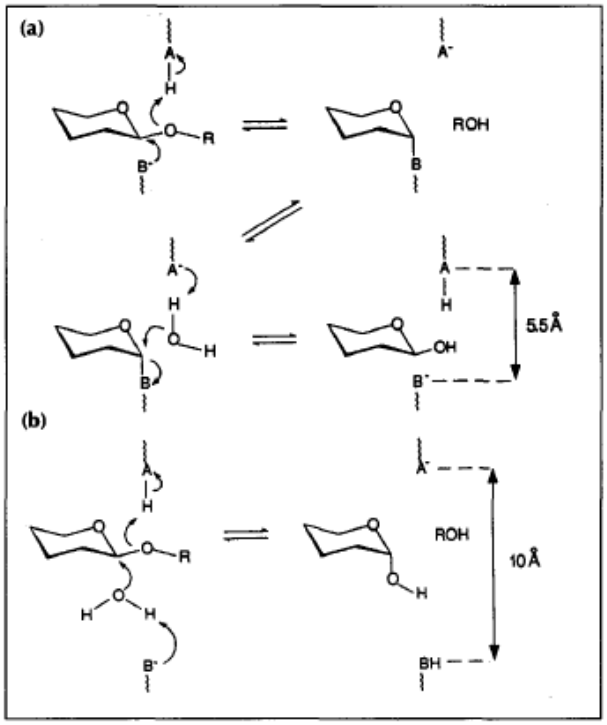


Figure 1.12 The two major mechanisms for glycoside hydrolases.
The mechanisms were proposed by Koshland (1953). (a) The retaining mechanism, which involves the protonation of glycosidic oxygen by the acid catalyst (AH) and departure of the aglycon assisted by the nucleophile base (B-). The formed glycoside enzyme gets hydrolyzed by a water molecule and results into a product with the same stereochemistry as the substrate. (b) The inverting mechanism, in which the glycosidic oxygen gets protonated. A water molecule that is activated by the base residue (B^-) attacks at the anomeric carbon as the aglycon departs. The resulting product has an opposite stereochemistry to the substrate. Copied from (Davies and Henrissat, 1995).

1.6.3 Active-site topologies.

The overall topologies of the active sites of glycoside hydrolases fall into three general classes. These three topologies are built on the same fold, with the same catalytic residues (Davies and Henrissat, 1995).

The first class of topology is called the pocket crater (Fig. 1.14A), in which the saccharide non-reducing end from cellobiose or cellodextrin is recognized, and is encountered in monosaccharidases such as β-galactosidase, β-glucosidase, sialidase and neuraminidase, and in exopolysaccharidases such as glucoamylase and β-amylase (Davies and Henrissat, 1995; Langston et al., 2006). The substrates for these exopolysaccharidases have a large number of available chain ends, such as granules, whose radial structure exposes all the non-reducing chain ends at the surface. Fibrous substrates such as cellulose have no free chain ends and are not efficiently broken down by these enzymes (Davies and Henrissat, 1995).

The cleft or groove is the second class of topology. The active site in this class is an open structure (Fig. 1.14B) which allows a random binding of several sugar units in polymeric substrates. It is commonly found in endo-acting polysaccharidases such as lysozymes, endocellulases, chitinases, α-amylases, xylanases, β-1,3(4)-glucanases and β-1,3-glucanases (Davies and Henrissat, 1995; Langston et al., 2006).

The third class of topology is called the tunnel, which is formed when part of the cleft or groove gets covered by long loops formed when the groove protein evolves. This class has been found in cellobiohydrolases (Davies and Henrissat, 1995; Kurasin and Vaeljamaee, 2011; Liu et al., 2011; Teeri, 1997; Vocadlo and Davies, 2008). The tunnel formed makes it possible for the polysaccharide chain to be threaded through it (Rouvinen et al., 1990). Members of the glycoside hydrolase families 5 (Zheng and Ding, 2013), 6 and 7 (Davies and Henrissat, 1995) have been reported to display this characteristic. Fig. 1.13 shows the loops that cause the catalytic centers of cellobiohydrolases to lie within enclosed tunnels, and these are also represented in Fig. 1.14C. This class creates suitable conditions for processivity because the enzymes are allowed to release the product while remaining firmly attached to the polysaccharide chain. The direction of the enzyme motion along the polysaccharide chain is specific and depends on the retaining or inverting mechanisms (Davies and Henrissat, 1995).

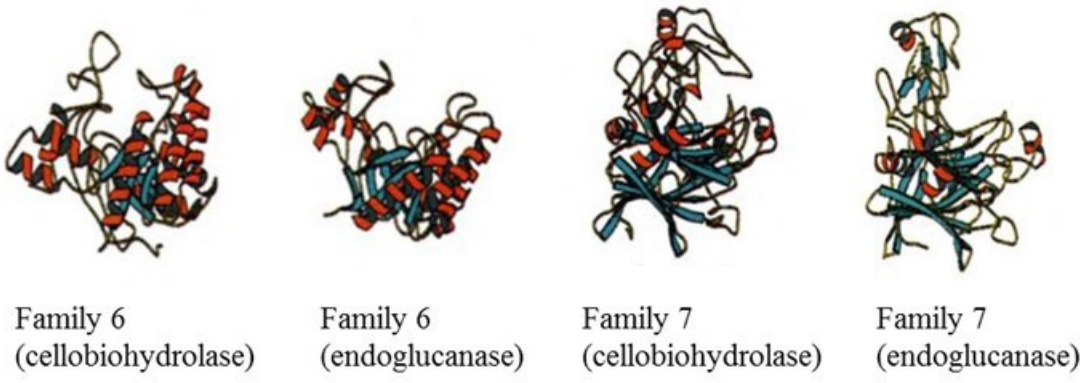


Figure 1.13 The main fold of the catalytic domain in glycosyl hydrolases families 6 and 7.
The folds are represented by ribbons. The cyan and the red colours represent the β strands and α helices, respectively. The figure was produced by using the program MOLSCRIPT (Kraulis, 1991). Copied from (Davies and Henrissat, 1995).

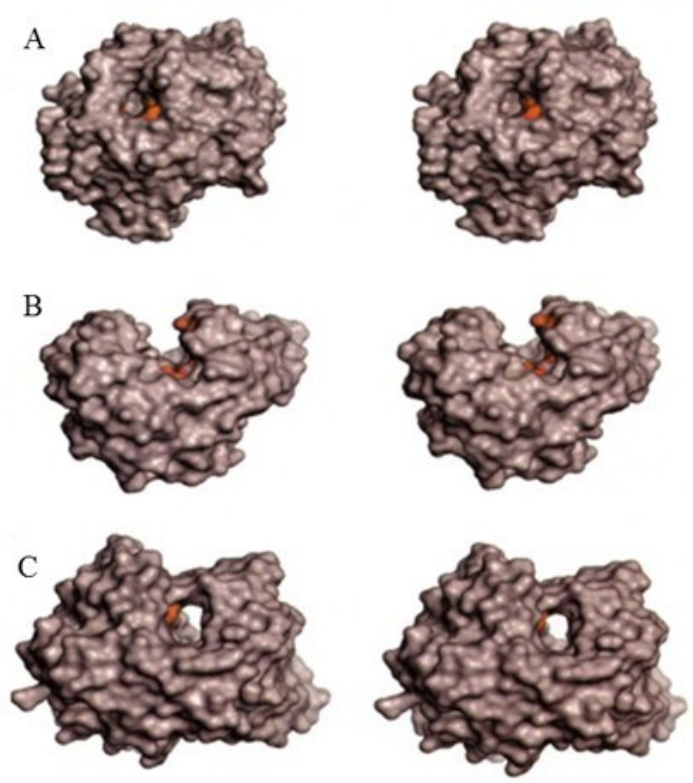


Figure 1.14 The three types of active site topologies found in glycoside hydrolases.
(A) The pocket (glucoamylase from *A. awamori*). (B) The cleft (endoglucanase E2 from *T. fusca*). (C) The tunnel (cellobiohydrolase II from *T. reesei*). The areas shaded in red are the proposed catalytic residues. (Molecular surface diagrams were prepared using the MOLVIEWER program by M Hartshorn). Source: (Davies and Henrissat, 1995).

1.6.4 Carbohydrate binding molecules (CBMs).

The enzymatic degradation of crystalline cellulose is initiated by the action of non-hydrolytic components (Shoseyov et al., 2006). Glycoside hydrolases that degrade cell walls are generally modular enzymes containing domains outside the catalytic domains (Davies and Henrissat, 1995; Henrissat and Davies, 2000). The catalytic and non-catalytic components of the glycoside hydrolases are joined via linker sequences (Bolam et al., 2004; Ohmiya et al., 1997). Most of the non-catalytic modules bind to specific carbohydrates and are defined as carbohydrate-binding modules (CBMs) (Bolam et al., 2004). The CBMs are rather ubiquitous but were first discovered associated with cellulose degrading enzymes (Michel et al., 2009).

The activity of glycoside hydrolase against plant composite structures is enhanced by CBMs, which increase the concentration of the enzyme on the surface of the mostly insoluble polysaccharide substrates (Bolam et al., 1998; Gill et al., 1999; Karita et al., 1996), or supply the catalytic module with an amorphous cellulose (Zhizhuang et al., 2001). The CBMs are also able to locate the detached cellulose chains and direct single cellulose chains into the active

site of an adjacent hydrolysing module to enable their hydrolysis (Gal et al., 1997; Irwin et al., 1998).

CBMs are thought to bring about the non-hydrolytic disruption of the cellulose fibres and provide the catalytic domains with cellulose that can easily be degraded (Din et al., 1991; Tormo et al., 1996). It is believed that the binding of the CBMs to the substrate helps to alter the non-covalent structure of the cellulose and disrupt the crystalline structures, creating a more easily degradable substrate (McCartney et al., 2004; Rigden, 2005). The cellulase CBMs fall into two groups, one group having an affinity for crystalline cellulose and the other group having an affinity for amorphous cellulose (Arai et al., 2003).

CBMs mediate binding of cellulolytic enzymes or whole organisms to the cellulosic substrate by either serving as targeting agents for the catalytic modules of free cellulases (Boraston et al., 2004; Tomme et al., 1995) or by acting as a separate targeting module as part of the non-catalytic scaffoldin subunit of the cellulosome (Bayer et al., 1998). The scaffoldin has been found to have numerous cohesion modules which bind selectively to a complementary dockerin modular component of the individual cellulosomal enzymes (Petkun et al., 2010).

The CBMs have been grouped into families based on sequence similarities (Bolam et al., 2004). Currently there are 69 CBMs families (http://www.cazy.org/Carbohydrate-Binding-Modules.html, 09/08/2014). Some of the CBM families, such as families 1 and 2, contain modules of invariant substrate specificity while others contain modules that bind to a range of different polysaccharides (Michel et al., 2009). Family CBM6 for example, contains not only modules of diverse specificity, but also has variations in its substrate binding site locations with respect to its 3D structure (Henshaw et al., 2004) as revealed by numerous studies (Boraston et al., 2003; Czjzek et al., 2001; Henshaw et al., 2004; Pires et al., 2004). CBMs which bind to individual polysaccharide chains always display the same substrate specificity as their attached catalytic domain (Boraston et al., 2004).

1.7 Ligninases.

Liginases are very important in the degradation of lignocellulosic biomass. Lignin degradation is necessary for successful biomass deconstruction. Lignin is very recalcitrant; degradation requires an oxidative process through activities of ligninolytic enzymes (Praveen et al., 2011). Additionally lignin undergoes structural modifications during physico-chemical lignocellulose

processing, complicating biocatalytic lignin degradation (Crawford, 1981; Emmel et al., 2003).

Chemical methods such as aqueous ammonia preatment, acid hydrolysis and steam explosion are being used for removal of lignin (Brodeur et al., 2011). Most of the lignin depolymerisation processes require large amounts of energy (Ward and Singh, 2002) and are therefore not efficient. Most of these processes occur under severe conditions and are environmentally harsh (Ward and Singh, 2002). Enzymes could be a better alternative for lignin depolymerisation. Biocatalytic processes generally take place under mild conditions which require low amounts of energy and are more environmentally friendly (Perez et al., 2002; Rahman et al., 2013; Sun and Cheng, 2002).

Bacterial and fungal ligninolytic enzymes include lignin peroxidases (LiP) (EC 1.11.1.4), manganese peroxidases (MnP) (EC 1.11.1.3), laccase (Lcc) (EC 1.10.3.2) (Chen et al., 2011; Praveen et al., 2011; Rahman et al., 2013), and versatile peroxidases secreted by white rot fungi (Arantes and Milagres, 2007; Hatakka, 1994; Kirk and Farrell, 1987; Shary et al., 2008).

It has been observed that less of the bacterial ligninolytic potential has been explored (Bandounas et al., 2011). Bacterial strains are capable of degrading lignin and lignin related model compounds (Bugg et al., 2011). Bacteria such as *Sphingomonas* sp. (Masai et al., 1999; Wenzel et al., 2002), *Pseudomonas* sp. (Delalibera et al., 2007) and the actinomycetes, *Rhodococcus* sp., *Nocardia* sp. and *Streptomyces* sp. (Bugg et al., 2011; Zimmermann, 1990) produce lignin degrading enzymes such as laccases, ring cleaving dioxygenases, glutathione s-transferases (Allocati et al., 2009; Masai et al., 2003), monooxygenases and phenol oxidases (Perestelo et al., 1989). Other bacteria including *Bacillus* sp., *Ochrobacterium* sp., and *Leucobacter* sp. were also reported to produce all the three main ligninases, namely lignin peroxidase, manganese peroxidase and laccase (Rahman et al., 2013).

Bandounas et al. (2011) have indicated that bacterial lignases could be better than the fungal lignases as they are said to have specific thermostability and mediator dependence (Kumar et al., 2008; Masai et al., 2007). It is difficult to commercialize lignin degradation by fungi due to problems related to fungal protein expression and genetic modification (Chandra et al., 2008). Fungi have been reported to show lack of stability in practical treatment under extreme environmental and substrate conditions involving oxygen limitation and high pH, extractive and lignin concentration (Daniel and Nilsson, 1998).

Activities of ligninolytic enzymes are said to be induced in the culture after exhaustion of nutrients such as S, N and C, usually after peak growth of the culture as observed in cultures of fungi *Phanerochaete chrysosporium* and *Trametes versicolor* (Das et al., 1997), and bacterium *Comamonas* sp. B-9 (Chen et al., 2012b), while in some organisms activities can also be induced even in medium with sufficient N (Faison and Kirk, 1985; Levin et al., 2002). Apart from culture conditions the lignolytic enzymes also depend on the type of producing organism (Praveen et al., 2011).

1.8 Improving microorganisms that are able to utilise the different types of sugars found in cellulosic biomass by genetic engineering.

1.8.1 Metabolic Engineering of microorganisms for lignocellulosic biofuel production.

In order to obtain renewable liquid transportation fuels from cellulosic biomass, it is necessary to develop microbes that will ferment all the available sugars, withstand the toxic by-products resulting from the preatment process, and will not get inhibited by the fuel being produced (Sommer et al., 2004). Thermophiles have been reported to be ideal microbes in this respect because many can utilize a range of hexoses and pentoses, and some can produce cellulases that function efficiently at high temperatures and at a broader range of pH than cellulases produced by mesophiles (Rastogi et al., 2010). The current cost of converting cellulosic biomass to sugar monomers which are required for fuel production is still very high, and is approximately double the cost of buying corn in the USA (Lynd et al., 2008).

Development of technologies that reduce the processing costs in biorefineries is necessary and can be achieved by reducing cellulase production cost, improving cellulase performance and increasing sugar yields (Zhang et al., 2006). Biotechnology can be used to convert lignocellulosic materials to meet societal energy challenges, mostly by genetic reprogramming of microorganisms (Lynd et al., 2008; Ragauskas et al., 2006). Metabolic engineering or extensive reprogramming of the physiology of the producing organisms could greatly contribute to the success of lignocellulosic ethanol production (Lovins et al., 2004).

The fermentation of xylose, a major constituent of hemicellulose (Fig. 1.3 B), is very important for the efficient biological conversion of lignocellulose to ethanol (Jeffries et al., 2007). Unfortunately, *Saccharomyces cerevisiae*, the favoured organism for first generation ethanol

production, is unable to utilise xylose, so genes for xylose metabolism have been engineered into *S. cerevisiae* (Table 1.2).

Table 1.2 Engineering of xylose metabolism genes into *S. cerevisiae*.
Genes have been engineered into *S. cerevisiae* to enable it to metabolise xylose.

Enzyme/gene	Organism (gene)	Reference
Xylose reductase (XR)	*Pichia stipitis* (*Ps*XR)	(Bengtsson et al., 2009; Watanabe et al., 2007)
Xylitol dehydrogenase (XDH)	*Pichia stipitis* (*Ps*XDH)	(Bengtsson et al., 2009; Watanabe et al., 2007)
L-arabinose isomerase,	*B. subtilis* (araA), *B. lichenformis* (araA)	(Becker and Boles, 2003; Wiedemann and Boles, 2008)
L-ribulokinase and L-ribulose-5-P 4-epimerase	*E.coli* (araB and araD), respectively	(Wiedemann and Boles, 2008)
Xylose isomerase (XI),	*Thermus thermophilus* and *Piromyces* sp. strain E2 (xylA)	(Kuyper et al., 2003; Walfridsson et al., 1996)
Xylose isomerase (XI),	*Piromyces* sp. strain E2 (xylA)	(Harhangi et al., 2003)
D-xylose isomerase (XI)	*Prevotella ruminicola* TC2-24 (xylA)	(Hector et al., 2013)
Xylose isomerase (XI)	*Bacteriodes thetaiotaomicron* (xylA)	(Smith et al., 2014)

Many other efforts have also been made to metabolically engineer mesophilic bacteria that naturally consume both hexose and pentose sugars to redirect the flow of carbon by expressing heterologous genes that can form ethanol and other biofuels (Dien et al., 2003; Kim et al., 2007; Liu et al., 2005; Romero et al., 2007; Talarico et al., 2005; Yanase et al., 2005; Yanase et al., 2007; Zhou et al., 2008). A number of strains have been developed that are able to produce ethanol from xylose and other sugars, although it is difficult to ascertain their industrial potential (Karakashev et al., 2007; van Maris et al., 2006).

1.8.2 Thermophiles and ethanol.

Bacteria that grow optimally at temperatures above 55ºC are called thermophiles. These have been reported to be ideal microbes for improved production of bioethanol and other more advanced biofuels. Thermophilic bacteria such as *Clostridium thermocellum, Thermoanaerobacter thermosaccharolyticum*, *T. thermohydrosulfuricus*, *T. ethanolicus, T. brockii*, *T. saccharolyticum* and *Geobacillus* spp., have been evaluated for biofuel production and have been found to have a number of advantages over mesophilic bacteria (Cripps et al., 2009; Shaw et al., 2008; Sommer et al., 2004). Thermophilic bacteria are capable of producing robust enzymes suitable for use in industrial processes (Bhalla et al., 2013a; Ng et al., 1981; Rhee et al., 2000; Wiegel, 1980).

i. Many thermophiles readily utilize pentoses, glucose, and other complex carbohydrates, and therefore have a high potential for producing ethanol from lignocellulose.

ii. A number of thermophiles express cellulase systems.

iii. They generally display high maximum specific growth rates (μ_{max}) and have high maintenance energies resulting in low cell yields and conversely high substrate conversion to product.

iv. High temperatures result in an increased vapour pressure of ethanol, facilitating ethanol removal and recovery.

v. Substrates are more soluble at high temperatures, allowing increased concentrations of carbohydrate to be used during the process.

vi. Many glycolytic thermophiles are able to use polymeric or short oligomeric carbohydrates.

vii. Oxygen is less soluble at high temperatures, thus facilitating the maintenance of anaerobic conditions required for the fermentation process.

viii. High temperatures reduce the risk of contamination by non-thermophiles during the process.

ix. There is no requirement for cooling during fermentation with thermophiles.

An earlier study (Rastogi et al., 2010) indicated that bacteria belonging to the genera *Geobacillus*, *Thermobacillus*, *Cohnella* and *Thermus* are capable of degrading amorphous cellulose, carboxymethylcellulose (CMC), or ponderosa pine saw dust. Previous studies have

reported isolation of cellulose-degrading bacteria from compost systems (Lu et al., 2005; Mayende et al., 2006; Ng et al., 2009), soils (Abdel-Fattah et al., 2007; Lee et al., 2008), wastewaters (Tai et al., 2004), and from deep biosphere of gold mines (Rastogi et al., 2009). Cellulase enzymes from these bacteria can probably withstand extreme conditions such as temperature and toxic inhibitors (Rastogi et al., 2010). Efforts to produce ethanol from thermophiles have been hampered due to limited biochemical knowledge, limited genetic tools, poorly understood host transformation systems and scarcity of sequence data for these microorganisms.

1.8.3 *Geobacillus* spp. as potential ethanologens.

Bacteria are a more promising group of microorganisms for the successful development of CBP because filamentous fungi are not able to ferment sugars (Fonseca et al., 2008; Nofsinger and Bothast, 1981; Zhang et al., 2014). *Geobacillus* spp. are a promising group of microorganisms for development of a successful CBP for producing biofuel at a low cost (Cripps et al., 2009).

Some *Geobacillus* spp., including *G. thermoglucosidasius* are facultative anaerobes (Nazina et al., 2001). Members of this genus are capable of growth between 40 and 70^{o}C and can ferment both hexose and pentose sugars and oligomers to generate lactate, formate, acetate and ethanol as fermentation products (Cripps et al., 2009). *G. thermoglucosidasius* has a rapid growth rate and is metabolically versatile using a range of carbohydrates as feedstocks. It can easily be genetically engineered due to the availability of genetic tools, including shuttle vectors, gene deletion strategies and reporter genes, as well as genome sequences. It has been engineered to produce ethanol as a major fermentation product (Cripps et al., 2009), but the engineered strain is unable to degrade the polymeric carbohydrates in lignocellulose materials to release the sugars.

Like other *Bacillus* spp, previously reported (Robson and Chambliss, 1984), *G. thermoglucosidasius* lacks a complete cellulase system, but may have endoglucanase or CMCase activity which does not hydrolyze crystalline cellulose. Successful engineering of *G. thermoglucosidasius* could create a strain which can both hydrolyze cellulose and ferment the resulting products to produce ethanol as the main product.

The importance of "*Bacillus stearothermophilus"* in the canned food industry, and relative ease of working with aerobic and facultative anaerobic thermophiles, means that *Geobacillus*

spp. have been extensively studied at a biochemical level and also in large scale fermentation for the production of thermophilic enzymes. Their fermentative metabolism was summarised by (Payton and Hartley, 1985). Furthermore, many strains are genetically transformable and plasmid shuttle vectors have been developed and improved upon over recent years, including tools for gene knockouts and reporter genes. Although relatively late in the scheme of things, a number of genome sequences for *Geobacillus* spp. have recently appeared in the literature.

G. thermoglucosidasius strains are able to withstand moderate concentrations of ethanol (Taylor et al., 2009) and, with the aid of developing genetic tools and extensive background fermentation experience with this species (Hartley and Shama, 1987; Payton and Hartley, 1985; Sanmartin et al., 1994), *G. thermoglucosidasius* is being developed into an industrial microorganism for development of consolidated bioprocessing, by improving its secretion of the glycosyl hydrolases. TMO Renewables have engineered a novel ethanol production pathway in *G. thermoglucosidasius* NCIMB 11955, with parallel work done at Imperial College on strain DL33. Their fermentative carbon flux has been diverted from a mixed acid pathway (Fig. 1.15), by elimination of the lactate dehydrogenase and pyruvate formate lyase pathways by knocking out the *ldh* and *pfl*B genes, respectively, and upregulating the expression of pyruvate dehydrogenase resulting in ethanol production in excess of 90% of the theoretical yield (Cripps et al., 2009).

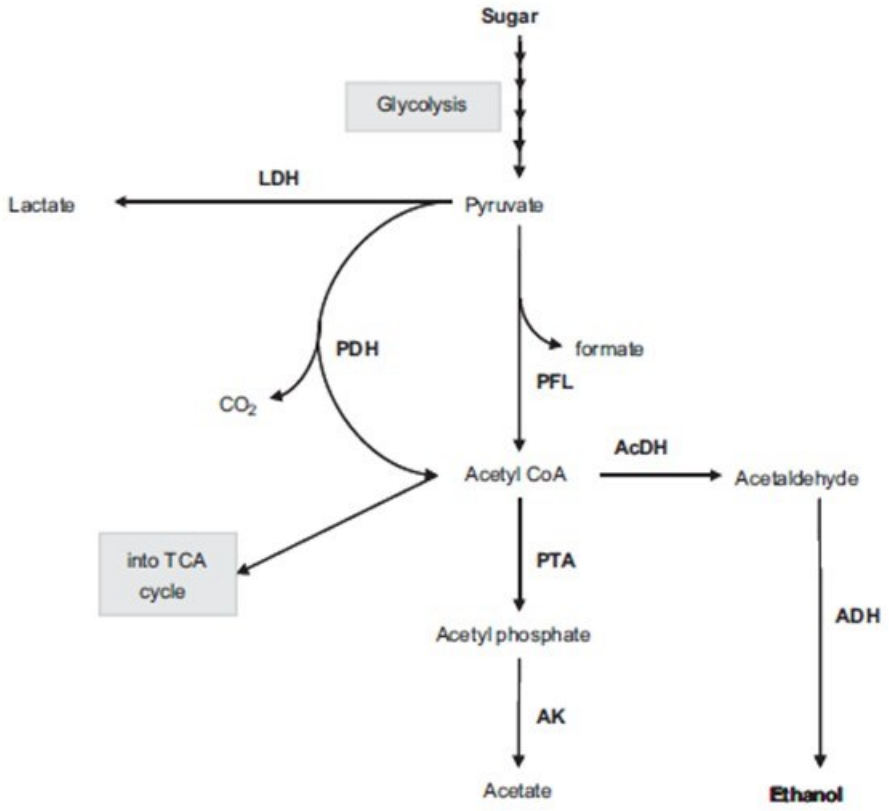


Figure 1.15 Major metabolic pathways associated with ethanol production in *Geobacillus* spp.
The first step involves conversion of glucose to pyruvate which subsequently gets converted to lactate by Lactate dehydrogenase (LDH), or to formate and acetyl CoA by Pyruvate-formate lyase (PFL). Acetaldehyde dehydrogenase (AcDH) and Alcohol dehydrogenase (ADH) converts acetyl CoA to ethanol or acetate by transacetylase and Acetate Kinase (AK). Pyruvate Dehydrogenase (PDH), is primarily an aerobic enzyme producing Acetyl CoA but has been shown to be active under anaerobic conditions. The abreviations PTA and TCA cycle stand for phosphotransace-tylase and tricarboxylic acid cycle, respectively. Source: (Cripps et al., 2009).

1.9 Cocultures.

The degradation of plant biomass in the natural environment is achieved by complex microbial communinities that employ hydrolytic and oxidative enzymes to deconstruct polysaccharides and lignin (D'Haeseleer et al., 2013; Li et al., 2011). A more comprehensive view of lignocellulose depolymerisation may be provided by studying lignocellulose deconstruction by microbial communities, rather than isolates, and new microbial groups and degradation mechanisims may also be uncovered (D'Haeseleer et al., 2013). The complex microbial communities may be simplified and the roles of the specific populations within the community may also be identified by the enrichment cultures established with defined substrates and at constant temperatures (D'Haeseleer et al., 2013).

Research on improvement of microbial and enzymatic processes on lignocellulosic biomass degradation is important for success in sustainable green biotechnology (Wongwilaiwalin et

al., 2010). Research on microbial consortia can help scientists to understand the complex interactions involved in lignocellulose degradation in nature. This information could inform studies on the biotechnological application of biomass degradation in composting, anaerobic digestion, enzymatic biomass saccharification and the direct microbial conversion of cellulosic biomass to products without the addition of saccharolytic enzymes (Lynd et al., 2005).The biomass degradation capability depends on the functional and structural stabilities of microbial consortia (Wongwilaiwalin et al., 2010).

Mixed microbial cultures have been developed during previous studies in an attempt to increase the production of biomass, fuels and enzymes for the digestion of cellulose (Haruta et al., 2002; Kato et al., 2004; Rattanachomsri et al., 2009). Successful cellulose utilization and hemicellulose hydrolysis can be achieved by a coculture of compatible organisms (Ng et al., 1981). Cocultures between cellulolytic and pentose utilizing microbes are common in nature and could provide better hydrolysis (Lynd et al., 2002), and yield higher amounts of products such as ethanol from cellulose than pure cultures (Svetlitchnyi et al., 2013).

Lignocellulose-utilising bacteria naturally exist in symbiotic relationships with one another, which contribute to the successful recycling of carbon in the environment (Zhang et al., 2014). Cellulose degradation by mixed microbial cultures has been demonstrated by symbiosis between cellulolytic and non-cellulolytic micoorganisms in previous studies (Pohlschroeder et al., 1994; Veal and Lynch, 1984). Use of cocultures or complex microbial communities can prevent problems of feeback regulation and metabolic repression (Delatorre and Campillo, 1984; Haruta et al., 2002; Soundar and Chandra, 1987). Cocultures of two thermophilic *Clostridium* strains, CS-3-2 and CS-4-4, on cornstalk showed synergism of glycoside hydrolase secretomes by producing much greater enzyme activities than the pure cultures or an artificial mixture of samples (Zhang et al., 2014).

A thermophilic lignocellulose degrading mixed microbial community from bagasse compost comprising of *Bacilli*, uncultured bacteria, an aerobic/facultative anaerobic *Rhodocycloceae, Clostridium* and *Thermoanaerobacterium* genera showed efficient degradation activity on bagasse, cornstover, rice straw and industrial eucalyptus sludge (Wongwilaiwalin et al., 2010). This mixed microbial group had lignocellulolytic activities such as endo-glucanase, xylanase and β-glucanase in the culture supernatant.

Clostridium thermocellum forms successful cocultures with saccharolytic organisms. It formed stable cocultures with the saccharolytic ethanol producing *Thermoanaerobacter ethanolyticus* due to beneficial syntrophic relations, resulting into production of higher amounts of ethanol than the pure cultures (Cann et al., 2001). Coculture of organic acid-deficient engineered strains of both *C. thermocellum* and *Thermoanaerobacterium saccharolyticum* resulted into high ethanol production from cellulose (Argyros et al., 2011). Cocultures of *Caldicellulosiruptor* DIB 087C and *Thermoanaerobacter* DIB 097X were also able to convert cellulose to higher ethanol concentrations than their monocultutures (Svetlitchnyi et al., 2013).

However, low production of ethanol from cocultures of *Caldicellulosiruptor saccharolyticus* DSM 8903 and *Thermoanaerobacter mathranii* subsp. *mathranii* DSM 11426 were also reported (Svetlitchnyi et al., 2013). These studies on cocultures of *Caldicellulosiruptor* strains with new *Thermoanaerobacter* strains showed that specific strain combinations are important to achieve high ethanol production for development of CBP (Svetlitchnyi et al., 2013).

1.10 Molecular phylogeny.

Research has shown that phylogenetic relationships of bacteria and all life-forms can be determined by comparing a stable part of the genetic code, which has a common function in all of the organisms under study (Woese, 1987; Woese et al., 1985). Over time, functionally neutral mutations will arise in the gene under study, such that the differences in sequence can be used as a measure of evolutionary divergence. Because function needs to be maintained the extent of mutation is limited so the size to be studied needs to be sufficient to allow this discrimination, but not too large to be cumbersome.

The 16S rRNA gene is the sequence which is most commonly used for taxonomic purposes for bacteria (Clarridge, 2004). Differences in sequence of the 16S rRNA gene marks the evolutionary distance and relatedness of organisms (Harmsen and Karch, 2004; Pace, 1997; Thorne et al., 1998), with the same species having at least 97% sequence identity over the whole gene. In this study 16S rRNA gene sequencing was used to identify bacterial strains isolated from compost to genus and probable species level.

1.11 Aim of project.

The main aim of this project was to enrich a thermophilic mixed culture capable of degrading ammonium hydroxide pretreated *M. x giganteus* in order to identify the enzymes, their genes and associated organisms involved in the process. This would act as a guide to important functions necessary for miscanthus degradation and hence the proteins that would need to be expressed from a recombinant *Geobacillus* sp. in order to convert it into a suitable CBP organism.

Specific objectives.

- Optimise pretreatment of *Miscanthus x giganteus* using alkaline treatment
- Determine the carbohydrates present in miscanthus after pretreatment
- Evaluate existing cellulolytic strains of *Geobacillus* spp.
- Use the pretreated miscanthus as a substrate to isolate thermophilic bacteria which can degrade the complex oligomers and polymers present in the pre-treated material
- Characterise lignocellulosic mixed cultures for enzymes that are responsible for producing enzymes that can hydrolyse the pretreated miscanthus
- Identify the bacteria detected or isolated from the mixed culture through analysis of their 16S RNA gene analysis
- Transfer genes encoding the enzymes identified (or similar genes from characterised strains) into ethanologenic *Geobacillus thermoglucosidasius* to create new recombinant strains with improved cellulolytic activities
- Evaluate the recombinant strains

2. Materials and methods.

2.1 Bacterial strains and Plasmids used in this study.

Table 2.1 Bacterial strains and plasmids used in this project.

Strain	Genotype/Description	Source/ Reference
Bacteria		
E. coli JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1mcrB$^+$ Δ(lac-proAB) e14- [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^-m_K^+$)	Sigma
Thermophilic bacteria		
WL3, WL6, WL14 and WL16	Novel thermophilic isolates from West London Composting Company, Middlesex.	previuos study
TR1A strains-1 to 13, 17	Novel thermophilic isolates from West London Composting Company, Middlesex.	this study
Other bacteria		
TR1A strains- 14 and 16	Novel isolates from West London Composting Company, Middlesex.	this study
Geobacillus thermoglucosidasius		
DL33 NCIMB 11955	TMO Renewables Ltd	Originally isolated by Simon Baker from compost at Wisley(RHS)
Plasmids		
pJET1.2/blunt	Cloning vector. Containing *bla* (ApR)- ß-lactamase gene conferring resistance to ampicillin , and *eco47IR* - Lethal gene *eco47IR* enables positive selection of the recombinants	Fermentas
pUCG4.8 1.1	Cloning vector containing cellobiose promoter	University of Bath

2.2 Sample Collection.

Miscanthus x giganteus was collected on April 4, 2010 and April 11, 2011, from Silwood Park, Ascot, UK, from a plot on the campus grounds (Fig. 2.1A), and from Rothamsted, respectively. It was harvested using branch/tree pruners, packaged in black plastic bags and transported to the lab (Fig. 2.1B). The stems were cleaned by removing the leaves and leafstalks and left for air drying in a large well aerated room for about two months. Then it was put in an air tight room for storage (Fig. 2.1C).

2.2.1 Preparation and pretreatment of *M. x giganteus*.

The dried miscanthus biomass was cut for grinding (Fig. 2.1D) and milled using a Retsch® Heavy-Duty Cutting Mill SM2000 followed by sieving (Fig. 2.1E) through mesh No. 20 (850 μm), No. 80 (180 μm), the sieves were stack on a sieving shaker in order, starting at the bottom: a solid catch pan, 80- mesh sieve, then 20-mesh sieve. To treat miscanthus a 1:20 ratio biomass:10% (wt/wt) NH_4OH mixture was prepared in Ace pressure tubes (from Sigma-Aldrich) as shown in Fig. 2.1F, and pretreated in an oven (from Pickstone Ovens, Thetford, England) for 14 h at temperature ranges from 60-200^oC (Fig. 2.1G). After cooling to room temperature, the pretreated miscanthus biomass was washed with distilled water and the water was drained out using a suction pump. The biomass was washed until the pH of the filtrate was about 7, and then dried at room temperature (Figures 2.1H and I).

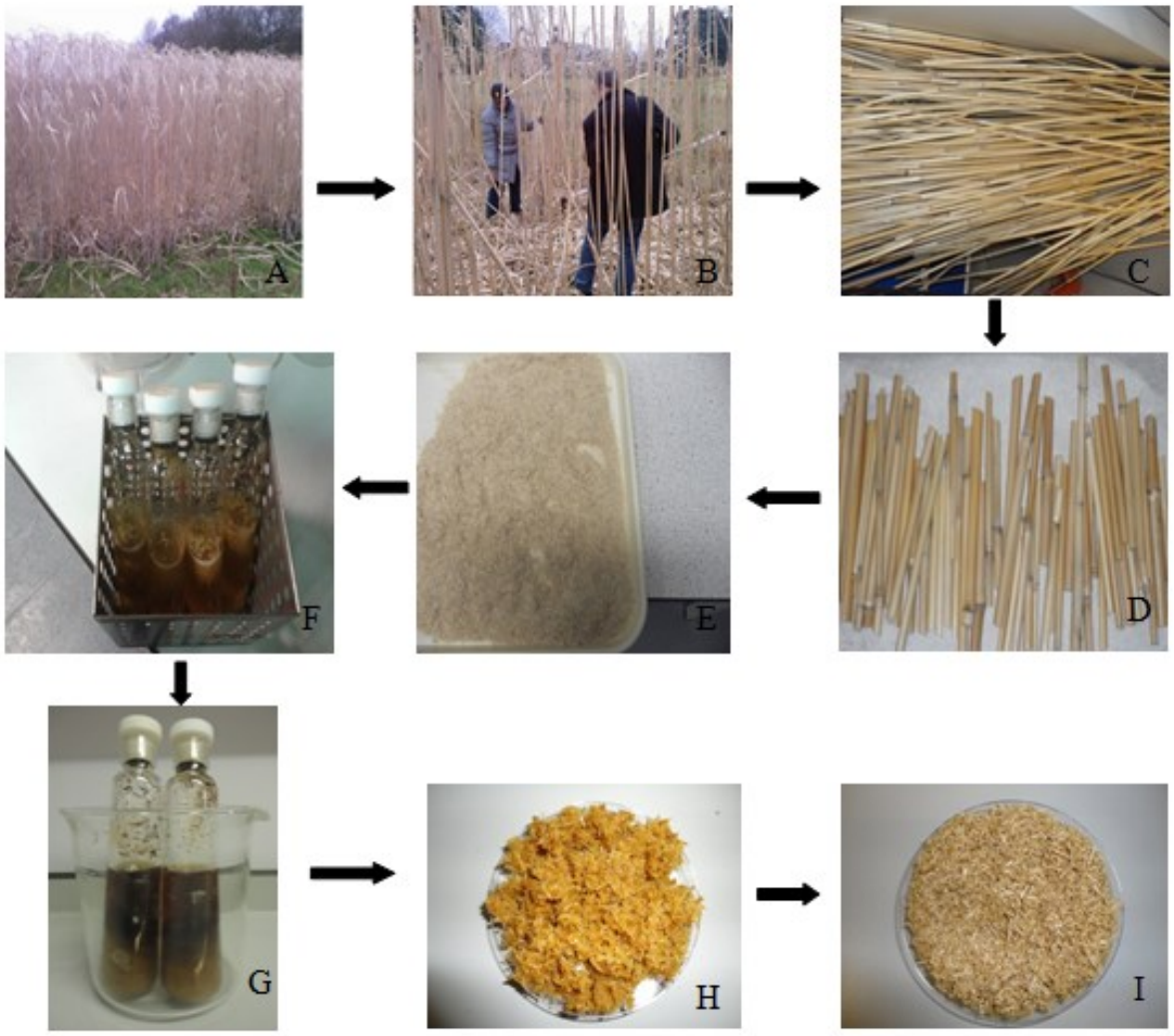


Figure 2.1 Pretreatment of *M. x giganteus*.
Flow diagram showing *M. x giganteus* in a field at Silwood Park (A), and the activities involved in the preparation of the miscanthus biomass; collection (B), cleaning and drying (C), cutting (D), grinding (E), soaking in 10% (wt/wt) NH_4OH (F), pretreatment at 120°C in an oven from Pickstone Ovens, Thetford, England (G), washing using distilled water until when the filtrate pH was about 7 (H) and drying at room temperature (I).

2.2.2 Composition analysis of untreated and pretreated *M. x giganteus*.

2.2.2.1 Extractives content.

Extractives were removed from the untreated biomass prior to composition analysis to prevent their possible interference with subsequent processes (Selig et al., 2008). Extractives are non-structural materials in biomass. The water soluble extractives include inorganic material, non-structural sugars and nitrogenous materials etc, while the ethanol soluble extractives include chlorophyll, waxes or other minor components (Sluiter et al., 2005). The inorganic material in the water soluble material may be from the biomass or any soluble material in the soil or fertilizer associated with the biomass (Sluiter et al., 2005).

The removal of extractives was carried out according to the National Renewable Energy Laboratory-Laboratory Analytical Procedure (NREL-LAP) (Sluiter et al., 2005). The *M. x giganteus* biomass was subjected to a two step extraction, first with distilled water, then with 95% EtOH. Extractives were recorded on a dry weight basis as weight percentage of the biomass.

2.2.2.2 Determination of structural carbohydrates, acid-insoluble and acid-soluble lignin in pretreated and untreated *M. x giganteus* biomass.

The pretreated and untreated miscanthus samples were analysed for their carbohydrate profile and lignin content by using the NREL-LAP method as outlined by Sluiter et al., (2008). The biomass pre-treated at 60ºC, 80ºC, 100ºC, 120ºC, 140ºC, 160ºC, 180ºC and 200ºC and untreated miscanthus biomass samples were used for the assay.

For each untreated and pre-treated sample, 0.30 $\pm$ 0.01 g (oven dry weight) was hydrolysed with 3.00 $\pm$ 0.01 ml (or 4.92 $\pm$ 0.01 g) of 72% sulphuric acid in an Ace pressure tube (from Sigma-Aldrich). The samples were mixed for 1 min or until they were thoroughly mixed by using a Teflon stirring rod, followed by incubation at 30ºC for 1 h. The samples were stirred using the stir rod, every 5 to 10 min without removal from the water bath, to ensure even acid to particle contact and uniform hydrolysis.

After the incubation period, the samples were removed from the water bath and diluted with 84.00 $\pm$ 0.04 ml deionised water using an automatic burette, to a final 4% sulphuric acid concentration. The Teflon caps were securely tightened before mixing the samples by inverting the tube several times to eliminate phase separation between high and low concentration acid layers.

A set of sugar recovery standards (SRS) [D-(+)glucose, D-(+)xylose, -L(+)arabinose, and D-(+)mannose was prepared at 0.1, 1, 2, and 4 mg/ml. The SRS were taken through the dilute (4%) acid hydrolysis step and used to correct for losses due to destruction of sugars during this step. The samples and the SRS were autoclaved at 121ºC for 1 h.

The samples were then slowly cooled at room temperature, filtered under vacuum and divided into acid soluble and acid insoluble residue using previously weighed filter crucibles and Buchner flasks. The acid insoluble lignin (AIL) was retained on the crucibles as part of the insoluble residue. The acid insoluble residue was further dried overnight at 105ºC in a

convection oven, allowed to cool in a desiccator, and weighed. The acid insoluble residue was further ashed in a muffle furnace at 575°C for 48 h. The ash was weighed, and the weight of the acid insoluble lignin was determined by subtracting the weight of ash from the weight of the acid insoluble residue. The acid soluble lignin in the filtrate was measured using a Light-wave II WPA UV-spectrophotometer at 240 nm, blanking with deionised water. The sample was diluted when necessary to bring the absorbance into the range of 0.7-1.0. The amount of acid soluble lignin was determined by using the following formula as described by Sluiter et al,. (2008):

$$\%\ ASL = \frac{UVabs\ x\ Volume_{filtrate}\ x\ Dilution\ x\ 100}{\varepsilon\ x\ ODW_{sample}\ \text{x Pathlength}}$$

Where:

UVabs = average UV-Vis absorbance for the sample at specified wavelength

Pathlength = pathlength of UV-Vis cell in cm

$\text{Volume}_{\text{hydrolysis liquor}}$ = volume of filtrate

$$\text{Dilution} = \frac{Volume_{sample} + Volume_{dilution\ solvent}}{Volume_{sample}}$$

ε = absorptivity constant of biomass at specific wavelength in L/g cm [table of values for different biomass types is provided in the protocol by Sluiter et al., (2008)].

The carbohydrates in the hydrolysis filtrate were neutralised with calcium carbonate to pH 5-6, and then centrifuged twice at 13 000 rpm to remove any small particulates from the aqueous phase. The amount of sugar was determined using an Agilent Technologies 1200 Series HPLC, equipped with a refractive index detector and a Bio-Rad Aminex HPX-87P carbohydrate analysis column (Hercules, CA, USA) with HPLC grade water as a mobile phase at the flow rate of 0.6 ml/min and a column temperature of 55-65°C. Each set of samples was run on the HPLC with standards of glucose, xylose, mannose, and arabinose at 0.1, 1, 2, and 4 mg/ml.

2.2.3 Enzyme saccharification.

The enzymatic saccharification was carried out according to the NREL-LAP (Selig et al., 2008) for pretreated and untreated miscanthus biomass. Samples were pretreated at the same temperatures as in part 2.2.2.2. In labelled sterilin tubes, for each sample, an equivalent of 100 mg of biomass on a 105°C oven dry weight (ODW) basis was mixed with 55 µl of 0.1 M sodium citrate buffer (pH 4.8), 30 µl of tetracycline (10 mg/ml in 70% ethanol), 40 µl of

cyclohexamide (10 mg/ml) (to prevent potential microbial growth), and distilled water to bring the volume to 10 ml. To each mixture, 118 µl of enzyme [1:1 blend of cellulase from *Trichoderma reesei* and β-glucosidase (Novozyme 188) from Sigma] was added. The enzyme loading was according to the specifications outlined by Mosier et al., 2005.

The samples were incubated in a New Brunswick Scientific Innova 4330 incubator shaker at 50^{o}C with shaking at 200 rpm for 7 days. The 7 day incubation period in this procedure allows determination of the maximum extent of digestability due to enzymatic saccharification of cellulose from the native or pretreated lignocellulosic biomass (Selig et al., 2008). After 7 days of incubation, the enzymatic hydrolysis samples were centrifuged for 10 min at 10 000 rpm (x2), using a 5424 Eppendorf centrifuge. 1 ml of each supernatant was transferred into labelled HPLC vials. The soluble glucose and xylose, and enzyme derived glucose and xylose were measured using HPLC as described in part 2.2.2.2 above. Each set of samples was run on the HPLC with glucose and xylose standards of 0.1, 1, 2, and 4 mg/ml. Glucose and xylose yields were expressed as amount of sugar (g)/g of the original ODW of miscanthus biomass and the correction for hydration (water incorporated upon hydrolysis of cellulose to glucose and xylose monomers) was applied (Selig et al., 2008).

2.3 Phosphoric acid swollen cellulose.

Regenerated amorphous cellulose was prepared with help fom Dr. Bartosiak-Jentys, by adding approximately 0.2 g of microcrystalline cellulose (FMC PH-105) to a 50 ml centrifuge tube, and 0.6 ml distilled water was added to wet the cellulose powder to form a suspended slurry. Ten ml of ice-cold 86.2% H_3PO_4 was slowly added to the slurry with vigorous stirring so that the final phosphoric acid concentration was ca. 83.2%. Before the last 2 ml of the phosphoric acid was added, the cellulose was mixed evenly. The cellulose mixture turned transparent within several min, and was left for an h on ice with occasional stirring.

Approximately 40 ml of ice-cold water was added at approximately 10 ml per addition with vigorous stirring between additions, resulting in a white cloudy precipitate. The precipitated cellulose was centrifuged at 5 000 x g and 4^{o}C for 20 min. The pellet was suspended by adding ice-cold water then centrifuged four times, to remove the supernatant containing phosphoric acid. Approximately 0.5 ml of 2 M Na_2CO_3 was added to neutralize the residual phosphoric acid, and then, 45 ml of ice-cold distilled water was used to suspend the cellulose pellet. After centrifugation, the pellet was suspended in distilled water and centrifuged twice or until pH 5-

7. The regenerated (homogeneous) cellulose can be kept at 4ºC for a long time by adding a small amount of sodium azide. The carbohydrate concentration of regenerated amorphous cellulose was calibrated by the phenol-H_2SO_4 method.

2.4 Routine maintenance and growth of bacterial strains.

2.4.1 Sterilisation.

All media, instruments and containers were sterilised by autoclaving at 121ºC/103 Pa for 20 min. Heat labile solutions were filter sterilised through Acrodisc 0.2 μm filters (Gelman Sciences, Northampton, UK). Loops and glass spreaders were sterilised by ethanol and flame.

2.4.2 Antibiotics.

Strains that contained plasmids were maintained on media containing the appropriate selective antibiotic. Stock antibiotic solutions were stored at –20ºC. Kanamycin and ampicillin (Sigma, Dorset, UK) stock concentrations were 12 mg/ml and 50 or 100 mg/ml, respectively. In *Geobacillus*, a concentration of 12 μg/ml kanamycin was required for plasmids coding for kanamycin resistance. In *E. coli*, a concentration of 50 or 100 μg/ml ampicillin was required for plasmids coding for ampicillin resistance. Antibiotics were prepared in autoclaved distilled water and stored at -20°C, for usage as required.

2.4.3 Luria-Bertani (LB) Medium.

LB broth contained 10 g/L bacto-tryptone; 5 g/L bacto-yeast extract; 5 g/L NaCl. The pH was adjusted to pH 7 with 5 M NaOH prior to autoclaving. For solid media 16 g/L bacto-agar was added.

2.4.4 2TY medium.

The 2TY broth was prepared by dissolving 16 g/L Bacto-tryptone, 10 g/L yeast extract and 5 g/L sodium chloride. The pH was adjusted to 7 using 5 M NaOH. To prepare agar plates, 15 g of bacto-agar was added. The medium was autoclaved.

2.4.5 TGP aerobic growth medium.

The first part was made by mixing, Bacto tryptone 17.0 g/L, Soy Peptone 3.0 g/L, NaCl 5.0 g/L, KH_2PO_4 2.5 g/L, and made up to 90% final volume. The resulting solution was corrected

to pH 7.0 with 3M NaOH then autoclaved for sterilization. The second part was made up to 10% final volume, by mixing Sodium Pyruvate 4.0 g/L, Glycerol 4.0 ml/L. The second part was filtered to sterilize and added to the first part.

Bacto-agar (16 g/L) was added to the first part before autoclaving for solid medium.

2.4.6 Modified Ammonium salts medium (ASM).

A litre of modified ammonium salts medium (ASM) contained 0.8 g K_2HPO_4; 0.2 g KH_2PO_4; 0.2 g $MgSO_4.7H_2O$; 0.2 g NaCl; 0.1 g $NaNO_3$; 0.01 g $CaCO_3$; 1 g yeast extract; and 0.15 g $(NH)_2SO_4$. The pH was adjusted to 7 using H_2SO_4.

Solid medium was prepared by adding 15 g of agar. The medium was autoclaved.

2.4.7 Rastogi isolation medium (RIM).

A litre of RIM contained 0.1 g Nitrilotriacetic Acid, 1 ml $FeCl_3$ solution (0.03%), 0.05 g $CaCl_2.2H_2O$, 0.1 g $MgSO_47H_2O$, 0.01 g NaCl, 0.01 g KCl, 0.3 g NH_4Cl, 1.8 g of 85% H_3PO_4, 0.005 g Methionine, 0.05 g Bacto-yeast extract, 0.01 g Casamino Acids, and 1 ml Nitsche's TE solution. One litre of Nitsche's TE Solution contained 2.2 g $MnSO_4$, 0.5 g $ZnSO_4$, 0.5 g H_3BO_3, 0.016 g $CuSO_4$, 0.025 g Na_2MoO_4, and 0.046 g $CoCl_2.6H_2O$. The pH was adjusted to 7 using 5 M NaOH. Solid medium was prepared by adding 15 g of agar. The medium was autoclaved.

To prepare buffered RIM medium, Bis-Tris (8.36 g), Hepes (9.532 g) and Pipes (12.09 g) were added to a liter of RIM medium before autoclaving.

2.4.8 Actinomycetes isolation medium.

One liter of actinomycetes isolation medium contained glycerol (or starch), 10 g; casein (vitamin-free), 0.3 g; KNO_3, 2 g; NaCl, 2 g; K_2HPO_4, 2 g; $MgSO_4.7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4.7H_2O$, 0.01 g; Bacto Agar, 18 g; distilled water 1 liter; pH7 (Kuester and Williams, 1964). The medium was autoclaved. The medium was amended with 0.3g/L of filter sterilized streptomycine sulphate.

2.5 Frozen glycerol stocks.

2.5.1 Preparation of 50% (v/v) glycerol.

The 50% v/v glycerol was prepared by mixing 25 ml of sterile 99.5% glycerol and 25 ml sterile water.

2.5.2 Preparation and storage of stocks.

Stocks were prepared by mixing 300 µl of sterile 50% (v/v) glycerol and 700 µl of the fresh culture and immediately stored in a -80ºC freezer.

2.5.3 Reviving of thermophilic bacterial strains from stock cultures stored at -80ºC.

A loopful of frozen stock culture was inoculated into 10 ml of pre-warmed 2TY in 50 ml falcon tubes. The inoculated culture was well mixed; 100 µl was immediately sampled and spread on pre-warmed 2TY agar plates with a spreader. The remaining culture was incubated at 55ºC with shaking at 250 rpm. Further samples were taken from the broth cultures after every h for 5 h and plated as above. The inoculated plates were incubated at 55ºC overnight and the remaining broth culture left to continue growing overnight at 55ºC with shaking at 250 rpm. Further 2TY plates were inoculated using the overnight broth cultures and incubated like the other plates. Bacterial population was recorded as colony forming units per 100 µl (CFU/100 µl).

2.5.4. Screening for possible factors that could be reviving cells from stock cultures after storing at -80ºC.

A loopful of WL14 stock culture was inoculated in pre-warmed 10 ml of 2TY medium in four 50 ml falcon tubes. Each inoculated culture was well mixed and the four inoculated tubes were then incubated at 55ºC with shaking at 250 rpm for 1, 2, 3 and 4 h. After the incubation period the culture was used to inoculate a pre-warmed 2TY plate by spreading 100 µl of the culture. The remaining culture from each tube was filtered through a sterile 0.2 µm filter. A 100 µl sample of each filtrate was spread on a pre-warmed 2TY plate to confirm that no cells had passed through the filter (control). Each of the remaining filtrate was inoculated with another loopful of WL14 -80ºC stock culture. The inoculated filtrate was well mixed, and then 100 µl

was sampled and spread on a 2TY plate. The remaining culture was incubated at 55ºC with shaking at 250 rpm for 1 h. After incubation, 100 µl of each culture was inoculated on separate plates. All plates were incubated overnight at 55ºC. Colony counts (CFU/100 µl culture) from the plates were recorded.

2.6 Preparation of working stocks.

For thermophilic bacteria, a loopful of frozen glycerol stock was inoculated in pre-warmed 10 ml of 2TY medium in 50 ml falcon tubes. The inoculated tubes were incubated for 1 h or overnight (approximately 16 h) at 55ºC with shaking at 250 rpm. On pre-warmed 2TY plates, (pre-warmed medium plates to avoid temperature shock during revival of the thermophilic strains), 100 µl of the overnight culture was inoculated by spreading using sterile plastic disposable spreaders (it has been found that *Geobacillus* spores can survive in ethanol). The plates were incubated overnight in the 50-60ºC incubator. Plates of working cultures were stored at -4ºC and were discarded after 2 weeks.

2.6.1 Preparation of overnight cultures.

Overnight cultures for thermophilic bacterial strains were inoculated from working culture agar plates and grown aerobically using 10 ml of media in a 50 ml falcon tube with shaking at 250 rpm in the Innova®44 Incubator Series, for approximately 16 h. Overnight cultures were grown at 55ºC in 2TY.

2.7 Microbiological Methods.

2.7.1 Isolation of cellulolytic strains.

The first set of thermophilic bacterial strains used for this study was isolated during a previous study by Prof. Leak's research group from wood compost collected from West London Composting, Uxbridge, Middlesex, and are referred to as West London (WL) strains. The temperature in the compost heap from where they were isolated was 60-70ºC. Compost (500 g) was collected in a plastic bag and sealed tightly.

In the lab, 25 g of compost was suspended in 250 ml of saline solution and incubated at 55ºC for 5 h. The samples were serially diluted with saline and 100 µl aliquots plated on carboxymethylcellulose (CMC) sodium salt (ASM + 0.5% CMC + 0.005% YE) agar and incubated at 55ºC. After 3 days, single colonies were picked and plated on fresh CMC agar

plates and incubated at 55ºC. The experiments for growth of WL strains on pretreated *M. x giganteus* were carried out by inoculating 0.5 ml overnight culture of the strain into 1% pretreated *M. x giganteus* in unbuffered RIM.

The second set of thermophilic bacterial strains was isolated from domestic compost collected from West London Composting, Uxbridge, Middlesex, and were referred to as TR1A strains. The temperature in the compost heaps (Fig. 2.2) is normally between 52-78ºC, while that of the sampled compost heaps was kept at 60ºC for 2 days before sampling. The compost samples were carried in sealed plastic bags. Inoculations were set up by adding 1 g compost in (i) 100 ml RIM + 1 g pre-treated *M. x giganteus* (1% w/v) (ii) 100 ml RIM + 1 g untreated *M. x giganteus*, in 200 ml inoculation bottles for isolations. Two pretreated experiments were set up, (TR1A and TR2A) and a control each for the pretreated *M. x giganteus* and the untreated *M. x giganteus* without inoculation. For the untreated experiments, two experiments were set up (UT1A and UT2A). The inoculation bottles were incubated at 55ºC with shaking at 250 rpm.



Figure 2.2 A domestic compost heap at the sampling site.
The compost samples were collected from West London Composting, Uxbridge, Middlesex.

2.7.1.1 Isolation of microbial colonies from the TR1A subcultures.

The TR1A culture was used for isolation of the new thermophilic strains, based on this culture's good growth on pretreated *M. x giganteus* in RIM. The TR1A was subcultured about every 6 weeks, TR1A subcultures 6 and 8 were used for isolation of individual colonies. Isolation was carried out on agar plates of swollen cellulose medium, 2TY, fine powder of

pretreated *M. x giganteus* in RIM, fine powder of untreated *M. x giganteus* in RIM, agar medium containing xylose, cellobiose, and glucose (0.5% w/v of each), and medium for isolating actinomycetes (Kuester and Williams, 1964; Pine and Watson, 1959).

One ml TR1A subculture (mixture of liquid portion and the solid particles of the pretreated *M. x giganteus*) were serially diluted in 9 ml of sterile 0.85% autoclaved saline water. The 0.85% saline water was prewarmed at 55ºC for 20 min before being used for the serial dilutions. Then 0.1 ml of the serial dilutions 10^{-3}, 10^{-4} and 10^{-5} were inoculated on agar plates for each medium and spread with an L-shaped disposable loop. The plates for actionomycetes were prepared in duplicate.The plates were incubated at 55ºC, one set of the plates for actinomycetes isolation was incubated at 26ºC. All the plates incubated at 55ºC were covered in aluminium foil to prevent drying up of the medium during the incubation. The 2TY, xylan, cellobiose and glucose plates were incubated overnight, while the swollen cellulose plates were incubated for 3 days. The isolation plates of the fine powder of *M. x giganteus* in RIM and those on the actinomycetes isolation medium were incubated for 1 week.

The developed colonies were purified by streaking each colony on an individual plate of fresh medium same as that used for the initial isolation. The purification was done several times to obtain pure colonies.

2.7.1.2 Monitoring microbial growth for *M. x giganteus* cultures by total cell protein.

Due to the presence of particulate material in the flasks, microbial growth was monitored by assaying for an increase in total cell protein of the cultures. The assay was carried out as outlined by Ishida et al., 1997, with modifications, by mixing 0.5 ml of the culture with 0.5 ml of 1 N NaOH. After incubation for 10 min at 95ºC, the mixture was well mixed and cooled at room temperature and allowed to settle. A clean tube was used to mix 0.5 ml of the clarified part of the sample with 0.1ml of 6 N HCL. The protein content was measured by the Bradford Protein Assay (Bio-Rad) following manufacturers protocols, or by using the biuret method if the medium contained buffers.

2.7.2 Determination of growth rates of WL strains on 2TY medium.

For each strain 50 ml of pre-warmed 2TY medium in a 500 ml flask was inoculated with 0.5 ml of an overnight pre-culture in the same medium, which had been inoculated from a freshly

grown colony. The flasks were incubated aerobically at 55°C with shaking at 250 rpm. The $OD_{600\ nm}$ was recorded over a period of 9 h. The growth curves were plotted and the growth rates were determined from the plot of natural log OD readings against time. The gradient of the linear part of the graph was used to record growth rate.

2.8 Centrifugation.

Centrifugations of volumes up to 1.5 ml were carried out using a bench top MSE Micro Centaur centrifuge (MSE scientific instruments, UK) at room temperature and 13 000 x g. Large scale centrifugations (2 to 50 ml) were performed using an Eppendorf 5810-R centrifuge at 4 000 x g.

2.9 Optical density analysis.

Optical density was measured on a Bio-TEK Synergy HT spectrophotometer or a Jasco V-530 UV/Vis spectrophotometer and the data analysed in the spectral manager programme. Optical density was read at 600 nm wavelength and was used as an indication of culture biomass. Samples were appropriately diluted with sterile water to obtain an absorbance reading between 0 and 0.7. All $OD_{600\ nm}$ readings were blanked against fresh media to obtain an accurate representation of absorbance due to cell content.

2.10 HPLC analysis.

High performance liquid chromatography (HPLC) was carried out to analyse the supernatant from TR1A subcultures for residual sugars, organic acids, butanol and ethanol. TR1A subculture (1.5 ml) was centrifuged at 10 000 x g for 10 min and the supernatant was transferred to a glass HPLC vial sealed with a septum and cap. Ten µl of sample was injected into the system and allowed to pass through the column over a 25 minute run time. Glucose, xylose, pyruvate, formate, lactate, acetate, succinate, 2-butanol and ethanol were used as standards, all at a concentration of 10 mM-50 mM.

The HPLC system (Jasco UK, Great Dunmow, UK) had a PU2080 plus pump, AS-2051 plus intelligent sampler, a UV-2075 plus UV/Vis spectrophotometer (set at 210 nm), a RI-2031 plus refractive index (RI) detector and a column thermostat jetstream plus oven which was maintained at 35°C. The pump used for separation was an Aminex HPX87H ion exclusion column (300 x 7.8mm) (BioRad, Hemel Hempstead, UK). Ten mM sulphuric acid at a flow

rate of 0.6 ml/min was used as the mobile phase. The data was relayed by detectors through an LCnetII ADC adaptor to the Chrompass data acquisition software which made chromatogram visualisation and automatic peak area integration possible.

2.11 Protein methods and enzyme assay protocols.

2.11.1 Preparation of 0.1% Congo red.

The 0.1% Congo red solution was prepared by dissolving 0.5 g of Congo red powder (Sigma) into 500 ml of distilled water.

2.11.2 Checking for cellulase activity on salts medium containing CMC, cellobiose and avicel.

Agar plates were prepared as follows: 1. ASM (control), 2. ASM + 0.5% CMC medium, 3. ASM + 0.5% avicel (a type of microcrystalline cellulose which has been purified and partially depolymerised) medium, and 4. ASM + 0.5% cellobiose medium.

Bacterial strains were inoculated on the plates containing different carbon sources and on the control plate. The plates were incubated in a 55^{o}C incubator for 24 h, then flooded with Congo red solution (0.1%) and left to stand at room temperature for 1 h. The Congo red was poured off and the plates were subsequently flooded with 1 M NaCl. After 5 min this was poured off and then flooding with 1 M NaCl (this step was repeated three times).

2.12 Determination of enzyme specific activity.

2.12.1 Reagents.

2.12.1.1 Preparation of 3,5-dinitrosalicylic acid (DNS) solution.

The DNS solution was prepared by dissolving 16 g of Sodium Hydroxide pellets in 50 ml of dH_2O and allowed to cool. In a separate beaker on a magnetic stirring platform, into 800 ml dH_2O at 35 (± 2) ˚C, 10 g 3,5-Dinitrosalicylic acid was added, 300 g of Potassium Sodium (+) – tartarate was gradually added until all solids dissolved. The NaOH solution was slowly added. The solution was made up to 1 L.

The aim was to get as pale a yellow solution as possible. This makes the DNS solution more sensitive to very low concentrations of reducing sugar. If the solution is orange it still works

but will be unable to detect low sugar concentrations. The solution can be stored at ambient temperature for up to 10 weeks.

2.12.1.2 Preparation of 2.5% w/v Carboxymethyl Cellulose (CMC).

CMC was dissolved in a 2.5% w/v solution in dH_2O. Citrate buffer and Acetate buffer (pH 4.8) in place of distilled water are both compatible with the assay. The water was heated to 80˚C prior to adding CMC and then incubated the solution in the 55˚C orbital shaker until all CMC had dissolved.

2.12.1.3 Avicel or xylan.

Avicel or xylan (0.01 g) was added to the assay tube and added 0.1 ml of water.

2.12.1.4 Lactose solution.

Lactose monohydrate (0.12 g) was dissolved in distilled water and made up to 1 L.

2.12.2 Enzyme assay.

The assay mixture for detecting enzyme activity was modified from Miller et al., (1960), and contained 100 µl of 2.5% CMC in distilled water, (or the avicel/the xylan and distilled water mixture preparations) and 100 µl of culture supernatant. A control for each substrate was prepared with no enzyme (100 µl dH_2O instead of culture supernatant was added) and incubate in the same way.

CMC assays can be incubated in a static oven. Avicel and xylan assays must be shaken at 250 rpm during incubation in order to keep the avicel or xylan in suspension. The reaction mixtures were incubated at 55ºC for 1 h.

Glucose standards of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.8, 1, 2, 4, 6, 8, 10 mM glucose were prepared.

Fresh DNS-Lactose Reaction Stop solution was prepared each time the assay was performed by mixing 3:1 DNS Solution:Lactose Solution. Each assay required 400 µl of the DNS-Lactose solution.

Standards: To a clean microcentrifuge tube 100 µl dH_2O and 100 µl of sugar standard were added. To this mixture 400 µl of DNS-Lactose Reaction Stop solution was added.

Samples and controls: To a clean microcentrifuge tube 200 µl of the incubated sample and 400 µl of DNS-Lactose Reaction Stop solution were added immediately after incubation.

The tubes (samples, controls and standards) were incubated in the heating block for 20 min, ensuring the temperature was above 90˚C at all times.

The samples were allowed to cool and, in triplicate, 150 µl was pipetted into a 96 well plate. The plate was read at 540 nm. The standard curve was used to work out the amount of reducing sugar liberated from each sample.

The time of incubation, the µM of sugar released and the protein concentration in the cellulase sample measured by BioRad DC assay (or Biuret protein assay for supernatants from cultures grown in buffered RIM medium) were used to work out the µmoles sugar released /min/mg protein.

2.13 Protein concentration by filtration.

The protein in the culture supernatants of strains was concentrated using a bench top accuSpin^{TM} Micro centrifuge (Fisher Scientific) at 10 000 x g for 15 minutes using a 3 000 MWCO PES Vivaspin centrifugal membrane separator from Sartoriuos Stedim Biotech.

2.14 Protein assay.

The Bio-Rad D_C protein assay method based on the Bradford method (Bradford, 1976) was used to determine protein concentrations as specified by the manufacturer's instructions. A standard curve was prepared using lysozyme from chicken egg-white (Sigma) at concentrations 0, 0.2, 0.4, 0.6, 0.8, 1 and 1.5 mg/ml which was used for calculating the experimental protein concentrations.

For protein concentration analysis for supernatants from cultures grown in buffered RIM medium Biuret reagent test method modified from (Guobing et al., 2001) was used for determining protein concentration. The Biuret reagent consisted of 2.25 g sodium potassium tartrate, 0.75 g copper sulfate x $5H_2O$, and 1.25 g potassium iodide. All these were dissolved in order in 100 ml 0.2 M NaOH (0.8 g/100 ml) and the volume brought to 250 ml with distilled water. The solution should not be used if black precipitate forms.

2. 15 SDS PAGE.

2.15.1 Preparation of gels.

The 12% resolving gel (5 ml) was prepared by first mixing 1.9 ml H_2O, 1.3 ml Tris-HCl (1.5 M, pH8.8), and 0.05 ml SDS (10%), and then adding 1.7 ml 30% acrylamide mix (ProtoGel^{TM}), 0.05 ml 10% ammonium persulfate and 0.002 ml TEMED (Fisher Scientific) in that order. The mixture was promptly pipetted into the assembled gel plates evenly from side to side up to about 1 cm from the marked edge of the gel comb. The gel was immediately covered by a thin layer of isobutanol to even the surface, and allowed to dry (5-30 min). The isobutanol was poured off from the resolving gel and the surface was blotted dry using paper roll.

The stacking gel (2 ml) was then prepared by mixing 1.4 ml H_2O, 0.25 ml Tris-HCl (1.5 M, pH 6.8) and 0.02 ml SDS (10%), and then adding 0.33 ml 30% acrylamide mix, 0.02 ml 10% ammonium persulphate and 0.002 ml TEMED in that order. The mixed components of the stacking gel were promptly pipette into the assembled gel plates on top of the resolving gel evenly from side to side. The plates were filled with stacking gel such that it overflew upon addition of the comb. The comb was inserted between the gel plates carefully to avoid bubbles from persisting. The stacking gel was allowed to solidify (5-30 min).

2.15.2 Preparation of coomassie blue stain.

A litre of coomassie blue staining solution contained 50% (v/v) ethanol, 10% (v/v) acetic acid and 0.2% coomassie blue.

2.15.3 Coomassie blue destainer.

Coomassie blue destainer contained 10% (v/v) acetic acid and 30% (v/v) ethanol.

2.15.4 Running buffer.

A litre of 10x running buffer contained 30 g Tris-base, 144 g glycine and 10 g SDS. No SDS was added for running buffer to be used for native PAGE gels.

2.15.5 Running SDS PAGE gels.

The samples were prepared by adding 12 µl loading buffer [2.5 ml 1M Tris-HCl (pH 6.8 at 25°C), 1 g SDS, 6 µg bromophenol blue, 5 ml glycerol + 100 µl 1M DTT per 10 ml] to 28 µl

of the sample to a 1.5 ml centrifuge tubes. To another 1.5 ml centrifuge tube, 7 µl of unstained protein molecular weight marker (from Fermentas) was added. The tubes were boiled at 100ºC for 10 min. The samples and protein marker were loaded into separate wells of an SDS-PAGE gel and electrophoresis was run at 120v power supply from a BioRad power tank by using BioRad Gel Electrophoresis tanks (BioRad, UK).

The gel was stained with coomassie blue staining solution overnight and destained using coomassie blue destainer. The bands were viewed on a short wave UV transilluminator, and photographed on a UV transilluminator BioDoc-it system with an attached analogue thermal printer. Mixtures for PAGE gels for analysing enzyme activity contained 0.05% CMC and were stained as described in section 2.16.2.

2.16 Preparation of native PAGE gels.

The 12% resolving gel (15 ml) was prepared by first mixing 5.05 ml H_2O, [0.05% CMC or xylan (depending on the enzyme activity to be analysed) was added to the water and dissolved or well mixed by incubating at 55ºC, and then cooled], and 3.8 ml Tris-HCl (1.5 M, pH 8.8), and then adding 6 ml 30% acrylamide mix (ProtoGel™), 0.15 ml 10% ammonium persulfate and 0.006 ml TEMED (Fisher Scientific) in that order. The mixture was promptly pipetted into the assembled gel plates evenly from side to side up to about 1 cm from the marked edge of the gel comb. The gels were immediately covered by a thin layer of isobutanol to even the surface, and allowed to dry (5-30 min). The isobutanol was poured off from the resolving gel and the surface was blotted dry using paper roll.

The stacking gel (5 ml) was then prepared by mixing 3.45 ml H_2O, 0.63 ml Tris-HCl (1.5 M, pH 6.8), and then adding 0.83 ml 30% acrylamide mix, 0.05 ml 10% ammonium persulphate and 0.005 ml TEMED in that order. The mixed components of the stacking gel were promptly pipetted into the assembled gel plates on top of each resolving gel as described in section 2.15.1 and was allowed to solidify (5-30 min).

2.16.1 Sample preparation and loading.

The harvested cultures were centrifuged at 4 000 x g at 20ºC for 20 minutes in an Eppendorf 5810-R. The collected supernatant (0.5 ml) was concentrated by centrifuging in a bench top accuSpin™ Micro centrifuge (Fisher Scientific) at 10 000 x g for 15 minutes using a 3 000 MWCO PES vivaspin centrifugal membrane separator from Sartoriuos stedim biotech. The

samples were prepared by adding 12 µl loading buffer [2.5ml 1M Tris-HCl (pH 6.8 at 25°C), 6 µg bromophenol blue, 5 ml glycerol + 100 µl 1M DTT per 10 ml] to 28 µl of the sample to a 1.5 ml centrifuge tubes. The samples were loaded into separate wells of duplicate native gels. Electrophoresis was run in a cold room (4^{o}C) at 70 v until all the dye moved out of the gel.

2.16.2 Staining of native PAGE gels.

One of the gels was stained with coomassie blue staining solution overnight and destained using coomassie blue destainer. The second gel was first soaked in 2.5% v/v Triton X-100 on a shaker at room temperature for 1 h. Then it was washed with Milli Q water, and incubated in 50 mM citrate buffer pH 5.5 for 1 h at room temperature. The buffer was drained out and the gel was stained with 0.1% Congo red stain for 15 min at room temperature. The gel was destained with 1 M NaCl until the clear bands were clearly visible, usually between 1 and 2 h, or sometimes in less than 1 h. The bands were viewed on a short wave UV transilluminator, and photographed on a UV transilluminator BioDoc-it system with an attached analogue thermal printer or viewed under light and photographed using a digital camera.

2.17 Mass spectrometry analysis.

2.17.1 In gel digestion of proteins.

2.17.1.1 Excision of bands/spots.

The gel picture was highlighted on the approximate area to be excised for each slice and the eppendorf tubes were carefully labelled. The gel was placed onto a rigorously cleaned glass plate for cutting. Round N0. 22 scapel blades were used for excising the band as they allowed a rocking motion as opposed to a slicing motion, decreasing the chance of ripping the gel. The excised gel band was sliced into about 1 mm cubes and placed into a fresh Eppendorf tube. The cut gel pieces were collected with the help of a 20 µl pipette tip. A fresh scapel blade, Eppendorf and tip were used for every band, and each band was cut on a clean region of the glass.

2.17.1.2 Preparation for reduction carboxymethylation.

The gel was prepared either by using the ProteaseMAX™ Surfactant, Trypsin Enhancer (Promega), following the instructions provided by the manufacturer, or by following the method outlined below.

2.17.1.3 Gel Preparation/removal of gel stain.

Ambic (50 mM), pH 8.4 (50 ml) was prepared by dissolving 0.198 g ultra-pure ambic in 40 ml ddH_2O, the pH was adjusted to pH 8.4 with 20% ammonia (diluted 1:1 with ddH_2O), then topped up to 50 ml.

To the gel pieces, 200 µl 50 mM Ambic pH 4.8 followed by 200 µl of MeCN was added and incubated at room temperature for 5 min. The tube was well mixed to disperse the concentration of stain from the bottom of the eppendorf. The supernatant was carefully removed using a 200 µl pipette. These 3 steps were repeated until no colour remained in the gel pieces. The pieces were dried by washing twice with 200 µl MeCN. The tube was warmed at 56^{o}C to help drying. Then the MeCN was carefully removed using a 200 µl pipette.

2.17.1.4 Reduction/carboxymethylation.

The gel pieces were swelled in 10 mM dithiothreitol (DTT) dissolved in 50 mM ambic pH 8.4 (10 mM DTT: 15.42 mg in 10 ml of ambic pH 8.4). Enough DTT solution was added to completely cover gel pieces (~200 µl). The tube was incubated at 56°C for 30 min. The DTT solution was removed and the gel pieces were washed briefly (10-15 secs) with MeCN (~200 µl). The MeCN was removed using a 200 µl pipette.

The pieces were re-swelled in ~200 µl of 55 mM (102.3 mg in 10 ml ambic pH 8.4) iodoacetic acid dissolved in 50 mM ambic pH 8.4 (stored in the dark), and incubated at room temp in the dark for 30 min. The iodoacetic acid solution was removed and the gel pieces were washed with 500 µl of 50 mM ambic pH 8.4 for 15 min. Then ambic solution was removed and the gel pieces were shrunk with 200 µl MeCN for 5 min. The gel pieces were dried by washing twice with 200 µl MeCN and warmed at 56°C to help drying. The MeCN was carefully removed using a 200 µl pipette.

2.17.1.5 Tryptic digestion.

Sequencing grade modified trypsin (Promega cat V5111) 20 µg (kept in freezer) was used for tryptic digestion. Each vial was reconstituted with 20 µl of the acidic trypsin buffer provided (1 µg/µl). The reconstituted trypsin was aliquoted in 5 µl (in 0.5 ml tubes) and frozen. To make a working solution of trypsin, 5 µl was diluted with 195 µl of 50 mM ambic pH 8.4 (25 ng/µl). The gel pieces were re-swelled in 20 µl of working solution (0.5 µg trypsin) and incubated at RT for 15 min. Further ambic was added to cover the gel pieces, if necessary (~20-50 µl). The gel pieces were then incubated at 37°C overnight in a water bath.

2.17.1.6 Elution of peptides from the gel pieces.

Preparation.

The preparation of the 0.1% Trifluoroacetic acid (TFA) (50 ml) was carried out by mixing 20 ml ddH_2O + 50 µl 100% TFA, then topped up to 50 ml with ddH_2O.

The supernatant (ambic containing hydrophilic peptides) was removed from the gel pieces, and placed in a clean, labelled Eppendorf by using a 200 µl pipette tip carefully to reduce the chance of accidentally drawing up pieces of gel. About 50 µl of 0.1% TFA was added to the gel pieces (to halt the digestion) and incubated at 37°C for 10 min. Two volumes (~100µl) MeCN were added and incubated at 37°C for 15 min. The supernatant was removed and pooled with the hydrophilic peptides in the previous supernatant. The steps involving the addition of the 0.1% TFA and MeCN were repeated once.

The pooled supernatant for each sample was frozen on dry ice for 5 min. When drying the sample the tubes were covered with perforated lids prepared by cutting off the lid from the top of a clean Eppendorf and punching 8-10 holes in it using a clean hypodermic needle.

The volume was reduced to 10-30 µl using a SpeedVac vacuum centrifuge (~90-120 min), with regular checking as some samples evaporated faster and dried. The perforated lid was replaced with a non-perforated lid for each Eppendorf tube, and the samples were stored at -20°C. If the sample dried up, 30 µl of 0.1% TFA was added to the bottom of the tube and kept in the fridge for 30 min before the sample was stored at -20°C.

2.17.1.7 Determination of the enzyme sequence.

The digested eluted protein was given to a facility in Biochemistry Department for sample analysis by LC-mass spectrometry, where the sample was run initially on MALDI MS/MS (and nanoLC MS/MS if required). The derived partial peptide sequences were searched against protein databases.

2.18 Molecular biology methods.

2.18.1 Agarose gel preparation.

Agarose gels (0.7% w/v) were used to separate PCR products. Appropriate amounts of agarose were weighed and dissolved in 1x TAE (made by diluting 50x TAE buffer of composition 242 g Tris Base, 57.1 ml Glacial acetic acid and 100 ml of 0.5 M EDTA, pH 8, 50-fold) by heating in a microwave oven (850 watts) until all the agarose had dissolved. The solution was then cooled at room temperature to about 40^{o}C and 5 µl of SYBR safe was added per 100 ml gel. The gel was poured into a mould and allowed to solidify. DNA samples were mixed with 10x gel loading buffer and loaded onto the gel alongside 5 µl Bioline hyperladder molecular marker. DNA fragments were visualised on a short wave UV transilluminator, and photographed on a UV transilluminator BioDoc-it system with an attached analogue thermal printer.

2.18.2 Isolation of Genomic DNA.

The SV genomic DNA purification system (from Promega) was used to isolate genomic DNA following the protocol provided by the manufacturer.

2.18.2.1 DNA extraction from compost sample cultures.

The DNA from compost cultures growing on 1% pretreated miscanthus + RIM, was extracted. Two methods were used for isolating DNA.

1. The first method used for extracting DNA followed a modified procedure from Yang et al., (2007) and required purification of the extracted DNA before analysis. The method was carried out following the steps below.

(a) Sample treatment

The compost cultures were centrifuged for 20 min at 4 000 x g at 25ºC. The supernatant was discarded, and approximately 1 g of compost culture pellet (x3) was weighed into 10 ml centrifuge tubes. For each 1 g, 4 ml phosphate-buffer (0.12 mol l^{-1}, pH 8) was added to wash the pellet. The tube was shaken for 5 min on an orbital shaker at room temperature at 150 rpm. The contents were then centrifuged at 4 000 x g for 10 min at 4ºC. Washing of the pellet was done only once.

(b) DNA extraction

To lyse the cells in the washed compost culture samples, three washed samples (approximately 1 g each) were mixed with 1.5 ml extraction buffer (0.1 mol l^{-1} Tris-Cl, 0.1 mol l^{-1} EDTA, 0.1 mol l^{-1} , 0.1 mol l^{-1} sodium phosphate,1.5 mol l^{-1} NaCl, 1% cetyltrimethylammonium bromide, pH 8) and 10 µl Proteinase K solution. This mixture was shaken at 37°C on an orbital shaker for 30 min at 225 rpm. After shaking, 200 µl of 10% SDS was added into the tubes which were then incubated at 65°C in a water bath for 1 h with agitation at 15 to 20 min intervals. The tubes were then centrifuged at 4 000 x g for 5 min at room temperature and the upper layers transferred to fresh tubes. Extraction buffer (0.5 ml) and 50 µl of 10% SDS were added to the residue in the primary tubes to wash the pellets and were mixed using a spinmix (Gallenkamp) for 30 seconds. The mixture was incubated in a 65°C water bath for 10 min, and then centrifuged at 4 000 x g for 5 min at room temperature. The pellets were treated once again.

The upper layers were mixed with 1x volume of chloroform/isoamyl alcohol (24:1) by shaking gently by hand. The aqueous layers were transferred to clean tubes after centrifugation at 4 000 x g for 5 min and the contents precipitated with 0.6 volumes of isopropanol for 1 h. The crude DNA pellets were washed twice with 0.7 ml ice-cold 70% ethanol and dried under vacuum after centrifugation at 16 000 x g for 5 min at 4°C. The crude DNA was dissolved in 600 µl TE buffer (10mM of Tris-HCL pH 7.5 and 1 mM of EDTA) and stored at -20°C for future use.

(c) DNA Purification

The crude DNA was precipitated by adding 0.5 volume of 50% (w/v) PEG 8 000 and 0.1 volume of 5 mol l^{-1} NaCl. The samples were mixed by inverting gently and incubated for more than 1 h or overnight at 4°C. The precipitated DNA was added into a spin-bind DNA

purification cartridge (in which the DNA binds to a microporous membrane in the presence of chaotropic salts buffer) and the cartridge centrifuged for 1 min at 12 000 x g at 4°C. The cartridge was washed twice with 0.7 ml ice-cold 70% ethanol then the DNA eluted from the air dried cartridge with 200 µl hot TE buffer (65^{o}C). Ten µl RNase A (10 mg ml^{-1}) was added and incubated at 37^{o}C for at least 2 h. The DNA was stored at -20^{o}C for future use.

2. The second method for DNA isolation was carried out by using the Zymo Research (ZR) Soil Microbe DNA MiniPrep kit by following the instructions as described by the manufacturer. This product was better than the procedure described above, as it is suitable for isolating DNA for a range of microorganisms including bacteria, algae, protozoa and fungi. The whole procedure could be done within minutes and the resulting DNA is pure and ready to use for analysis.

2.18.3 DNA fragment purification.

After separating the DNA, the band of interest was excised from the agarose gel under ultra violet (UV) light (making sure that the DNA was not exposed to UV light for too long to avoid DNA damage). The DNA was extracted from the gel and purified by using a Qiagen gel extraction kit following the microcentrifuge method instructions given by the manufacturer. The purified DNA was stored in a freezer at -20^{o}C.

2.18.4 DNA Quantification.

DNA quantification was carried out by measuring the absorbance of samples at 260/280 nm. This was done by adding 2 µl of DNA sample to 18 µl of distilled water and loaded into a 384 UV/Vis multiwell plate (Corning). The absorbance was read at 260 nm and 280 nm on a Synergy HT multi-detection microplate reader.

2.18.5 Polymerase Chain Reaction (PCR).

The sequences of primers used for PCR are displayed in the table 2.2. Sterile 0.5 ml PCR tubes (Scientific Specialities Inc) were used to carry out PCR reactions for 16S rRNA gene for bacteria (primers 27F and 1492R), and actinomycetes (primers F243 and R513GC), using a thermal cycler (Eppendorf mastercycle gradient) and a total volume of 50 µl (made up of neutral water (nH_2O), 10 µl of HF buffer, 1 µl of 10 mM dNTPs, 1 µl of forward primer (10

pmol/µl), 1 µl of reverse primer (10 pmol/µl), 5 µl of template, 1.5 µl of DMSO (3%) and 0.5 µl Phusion hot start DNA polymerase (2 U/µl).

For analytical and routine PCR, *Taq* based Biomix red solution (Bioline) was used. When the amplified DNA was to be used for cloning, Expand hi-fidelity polymerase was used (Roche-diagnostics), which contains a Taq polymerase with a proof-reading enzyme. PCR reactions were carried out with an initial denaturation for 2 min at 94^{o}C followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 52^{o}C (or at temperature 5^{o}C less than the melting temperature of the primers) for 1 min, extension at 72^{o}C for 2 min and a final extension at 72^{o}C for 5 min. Samples were then held at 20^{o}C until processing.

The reaction mixture for amplifying protein sequences for routine 50 µl reaction *Taq* PCR consisted of 10x *Taq* reaction buffer (5 µl), 10 mM dNTPs (1 µl), 10 µM forward primer (1 µl), 10 µM reverse primer (1 µl), variable template DNA volume, *Taq* DNA polymerase (0.25 µl) and nuclease-free water was added to make up to 50 µl. The cycling conditions for *Taq* PCR were carried out with an initial denaturation for 30 s at 95^{o}C followed by 30 cycles of denaturation at 95^{o}C (15-30 s), annealing at 45-68^{o}C (15-60 s) and 68^{o}C (1 min), final extension at 68^{o}C for 5 min. The reaction was held at 4-10^{o}C.

2.18.6 DNA Ligation.

The Fermentas CloneJET (pJET1.2/blunt) vector was used for molecular cloning of the 16S rDNA PCR products. DNA fragments were ligated using T4 DNA Ligase (Promega, Southampton, UK). The molar ratio of plasmid to insert was typically 3:1 for cohesive end ligation. The ligation reaction mixture contained plasmid /insert mix, 10 µl of 2x buffer, 1 µl of T_4 DNA ligase, and made up to a volume of 20 µl with nuclease free water. The ligation mixture was incubated overnight at 4^{o}C.

2.18.7 Preparation of chemically competent *E. coli* cells.

Chemically competent *E. coli* cells were made using the method described by (Chung et al., 1989). *E. coli* strains were grown in 20 ml LB broth to an $OD_{600\,nm}$ = 0.4. Cells were harvested by centrifugation at 10 000 x *g* for 10 min at 4°C and resuspended in 2 ml ice cold transformation and storage solution (TSS) [LB broth with 10% (wt/vol) polyethylene glycol (PEG, molecular weight 3 350 or 8 000), 5% (vol/vol) dimethylsulfoxide DMSO, and 50 mM

Mg^{2+} ($MgSO_4$ or $MgCl_2$), at a final pH of 6.5]. Aliquots (50 µl) of chemically competent cells were transferred to 1.5ml microcentrifuge tubes and stored at -80°C.

2.18.7.1 Transformation.

To carry out transformation, 50 µl of *E.coli* (JM109) competent cells (from Invitrogen or promega) were thawed on ice for 10 min. Keeping the ligation mixture on ice, 2 µl of the ligation mixture was added slowly, well mixed using the pipette tip, and incubated on ice for another 20 min. The cells were then heat shocked at 42ºC for 90 s using a heating block then the mixture returned to ice for 2 min. Subsequently 700 µl of 2TY was added to the mixture and the cells incubated at 37ºC with shaking at 250 rpm for 1 h. The culture was spun at 800 x g for 1 min. About 550 µl of the supernatant was removed and discarded. The cells were gently mixed in the remaining supernatant by pipetting up and down; 200 µl was plated on an LB + Ampicillin plate which had been pre-warmed at 37ºC for 20 min. The plates were then incubated overnight at 37ºC.

Table 2.2 Sequences of primers used for PCR.
The oligonucleotide primers for amplification were from Invitrogen, pJET1.2 forward and pJET1.2 reverse were from Fermentas.

Primer	Sequence (5' → 3')
27F	GAGAGTTTGATCCTGGCTCAG
1492R	GGTTACCTTGTTACGACTT
pJET1.2 forward	CGACTCACTATAGGGAGAGCGGC
pJET 1.2 reverse	AAGAACATCGATTTTCCATGGCAG
F243	GGATGAGCCCGCGGCCTA
R513GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGCGGCCGCGGCTGCTGGCACGTA
ENDO-H_F	CATCCCGTTGGGAGACCATC
ENDO-H-R	CGGCACCTTCGTACTGGACT
Endo F1	ATGTTCACTCCGGTTCGCAG
Endo R1	CCGACGCCCCTACGGCGTCC
Endo F2	CACTCCGGTTCGCAGAAGGG
Endo R2	CTACGGCGTCCGGACCGCCT
Csac0137FWD	GTTATTGTTGCCTATAGGAGTGAATGACACATCTGCTGCAAGGCCTTTTTTTGAAGACGATTTTAAG
Csac0137RVR	aatttcacacaggaaacagctatgacatgattacgaattcGAGCTCGCAAAAAAACGCCCCTTTCGGGGCGCGActaaaatctcatca aaagct

2.18.8 Preparation of electrocompetent *Geobacillus* strains.

The *Geobacillus* cells were grown at 55°C overnight on pre-warmed TGP agar plates. One colony from the overnight plate was inoculated into 50 ml of pre-warmed TGP medium. The culture was incubated at 55°C. The culture $OD_{600\ nm}$ was monitored to approximately 1.4. The culture was cooled on ice for 10 minutes and the cells were harvested by centrifugation for 20 min at 9 000 x g at 4°C.

The pellet of the cells was re-suspended in 20 ml of ice cold, sterile, electroporation buffer (0.5 M sorbitol, 0.5 M mannitol and 10% v/v glycerol) and centrifuged at 9 000 x g for 20 min at 4ºC to pellet the cells. The washing step was repeated with 1x10 ml and 2x5 ml ice cold electroporation buffer, and centrifuged at 9 000 x g for 20 min. After the final wash the pellets were put on ice. Each pellet was re-suspended in 875 µl ice cold electroporation buffer. The

competent cells were divided into 60 µl aliquots in pre-chilled 1.5 ml microtubes and stored at -80ºC.

2.18.8.1 Electroporation of electrocompetent *Geobacillus* strains.

One 60 µl aliquot of electrocompetent cells from the -80ºC was thawed on ice and mixed with 1 µl of plasmid DNA, gently by using the pipette tip. The mixture was transferred to a 1 mm gapped ice cold electroporation cuvette (Yorkshire Biosciences, York, UK) and incubated on ice for 5 minutes. This was followed by a single exponential pulse (10 µF, 2500 V and 600Ω) applied using the Xcell gene pulser (Bio-Rad, Hemel Hempstead, UK).

Part of the 1 ml pre-warmed (52°C) TGP broth was added to the cuvette and mixed gently by pipetting up and down then transferred the electroporation mixture to the rest of the pre-warmed TGP. The cells were incubated at 52°C and 250 rpm shaking for 1 h. After 1 h incubation the suspension was centrifuged at 8 000 rpm for 2 min. About 700 µl of the supernatant was pipetted out. The remaining supernatant was mixed with the pellet and the mixture was plated out on pre-warmed TGP agar plates containing the appropriate selective antibiotic.

2.18.9 Colony PCR.

The colony PCR reaction mixture for each reaction contained H_2O (8 µl), primer (10 µM/µl) pJET1.2 F(5'-CGACTCACTATAGGGAGAGCGGC-3'), (1 µl), primer (10 µM/µl) pJET1.2 R(5'-AAGAACATCGATTTTCCATGGCAG-3'), (1 µl), (from Fermentas) and 10 µl of Biomix Red (2x). Samples from the selected transformed colonies from cloning were introduced by touching the colony with a pipette tip and mixing with the reaction mixture in separate PCR tubes. A PCR was run using *Taq* based Biomix red solution conditions, using an annealing temperature of 52ºC, for 30 cycles. The colony PCR products were then analysed on a 0.7% agarose gel.

2.18.10 Isolation of plasmid DNA.

The selected transformants were grown by transferring a portion of each colony into pre-warmed 10 ml LB + medium containing appropriate antibiotic and incubating overnight at 37ºC or 55ºC, for *E.coli* and *Geobacillus* strains, respectively, with shaking at 250 rpm. Plasmids were extracted from the cells using QIA prep spin Miniprep kits (from Qiagen) using

the microcentrifuge method as specified in the manufacturer's protocol. The samples were run on a 0.7% agarose gel.

2.18.11 Plasmid digestion.

The following digestion mixture was prepared for each isolated plasmid: 8 µl plasmid; 1 µl of 10x Buffer H; 0.5 µl of appropriate restriction enzyme (restriction enzymes were obtained from New England Biolabs (Hertfordshire, UK) or Promega (Southampton, UK). Digests were performed for 4 h or overnight at appropriate temperature, using the supplied buffers and 0.5 µl of 3% bovine serum albumin (BSA) when required. After incubation, the products were analysed on a 0.7% agarose gel.

2.18.12 Preparing DNA for sequencing.

Samples of plasmid were diluted to give 100 ng in a final volume of 15 µl, of nH_2O + 1.5 µl of sequencing primers (10 µM/µl) pJET1.2 F(5'-CGACTCACTATAGGGAGAGCGGC-3') /(10 µM/µl) pJET1.2 R(5'-AAGAACATCGATTTTCCATGGCAG-3'). Two reactions were set up for each transformant, one with a forward primer and another one with reverse primer. The reaction mixtures were sent for DNA sequencing to Eurofins Genomics.

3. Pretreatment of *M. x giganteus.*

The recalcitrance of lignocellulosic biomass to enzyme deconstruction is considered to be the major obstacle to production of sustainable energy and other important chemicals. The biomass has to be pretreated to overcome the recalcitrance caused by the presence of lignin which embeds the sugar polymers, preventing their access to the degrading enzymes. Grass pretreatment has been reported to improve sugar yields to more than 90% theoretical yield (Brodeur et al., 2011).

The *M. x giganteus* was pretreated by soaking in aqueous ammonia (10% w/w). Alkaline pretreatment is gentle on biomass. Brodeur et al., (2011) indicated that alkaline pretreatment causes deconstruction of ester and glycosdic side chains causing alteration to the lignin structure, partial decrystallization and swelling of cellulose, as well as partial dissolution of hemicellulose (McIntosh and Vancov, 2010; Sills and Gossett, 2011). The composistional analysis of the pretreated *M. x giaganteus* was carried out to assess the effectiveness of pretreating the biomass by SAA method and to check its suitability for use in consolidated bioprocessing.

Figure 3.1 shows *M. x giganteus* preteated by soaking in 10% (w/w) aqueous ammonia (SAA) for 14 h. The samples pretreated at temperatures over the range 100ºC to 200ºC were compared with the untreated (UT) *M. x giganteus*.

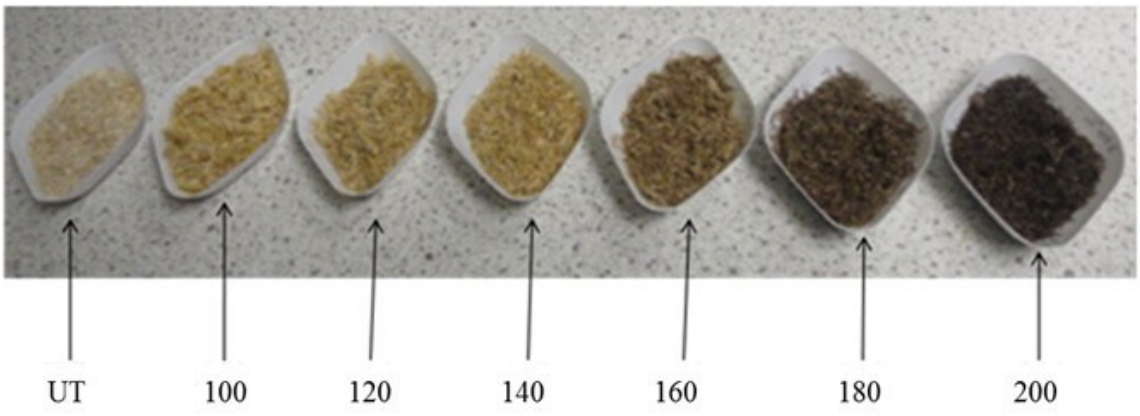


Figure 3.1 Pretreated and untreated *M. x giganteus*.
Dried, milled and sieved (through mesh No. 20 (850 µm) and No. 80 (180 µm) using a sieving shaker). The sieves were stack in the following order, starting at the bottom: a solid catch pan, 80- mesh sieve, and then 20-mesh sieve. The milled *M. x giganteus* was placed in the 20-mesh sieve. The covered sieve stack was secured in the sieve shaker and shaken for 15 min. The milled *M. x gianteus* that remained in the 80-mesh sieve was pretreated by SAA at 100ºC, 120ºC, 140ºC, 160ºC, 180ºC and 200ºC for 14 h, washed to neutrality and then dried.

The colour intensity of the pretreated material increased with increase in pretreatment temperature, an indication that the biomass might have been getting charred at high temperatures.

3.1 Compositional analysis of pretreated and untreated *M. x giganteus*.

The composition of pretreated and untreated *M. x giganteus* was analysed using standard NREL protocols (Sluiter et al., 2008) in order to determine the effectiveness of the alkaline pretreatment process.

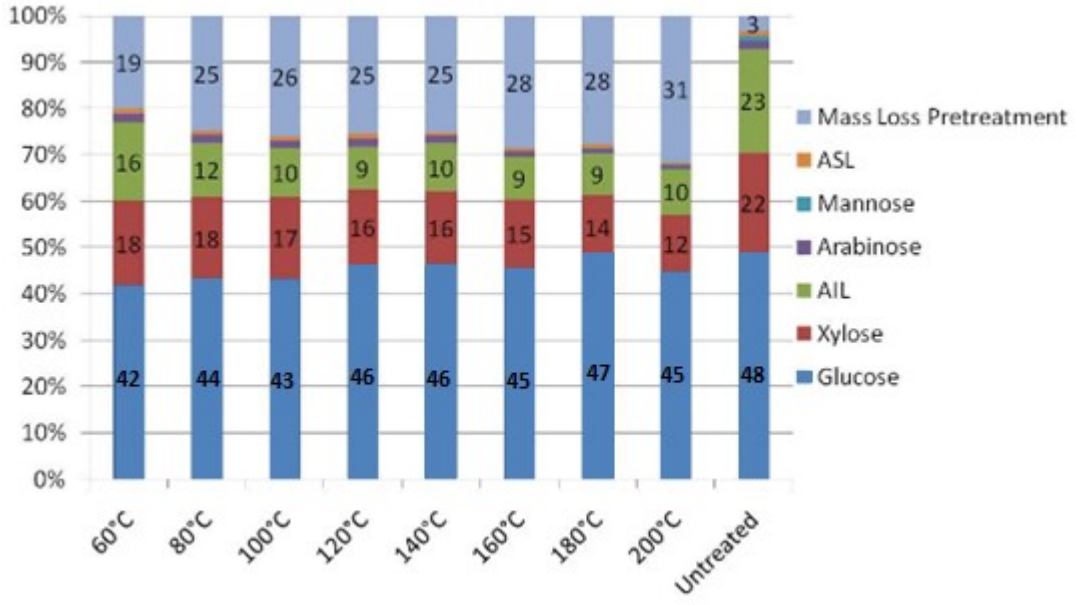


Figure 3.2 Chemical composition of untreated and pretreated *M. x giganteus* biomass.
The extractives were removed from the untreated *M. x giganteus*. Mass loss due to pretreatment and removal of extractives (recorded as "mass loss pretreatment") was used to adjust the composition values. The pretreatment was done at 60°C, 80°C, 100°C, 120°C, 140°C, 160°C, 180°C and 200°C. This data was produced with help from Dr. Bartosiak-Jentys.

The mass loss in the untreated samples is due to removal of extractives from the biomass prior to compositional analysis. The extractives in *M. x giganteus* are mostly aromatic compounds which contain simple phenols and sterols (Villaverde et al., 2009). They are water soluble and ethanol soluble non-structural materials in the biomass (Sluiter et al., 2008). Mass loss due to pretreatment increased with increasing pretreatment temperature; compositional analysis suggested that, up to 100°C, this was primarily due to lignin removal, whereas at higher temperatures increasing amounts of xylose (hemicellulose) was lost. The glucose values seem

to be consistently in the range of 42-47% (Fig. 3.2), suggesting that the bulk of the cellulose remained intact throughout this treatment.

3.2 Enzymatic saccharification of the pretreated and untreated *M. x giganteus* biomass.

The effectiveness of the SAA pretreatment process was analysed by carrying out enzymatic saccharification of the untreated and pretreated *M. x giganteus* biomass. Saccharification of the pretreated *M. x giganteus* was carried out by using the procedure described in the NREL-LAP (Selig et al., 2008). This procedure allows determination of the maximum extent of digestability due to enzymatic saccharification of cellulose from the native or pretreated lignocellulosic biomass (Selig et al., 2008). The amounts of glucose and xylose released per gram of biomass were compared.

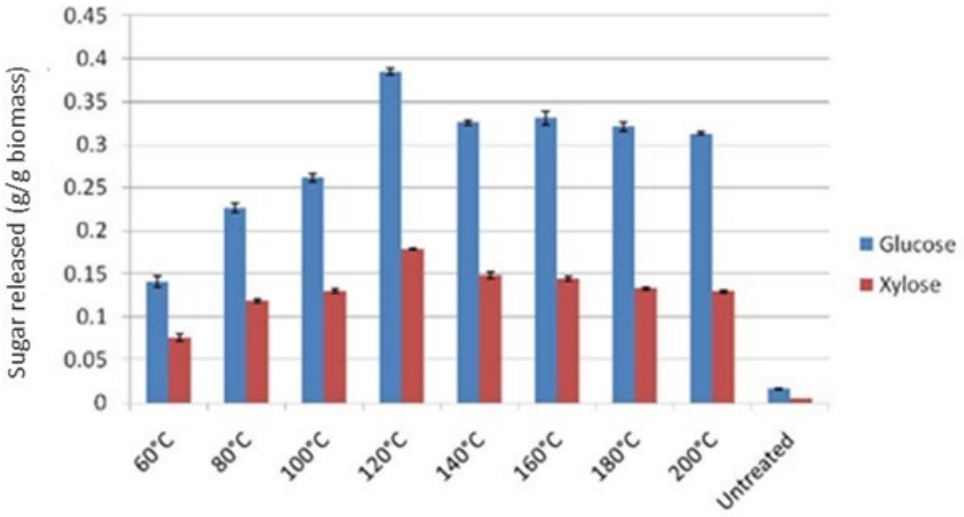


Figure 3.3 Saccharification of pretreated and untreated *M. x giganteus* biomass.
The graph shows the amount of glucose and xylose released per gram biomass after saccharification. Biomass had been pretreated by SAA for 14 h at temperatures ranging from 60-200°C. Each sample contained an equivalent of 100 mg of oven (105°C) dry weight biomass mixed with 55 µl of 0.1 M sodium citrate buffer (pH 4.8), 40 and 30 µl of cyclohexamide (10 mg/ml) and tetracycline (10 mg/ml in 70% ethanol), respectively, and 118 µl of 1:1 enzyme blend of β-glucosidase (Novozyme 188) and cellulase from *Trichoderma reesei* from Sigma. The samples were incubated for 7 days in a New Brunswick Scientific Innova 4330 incubator shaker at 50°C with shaking at 200 rpm. The enzyme hydrolysed samples were centrifuged for 10 min at 10 000 rpm (x2), using a 5424 Eppendorf centrifuge. Each sample was saccharified and analysed in triplicate by HPLC. This data was produced with help from Dr. Bartosiak-Jentys.

The results clearly showed that SAA pretreated *M. x giganteus* biomass was more susceptible to saccharification than untreated biomass. The untreated samples gave less than 0.05 g/g biomass each of both glucose and xylose after saccharification, while the pretreated samples

gave glucose and xylose levels of more than 0.35 and 0.15 g/g biomass, respectively, for biomass pretreated at 120ºC (Fig. 3.3). Saccharification efficiency appeared to be optimum with biomass pretreated at around 120ºC, with a gradual drop in monomeric carbohydrate release at increasing temperatures above this value.

3.3 Discussion.

Compositional comparison of pretreated and untreated *M. x giganteus* indicated that the alkaline pretreatment method used was good for subsequent use in consolidated bioprocessing, because there was very little loss of glucose and xylose, suggesting that the xylan and cellulose remained largely intact. The amounts of glucose and xylose ranged from 42–48% and 12-22%, respectively, for the untreated and pretreated *M. x giganteus* samples (pretreated at temperature range from 60-200ºC).

The subsequent test of saccharification clearly indicates that the pretreatment procedure used was effective in opening up the structure of the miscanthus particles because more glucose and xylose was released from the pretreated *M. x giganteus* than from the untreated *M. x giganteus*. These initial results showed that the optimum temperature for a 14 h pretreatment of the *M. x giganteus* samples combined with subsequent saccharification was 120ºC. The gradual decrease in the sugars released from *M. x giganteus* pretreated at temperatures above 120ºC could be due to the released monomers being degraded at increased temperatures (using the fixed time pretreatment) and affecting enzymatic hydrolysis or the polymers being modified making them more difficult to degrade.

A 14 h pretreatment was chosen because this could conveniently be done overnight and it is probable that shorter pretreatments would have different temperature optima. Clearly this would not be ideal for an industrial process, where shorter times and higher temperatures are typical. However, the prime purpose of this study was to create a feedstock which was useful for evaluating consolidated bioprocessing, ie one in which the carbohydrates remained largely in polymeric form but which was more accessible to enzymes than the starting material. Based on these findings, the *M. x giganteus* biomass used in this project was pretreated by SAA at 120ºC.

4. Preliminary studies of the WL isolates.

4.1 Introduction.

The WL strains 3, 6 14 and 16 were isolated from wood compost from West London Compost Company by members of the Leak lab during a previous study. The work with the WL strains was carried out to characterise these strains by 16S rRNA gene sequence analysis and individual strains for their ability to degrade cellulose, avicel, xylan and pretreated *M. x giganteus* to characterise the enzymes that appear to be active in degrading these carbon sources. The enzymes were characterised as extracellular enzymes induced during degradation of carbon substrates. They were purified from 1 dimensional native-PAGE gels and sufficient sequence information was determined by LC-mass spectrometry (LC-MS) to allow PCR amplification of a fragment of the gene. The relevant genes would then be cloned and expressed in *G. thermoglucosidasius* (DL33 and NCIMB 11955) and transformed *G. thermoglucosidasius* ultimately examined for growth on pretreated *M. x giganteus.*

4.2. Standardisation of the revival procedure for WL strains from stock cultures stored at -80^{o}C.

The WL strains used in this study had been isolated from high intensity compost heaps at West London Composting on ASM + 0.5% CMC. Initially it was not easy to revive them from glycerol stock cultures stored in the -80^{o}C freezer, with few, and frequently no colonies appearing on 2TY agar plates when spread directly with samples taken from frozen stocks. A study was carried out to standardize a reliable revival procedure for these strains, in which cells were incubated in 2TY liquid medium for a short time before spreading on plates (Table 4.1).

Table 4.1 Time course of cell revival from stock cultures stored at -80ºC.
The stock cultures were inoculated into 2TY liquid media before spreading on pre-warmed 2TY agar plates. The number of colonies reported represents CFU/100 µl of culture. TNTC stands for too numerous to count, while "mat" represents plates where colonies formed a mat on the agar surface.

Strain	Duration of incubation (hours)						
	0	1	2	3	4	5	Overnight
WL3	0	5	TNTC	TNTC	TNTC	TNTC	TNTC
WL6	0	30	TNTC	TNTC	TNTC	TNTC	TNTC
WL14	1	TNTC	TNTC	TNTC	TNTC	Mat	Mat
WL16	0	162	TNTC	TNTC	TNTC	TNTC	Mat

The results of this experiment showed that after 2 h incubation in liquid media, the number of colonies revived on 2TY agar plates dramatically increased, for all WL strains. The numbers of colonies obtained, and the calculated growth rates in 2TY liquid media (Fig. 4.1) indicated that this was unlikely to be due to growth alone.

4.2.1 Screening for possible factors that could be reviving cells from stock cultures stored at -80ºC.

Recognising that the rapid increase in colony numbers observed in Table 4.1 could not be derived from growth alone it was considered that either cells could individually be revived from some form of dormancy, or that a small number of growing cells could be producing a soluble "factor" that revived the other cells. To test for the latter possibility a study was carried out to look for evidence of cells releasing growth inducing factors into the medium. WL14 was selected for this study due to its rapid revival.

A culture of WL14 was inoculated and sampled from time 1 to 4 h incubation. After incubation 100 µl of each culture was inoculated on prewarmed 2TY plates. Each culture was then filtered using a 0.2 µm filter disc and 100 µl of the filtrate spread on a prewarmed 2TY plate (control). The filtrate containing the possible revival factor was then inoculated with a loopful of WL14 stock culture from -80ºC, and 100 µl was inoculated on a prewarmed 2TY plate. Each culture was incubated at 55ºC for 1 h. After the 1 h incubation 100 µl of each culture was plated on seperate prewarmed 2TY plates.

All the plates were incubated at 55°C overnight. The developed colonies were counted and recorded in table 4.2.

Table 4.2 Screening for possible soluble factors produced in liquid media which can revive cells previously stored at -80°C.
Cultures of WL14 were incubated in 10 ml of 2TY liquid media at 55°C with shaking at 250 rpm for: **A**: 1 h; **B**: 2 h; **C**: 3 h; **D**: 4 h. For each incubation: **1** represents number of colonies growing on 2TYplates after spreading 100µl from the liquid 2TY culture after each incubation period; **2** is the control experiment, in which uninoculated filtrate was spread on 2TY plates; **3** represents growth from filtrate inoculated with WL14 stock culture from -80°C sampled before incubation of the inoculated filtrate; and **4** represents growth after incubating the inoculated filtrate for 1h at 55°C with shaking at 250 rpm. TNTC stands for too numerous to count, while mat represents plates where colonies covered the agar surface.

Experiment treatment	Number of colonies for experiment type (CFU/100µl culture)			
	A	B	C	D
1	TNTC	TNTC	TNTC	Mat
2	0	0	0	0
3	0	0	0	0
4	TNTC	TNTC	TNTC	TNTC

After the initial incubation, all the experiments (A, B, C, and D) gave more than 300 colonies (TNTC/mat). There were no colonies on the plates spread with uninoculated filtrate, or the plates which were inoculated with samples taken immediately after inoculating the filtrate (Table 4.2 experiments 2 and 3, respectively), suggesting that, if a soluble "revival" factor was being produced it was not acting instantaneously. All the plates for experiment 4 gave large numbers of colonies.

4.2.2 Determination of the time at which cells of the WL14 strain start reviving from stock cultures stored at -80°C.

An experiment was carried out to determine the time taken for cells of WL14 to begin to revive to a culturable state from stock cultures stored at -80°C when incubated at 55°C with shaking at 250 rpm. A loopful of WL14 stock culture from -80°C was inoculated into 10 ml of pre-warmed 2TY medium in a 50 ml falcon tube which was incubated at 55°C with shaking at 250 rpm. An initial sample of 100 µl was taken immediately from the inoculated tube after mixing well but before incubation, and subsequent samples taken at 15 min intervals thereafter. They were spread on pre-warmed 2TY plates which were incubated overnight at 55°C.

After incubating the plates overnight no colonies were observed on the plate inoculated with a sample taken before incubation, while the plates spread with samples taken after 15 and 30 min incubation had 7 colonies and 100 colonies, respectively, and the plates spread after 45 min and 1 h had more than 300 colonies (TNTC). The results showed that for WL14, incubation of cells for about 15 to 30 min at 55^{o}C with shaking at 250 rpm was sufficient to obtain successful revival of cells from stock cultures stored at -80^{o}C, but that revival (as opposed to growth) appeared to continue for at least an hour.

A further experiment was set up to check for the possible production of a "revival" factor by strain WL14. A culture of WL14 was inoculated and sampled at time 0 and after 1 h incubation as previously described. The culture was then filtered as described above, 100 µl of the filtrate spread on a prewarmed 2TY plate (control). The filtrate containing the possible revival factor was then inoculated with a loopful of WL14 stock culture from -80^{o}C, and the revival time analysed by sampling at 0, 15, 30, 45 and 60 min. as described above.

No colonies were observed on the plates inoculated before the first incubation, with the uninoculated filtrate, or with the inoculated filtrate before incubation. After the second incubation, the plates spread after 15 min and 30 min had 1 and 77 colonies, respectively, while those spread after incubation for 45 min and 1 h had more than 300 colonies (TNTC). The low rate of revival after 15min reinforces the earlier suggestion that the revival of cultures in 2TY liquid medium is not due to transmission of an extracellular revival factor.

4.3 Growth profiles.

4.3.1 Determination of growth rates of strains WL3, WL6, WL14 and WL16 on 2TY medium.

The growth rates of the WL strains 3, 6, 14 and 16 on 2TY medium were determined for future reference. For this, 0.5 ml of a 2TY overnight culture of each WL strain grown from a freshly grown colony were inoculated into 50 ml of pre-warmed 2TY medium in separate 250 ml flasks. The cultures were grown at 55^{o}C with shaking at 250 rpm. Optical density readings were recorded over a period of 9 h (Fig. 4.1).

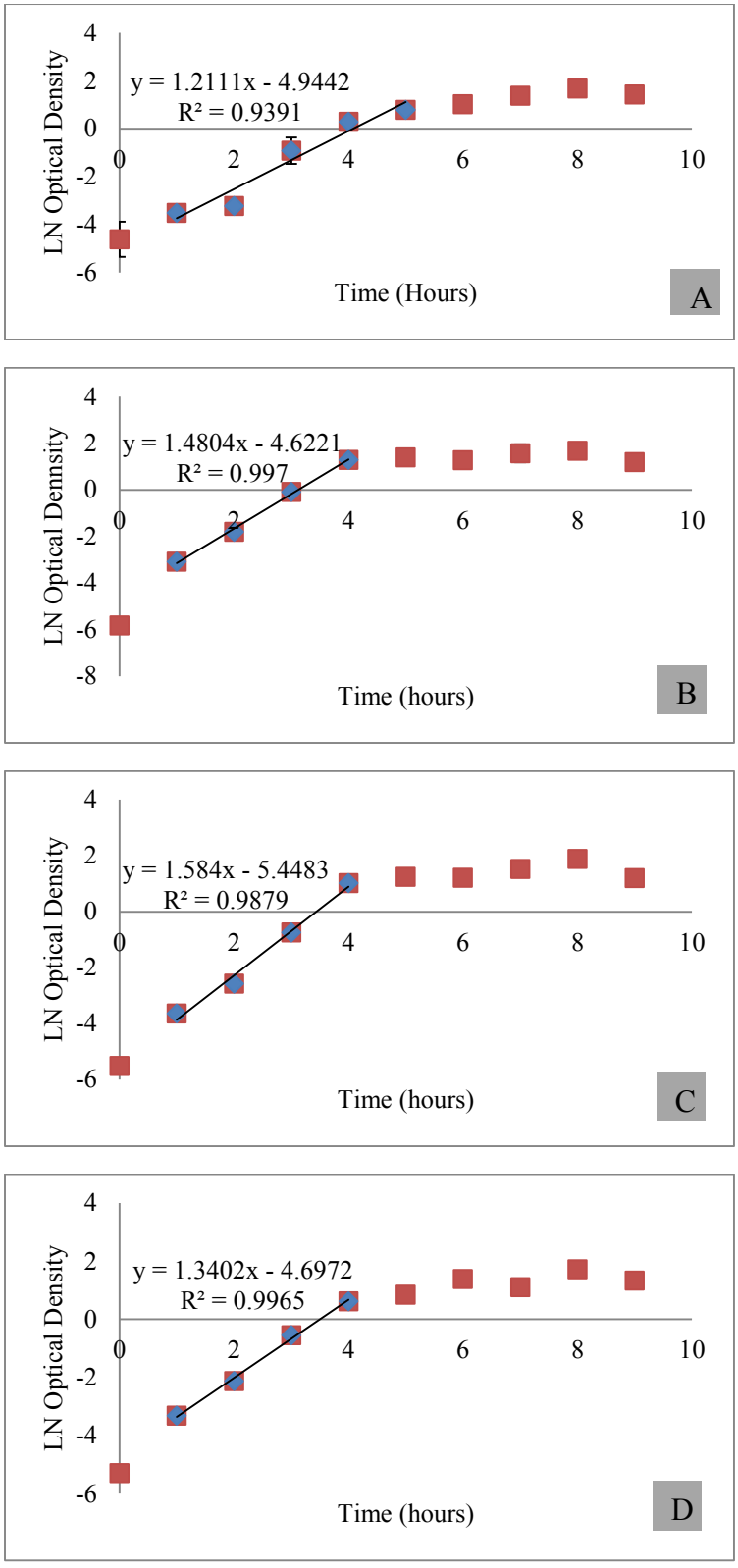


Figure 4.1 Growth curves of WL strains on 2TY medium.
(A) WL3, (B) WL6, (C) WL14 and (D) WL16. Three samples were taken at each time point for OD measurements at 600 nm.

From the slopes of graphs 4.1A-D the growth rates were calculated as 1.21 h^{-1} (WL3), 1.48 h^{-1} (WL6), 1.58 h^{-1} (WL14) and 1.34 h^{-1} (WL16).

4.3.2 Growth tests for the production of cellulases by WL strains.

The WL strains were tested for cellulase production by growing on plates containing ASM + 0.1% YE + 0.5% CMC, ASM + 0.1% YE + 0.5% cellobiose, ASM + 0.1% YE + 0.5% avicel, and on ASM +0.1% YE (control), and staining the plates with Congo red (Figures. 4.2 and 4.3). The presence of halos around the colonies after staining indicates zones of substrate clearance.

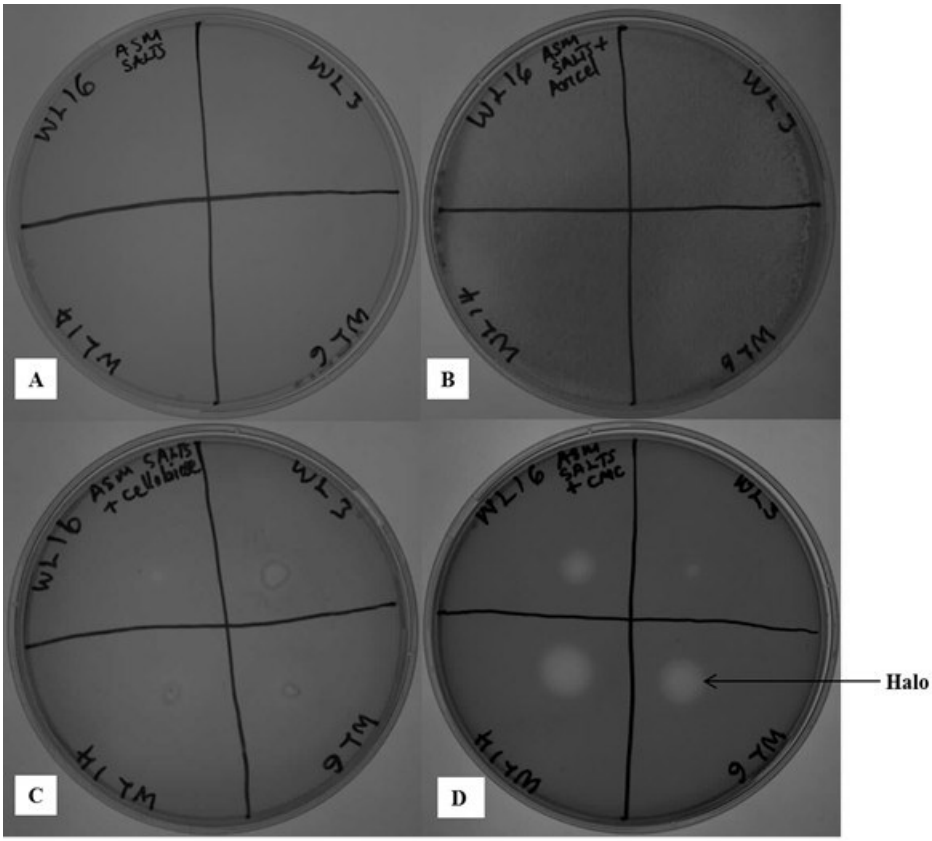


Figure 4.2 Congo red screen for cellulase activity in 24 h old WL strains cultures.
The cultures were screened for cellulase activity after growth on A: ASM + 0.1% YE, (control) B: ASM + 0.1% YE + 0.5% avicel, C: ASM + 0.1% YE + 0.5% cellobiose and D: ASM + 0.1% YE + 0.5% CMC. The inoculation was carried out by touching an overnight culture grown on 2TY with an inoculating loop and then spotting on the plates containing specific substrates. The order of the spotted strains from the top right quarter of the plate following the clockwise direction was WL3, WL6, WL14 and WL16. The plates were incubated for 24 h at 55ºC before being flooded with Congo red, and then rinsed with 1 M NaCl to show the zones of clearance around the colonies.

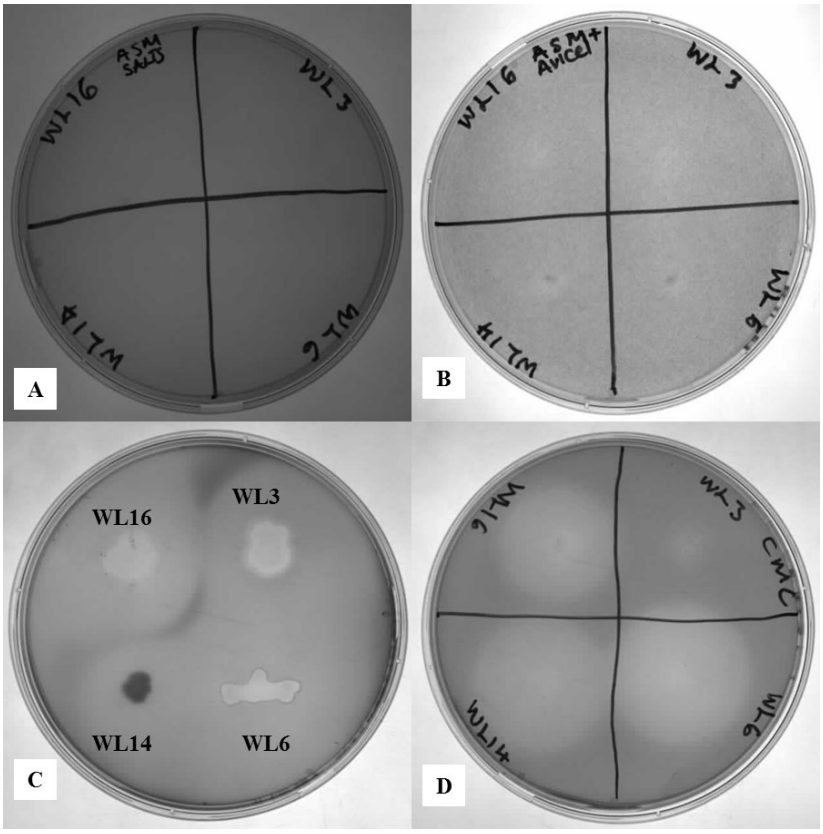
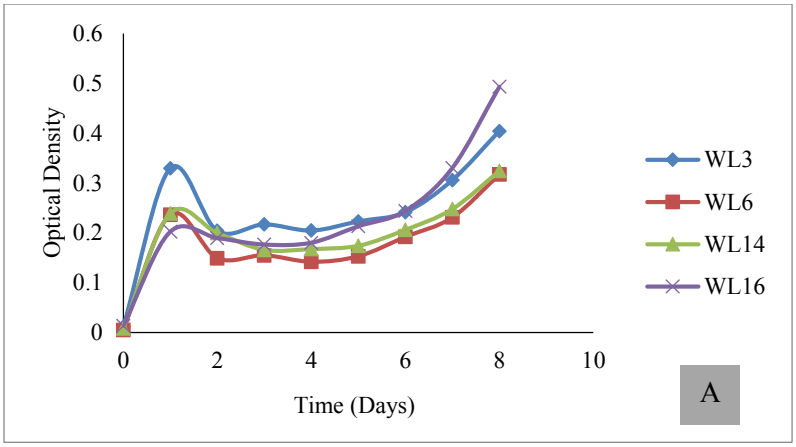


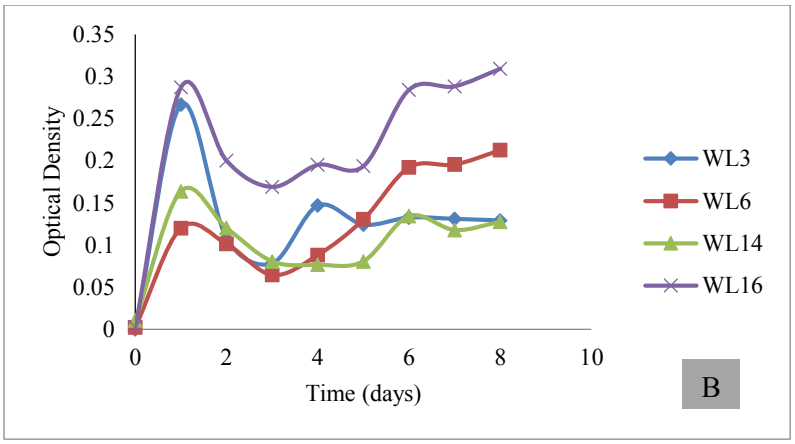
Figure 4.3 Congo red screen for cellulase activity in 8 d old WL strains cultures.
WL strains were growing on A: ASM + 0.1% YE, (control) B: ASM + 0.1% YE + 0.5% avicel, C: ASM + 0.1% YE + 0.5% cellobiose and D: ASM + 0.1% YE + 0.5% CMC. The inoculation procedure and the order of strains on the plates were as indicated in Fig. 4.2. After 8 days incubation at 55°C, plates showed zones of clearance after being stained with Congo red stain and then rinsed with 1 M NaCl.

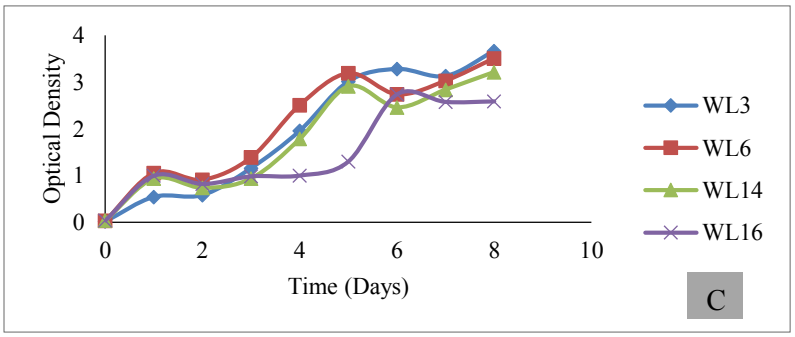
Bacterial colonies developed on all the plates. All four WL strains showed some activity against avicel, cellobiose and CMC, with most of the strains showing the largest zone of clearance on CMC and the smallest on avicel. There were no halos evident around the colonies on the control plates (ASM alone, Figures. 4.2A and 4.3A), consistent with the reaction with Congo red being specific for carbohydrates. Halos were evident on ASM + 0.1% YE avicel after 8 days incubation, though they are barely visible in Fig. 4.3B due to the opaqueness of avicel.

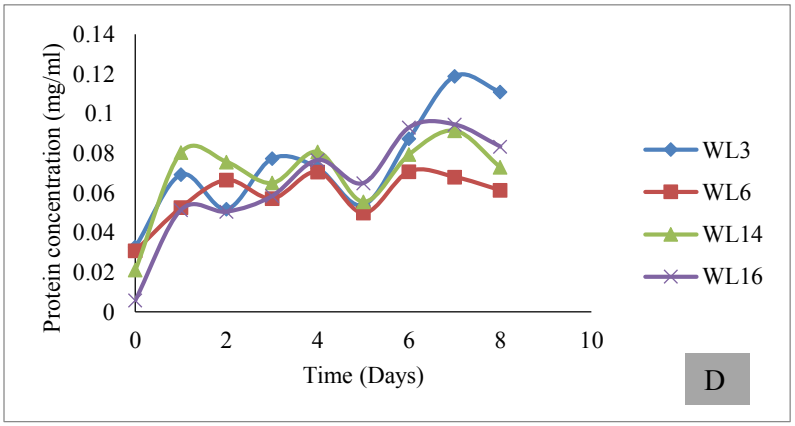
4.4 Growth of WL3, WL6, WL14 and WL16 in liquid medium on CMC, avicel and xylan in modified ASM.

The growth of the WL strains in liquid medium on CMC, avicel and xylan as substrates was assessed by growing the strains on 0.5% of the substrate + 0.1% YE + ASM. The strains were also grown on ASM + 0.1% YE (control for the experiments).


0.6
0.5
0.4
0.3
0.2
0.1
0
Optical Density
0
2
4
6
8
10
Time (Days)
WL3
WL6
WL14
WL16
A


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Optical Density
0
2
4
6
8
10
Time (days)
WL3
WL6
WL14
WL16
B


4
3
2
1
0
Optical Density
0
2
4
6
8
10
Time (Days)
WL3
WL6
WL14
WL16
C


0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Protein concentration (mg/ml)
0
2
4
6
8
10
Time (Days)
WL3
WL6
WL14
WL16
D

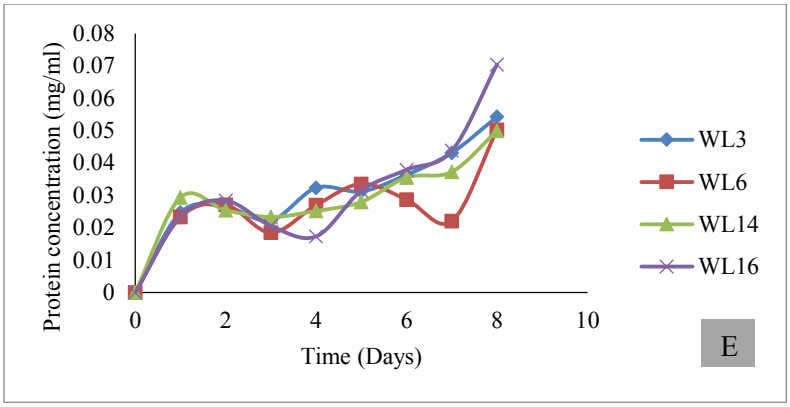


Figure 4.4 Growth profiles of WL strains on avicel, CMC and xylan.
WL3, WL6, WL14 and WL16 after growth on (A) ASM + 0.1% yeast extract (YE), (B) ASM + 0.5% CMC + 0.1% YE, (C) ASM + 0.1% YE + 0.5% xylan, (D) ASM + 0.1% YE + 0.5% avicel (by total protein concentration), and (E) ASM + 0.1% YE (by total protein concentration). The cultures were incubated for 8 days. Optical density was measured at 600 nm. Growth on avicel and ASM control for avicel was determined by measuring the total suspended protein concentration by Bio-Rad protein assay using the method of Bradford (Bradford, 1976) following the manufacturers protocols.

The WL strains did not show any significant growth on ASM (Fig. 4.4A) and growth on CMC did not seem to be greater than on ASM alone (Fig. 4.4B). Later on growth increased, but it is not clear whether the cells were growing or the increased OD was due to evaporation. All four WL strains showed good growth on xylan (Fig. 4.4C) while none of the strains showed any significant growth on avicel (Fig. 4.4D). Different results for the cultures on CMC and avicel might have been obtained by increasing the period of growth.

4.5 Enzyme assays.

4.5.1 Determination of glycoside hydrolase activity.

Cellulase assays were carried out on the secreted protein (after removing the cells by centrifugation) from cultures of strains WL3, WL6, WL14 and WL16 grown on ASM + 0.1% (YE) + 0.5% CMC, ASM + 0.1% YE + 0.5% xylan, ASM + 0.1% YE + 0.5% avicel and ASM + 0.1% YE (Fig. 4.5), grown for 8 days at 55^{o}C with shaking at 250 rpm.

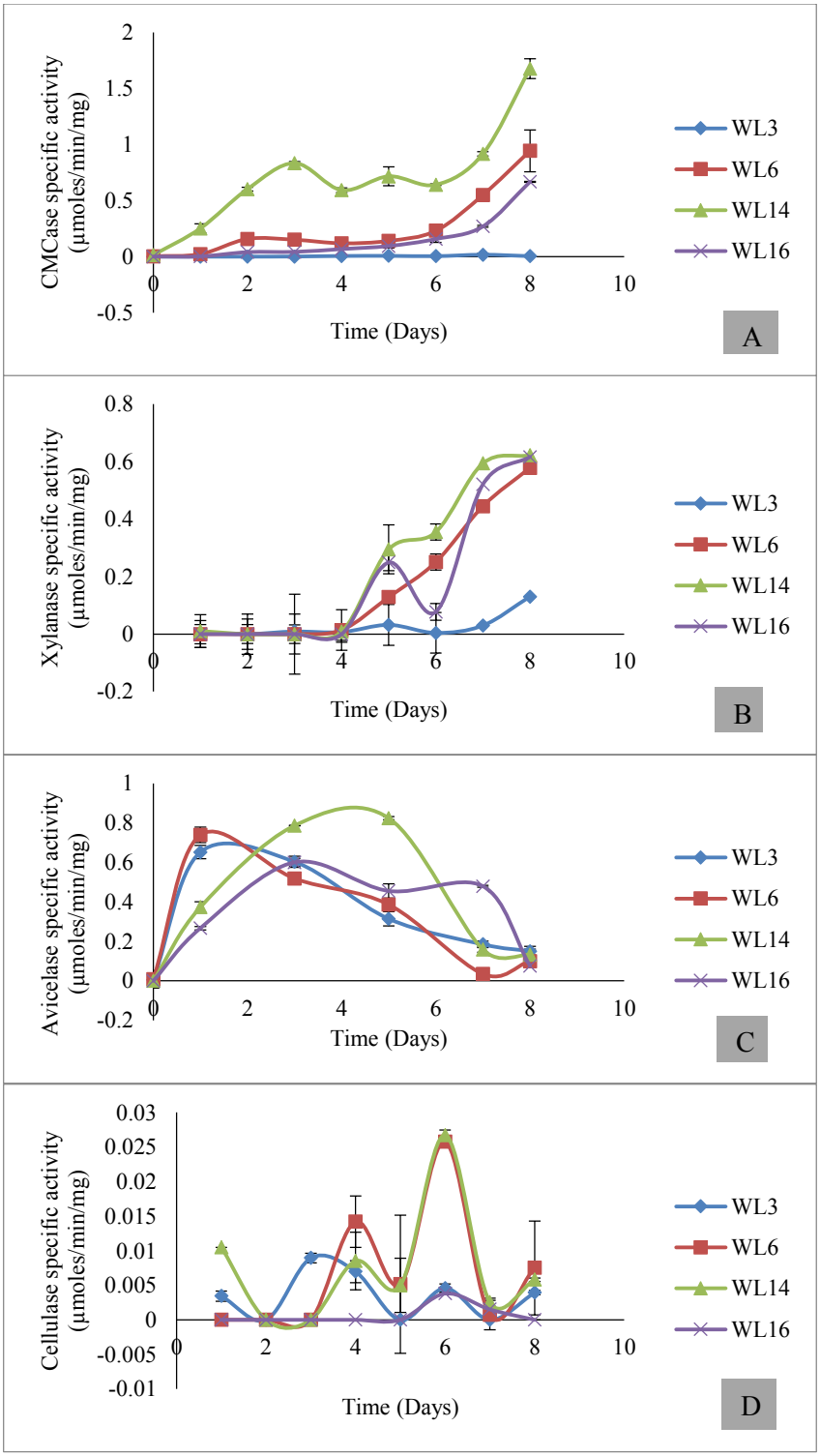


Figure 4.5 Cellulase, xylanase and avicelase specific activity of WL strains.
Enzyme specific activity was assessed in the culture supernatants of WL3, WL6, WL14 and WL16 growing on (A) ASM + 0.1% YE + 0.5% CMC (B) ASM + 0.1% YE + 0.5% xylan (C) ASM + 0.1% YE + 0.5% avicel, respectively, and (D) cellulase specific activity of the control (ASM + 0.1% YE). The supernatant from each culture was assayed for protein concentration and avicelase cellulase, or xylanase specific activity for the cultures grown on avicel, CMC, and on xylan, respectively. The protein concentration was determined by the method of Bradford (Bradford, 1976). Enzyme activity was measured by the DNS (3,5-dinitrosalicylic acid) method (Miller et al. 1960). The enzyme specific activity was determined by dividing the enzyme activity by the protein concentration. The samples were analysed 3 times.

WL3 showed no cellulase activity after growth with CMC throughout the experiment (Fig. 4.5A), while WL6 and WL16 started showing activity from the sixth day, and by day 8, had a CMCase specific activity of 0.94 and 0.67 μmoles/min/mg, respectively. WL14 showed higher CMCase activity than the other WL strains throughout this experiment, reaching a specific activity of 1.68 μmoles/min/mg on day 8. Except for WL3, all the WL strains showed a CMCase specific activity that increased from day 6, despite showing no evidence of growth (Fig. 4.4B).

Xylanase specific activity measurements were also recorded from day 1 (Fig. 4.5B). After day 5 all four WL strains showed xylanase activity, with WL3 having the lowest. WL6, WL14 and WL16 showed a trend of increasing xylanase specific activity from day 5. For WL3 activity increased after day 6.

In experiment (C) of Fig. 4.5, some avicelase enzyme activity was evident with all four strains by day 1. By day 2, the enzyme activity for WL3 and WL6 started decreasing, while that of WL14 and WL16 started decreasing after day 5 and 3, respectively, marking their highest avicelase specific activity of 0.82 and 0.60 μmoles/min/mg, respectively. By day 8 avicelase specific activity for all the four strains was close to 0 μmoles/min/mg.

Experiment (D) (Fig. 4.5) was used as a control, to check cellulase (avicelase or CMCase) activity in modified ASM medium without addition of an extra carbon source. Cellulase activity was recorded from day 1 of incubation. None of the WL strains showed any significant cellulase activity, until day 6, when WL6 and WL14 showed a slight increase, but dropped by day 7.

4.5.2 SDS PAGE and native PAGE gel analysis.

SDS PAGE gels (12%) were run to look for extracellular proteins produced by the WL strains during growth on avicel. The cultures on the carbon sources were left to grow on for 18 days. Samples used for protein analysis on gels were from day 8 and day 18 after growth on avicel. The resulting SDS-PAGE gels did not show good activity after staining with Congo red (results not shown). Therefore native-PAGE gels were run instead, and the enzyme activity was much better than the activity on the SDS-PAGE gels (Fig. 4.6).

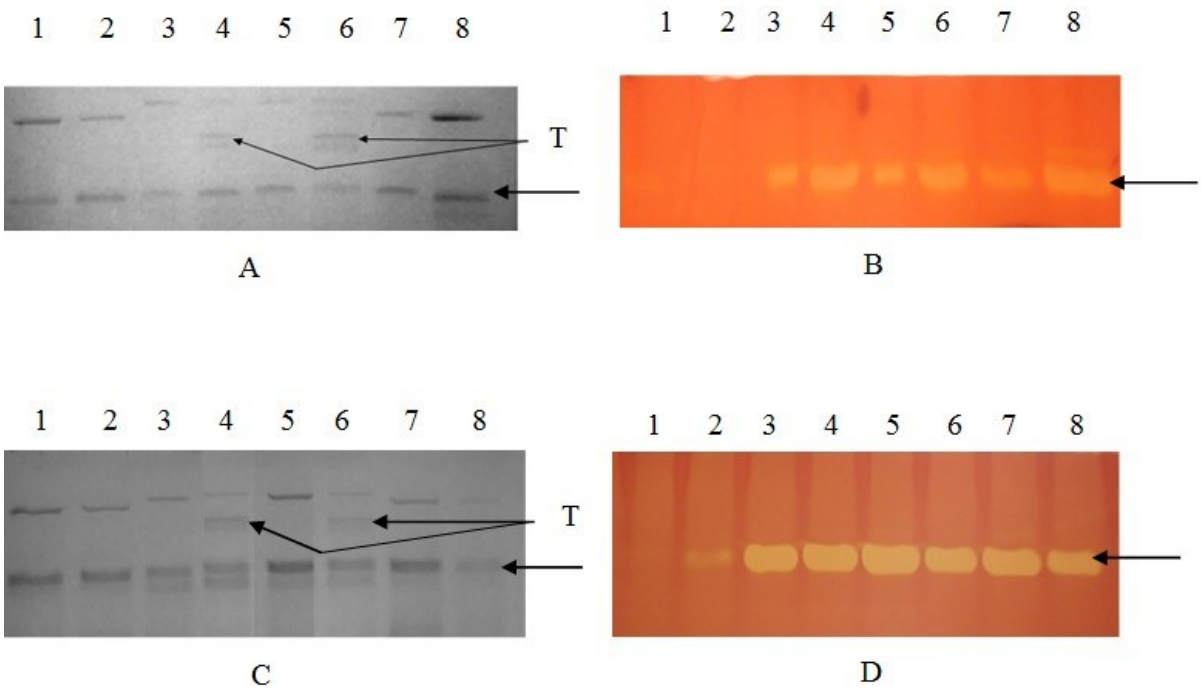


Figure 4.6 Native CMC PAGE gels of protein from the cultures of WL strains.
The cultures were grown on 0.5% avicel + 0.1% YE + ASM, and stained with Coomassie brilliant blue (gels A and C) and Congo red (gels B and D). The native PAGE gels contained 0.05% CMC (from Sigma). For gel B only, lanes, 2, 4, 6 and 8, were loaded with supernatant from WL3, WL6, WL14 and WL16, respectively, and their controls in lanes 1, 3, 5 and 7, respectively. The samples loaded in lanes 1, 3, 5 and 7 of gels A, C and D were supernatants from WL3, WL6, WL14 and WL16 cultures, respectively, while lanes 2, 4, 6 and 8 were loaded with supernatant from their controls, respectively. Gels A and B were loaded with supernatant from the 8 day old cultures, while gels C and D were loaded with supernatants from the 18 day old cultures. For each sample, 28 µl was mixed with 12 µl of loading buffer for native gels. The protein concentration was standardized to approximately 0.5 mg/ml for all samples. The single arrow on each gel point to the set of clear bands on the Congo red stained zymograms and the corresponding set of bands on the Coomassie brilliant blue stained gels.

Fig 4.6B and D show that cellulase activity was constitutive in strains WL6, 14 and 16. However the enzyme activity of the supernatant from the avicel cultures was higher than that of the supernatant from their controls as strains' cultures clear bands in the Congo red stained gels were in most cases larger than the clear bands of the control (Fig. 4.6B and D).

Two sets of 2 bands labelled T in Figures 4.6A and 4.6C, lanes 4 and 6, are visible in the lanes loaded with supernatant from controls of WL6 and WL14 8 day old cultures, respectively, (Fig. 4.6A), and similar bands were observed in the 18 day old controls of the same strains (Fig. 4.6C). However, no cellulase activity was evident in the locations of these protein bands. Mass spectrometric analysis of bands excised from the gels identified these as aconitate hydratase from *Geobacillus kaustophilus* (the closest strain being HTA426). It was not clear why the proteins in the two bands were inhibited from being secreted by the WL6 and WL14 cultures grown on avicel.

All the clear areas in gel D (Fig. 4.6) were analysed by mass spectrometry. The enzymes xylan-1,4-β-xylosidase from *Geobacillus* sp. (closest strain being *stearothermophilus*) was detected. Other identified enzymes from the same clear bands included NAD-dependent aldehyde dehydrogenase from *Geobacillus* sp. (closest strain being *kaustophilus* HTA426) and Δ-1-pyrroline-5-carboxylate dehydrogenase from *Geobacillus* spp. (closest strains being *kaustophillus* HTA426, Y412MC52 and WCH70). The faint clear bands visible just above the larger clear bands in lanes 6 and 8 of gel B were both identified as oligoendopeptidases from *Geobacillus* spp. (closest strains being Y412MC52, WCH70 and *kaustophillus* HTA426).

4.6 Growth of WL3, WL6, WL14 and WL16 on pretreated and untreated *M. x giganteus* in unbuffered RIM.

WL3, WL6, WL4 and WL16 were inoculated into media containing either 1% (w/v) untreated *M. x giganteus* in RIM, 1% (w/v) pretreated *M. x giganteus* in RIM or RIM alone. Because of the presence of suspended solids, growth was followed by measuring the protein concentration in the media (Fig. 4.7).

The results showed that none of the WL strains used was able to grow in RIM alone, which is unsurprising given the low carbon content of this medium. However, there was apparent growth on both pretreated and untreated *M. x giganteus*, with growth on untreated *M. x giganteus* apparently greater than that on pretreated *M. x giganteus*. Growth of all strains on untreated *M. x giganteus* showed a similar profile, increasing over the first 2 or 3 days but then apparently declining. This could reflect growth on easily available carbohydrate or other carbon sources, which may have been washed out of the treated *M. x giganteus* during preatment. There is some evidence that growth on pre-treated miscanthus might have been starting after a few days incubation.

The highest total protein concentration recorded during the 5 days growing period on pretreated *M. x giganteus* was 0.22, 0.52, 0.36, and 0.22 mg/ml, while on untreated *M. x giganteus* the highest values recorded were 0.66, 0.53, 0.54 and 0.55 mg/ml for WL3, WL6, WL14 and WL16, respectively. No physical degradation of pretreated or untreated *M. x giganteus* was visible in any of the cultures even when the cultures were left to grow on for more than 6 weeks.

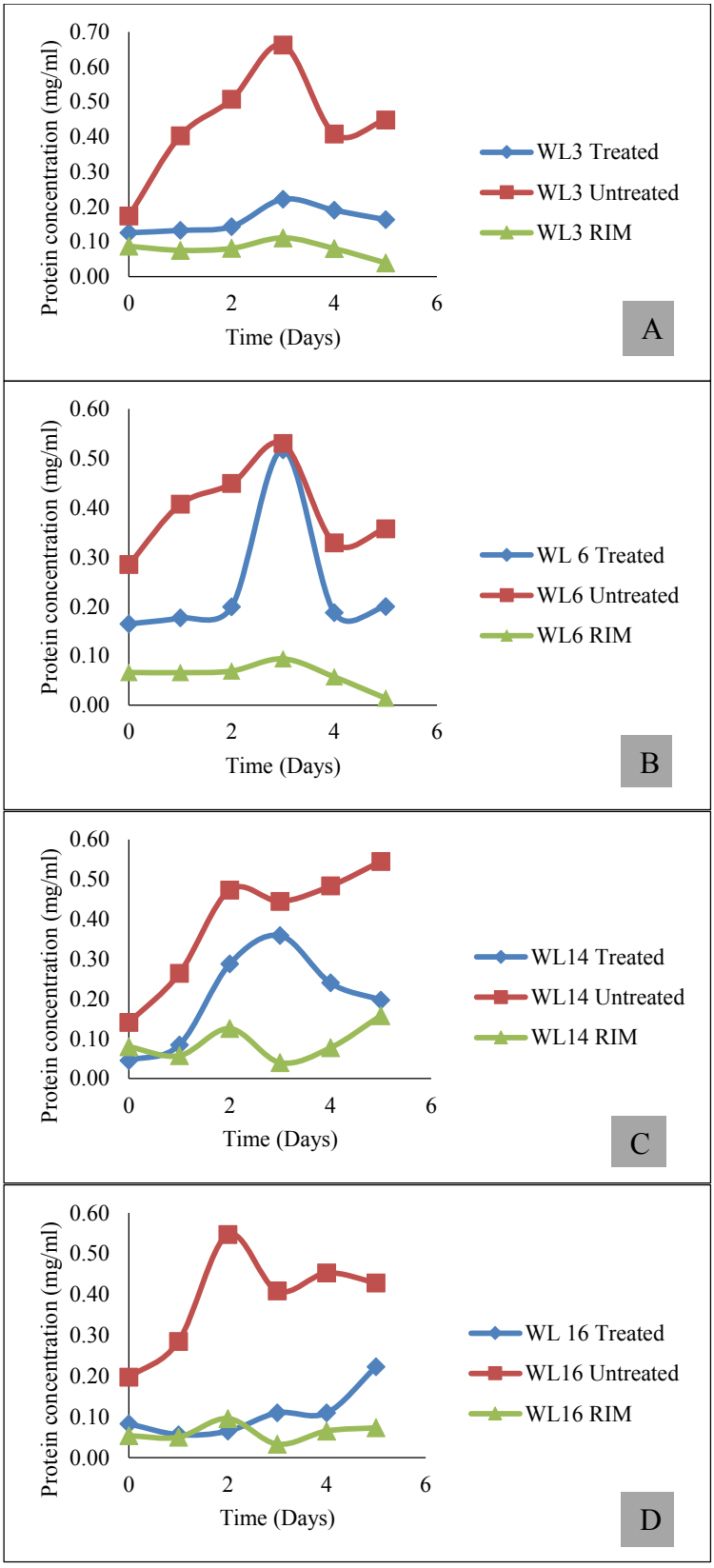


Figure 4.7 Growth of WL strains on 1% pretreated and untreated *M. x giganteus* in unbeffered RIM. (A) WL3, (B) WL6, (C) WL14, and (D) WL16. The strains were grown on 1% pretreated *M. x giganteus* in RIM, 1% untreated *M. x giganteus* in RIM and RIM alone, measured by protein determination in the culture supernatant after settling of larger particles as outlined by Ishida et al., 1997, using the Bradford assay (BioRad). The cultures were started with 0.5% inoculum from an overnight 2TY culture and incubated at 55°C with shaking at 250 rpm for 5 days.

4.6.1 Determination of enzyme activity of WL14 after growth on pretreated *M. x giganteus*.

WL14 showed some evidence of growth on the pre-treated *M. x giganteus* based on the increase in protein concentration and was, therefore, used to assess for secretion of glycoside hydrolases. The supernatant used for the analysis was from 3 day old cultures at which point the culture had the highest suspended protein concentration (Fig. 4.7C). The results showed that there was a low level (less than 0.2 µmoles/min/mg) avicelase and CMCase activity present in the supernatant, while xylanase activity was high (Fig. 4.8).

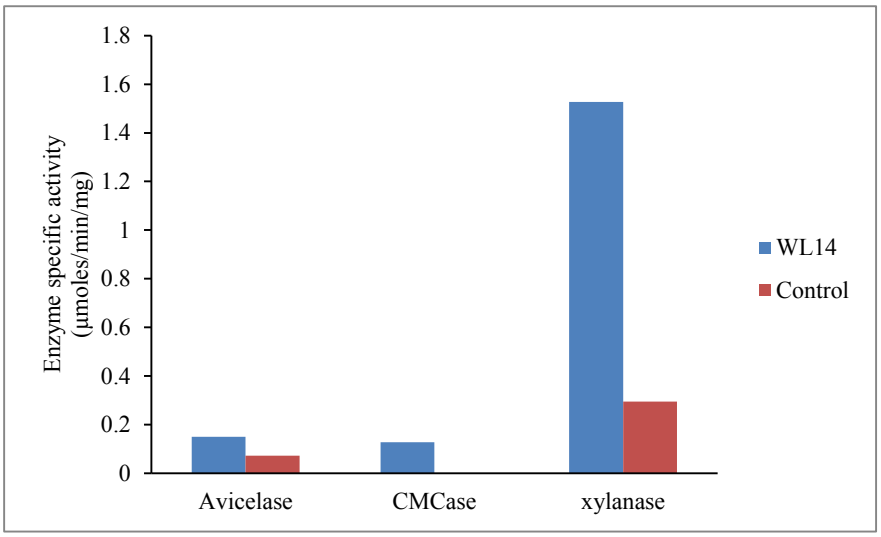


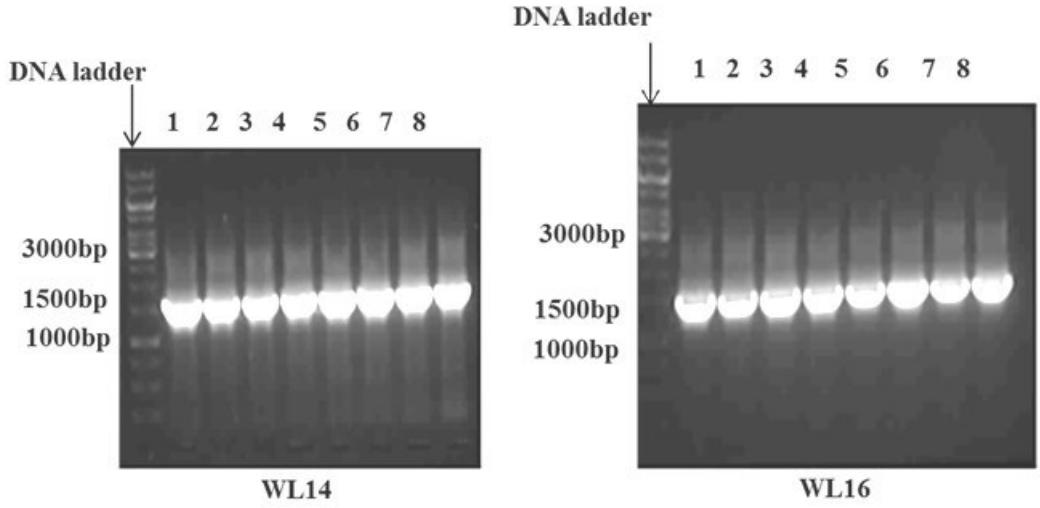
Figure 4.8 Avicelase, CMCase and xylanase specific activity in WL14 culture broth after growth on pretreated *M. x giganteus* in unbuffered RIM.
The supernatants analysed were taken from a 3 day old culture and the control. The cells were removed by centrifugation at 4 000 x g at 20ºC for 20 minutes in an Eppendorf 5810-R. The collected supernatant (0.5 ml) was concentrated to approximately 0.1 ml by centrifuging in a bench top accuSpin™ Micro centrifuge (Fisher Scientific) at 10 000 x g for 15 minutes using a 3 000 MWCO PES vivaspin centrifugal membrane separator from Sartorious stedim biotech. The protein concentration was determined by the Bradford method. The DNS (3,5-dinitrosalicylic acid) method (Miller et al., 1960) was used to measure the enzyme activity.

4.7 Identifying the WL strains based on their 16S rRNA gene sequences.

4.7.1 PCR amplification of 16S rRNA gene sequences.

Optimisation was carried out using chromosomal DNA from strains WL14 and WL16 (Fig. 4.9) to find the right annealing temperature for successful 16S rRNA gene amplification of the WL strains.

Experiment 1



Experiment 2

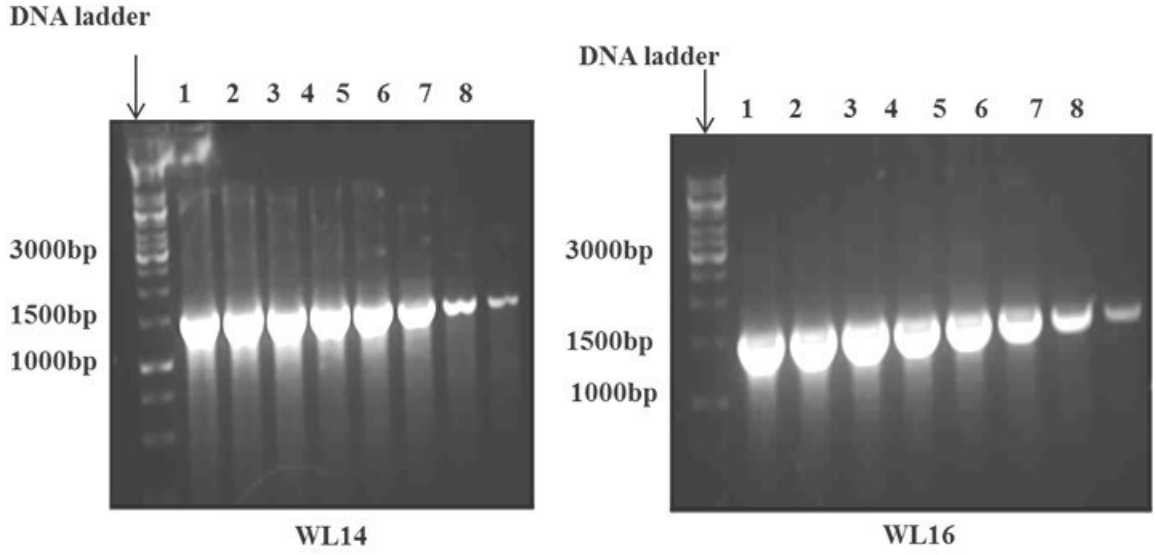


Figure 4.9 PCR optimisation experiments using WL14 and WL16 strains.
Chromosomal DNA was isolated from overnight cultures grown on 2TY, using the EZNA Bacterial DNA kit (Omega Bio-Tek). An annealing temperature range of 46.9 to 58.8ºC was used for experiment 1 and 58.2 to 69.9ºC for experiment 2, with annealing temperature increasing from samples 1 to 8. Products were analysed on a 0.7% agarose gel. The PCR reaction mixture contained 1 µl DNA, 1 µl of primer 27F (10 pmol/µl), 1 µl of primer 1492R (10 pmol/µl), 7 µl neutral water (nH_2O) and 10 µl Biomix Red (2x) (Bioline). The temperature for each specific lane was 46.9, 48.2, 49.8, 51.6, 53.5, 55.5, 57.2, and 58.8ºC for lanes 1 to 8, respectively, in experiment 1, and 58.2, 59.9, 61.7, 63.6, 65.6, 67.3, 68.8, and 69.9ºC, respectively, for lanes 1 to 8 in experiment 2.

It was clear that products were obtained at all annealing temperatures, although the amounts decreased at higher temperatures. The median temperature of experiment 1 was about 52ºC,

and was chosen as the annealing temperature for 16S rRNA gene amplification for all WL strains.

4.7.2 Phusion polymerase chain reaction.

To improve the fidelity of the PCR products, Phusion polymerase was used because it has the ability to proof read and correct errors in DNA synthesis. The concentration of the isolated DNA of WL3, WL6, WL14 and WL16 was 9.5 ng/µl, 6.75 ng/µl, 7.5 ng/µl and 5 ng/µl, respectively. These concentrations were used to estimate the amount of template DNA to be used for the Phusion PCR for each strain. The 16S rRNA gene PCR products for the WL strains are shown in Fig. 4.10A and B.

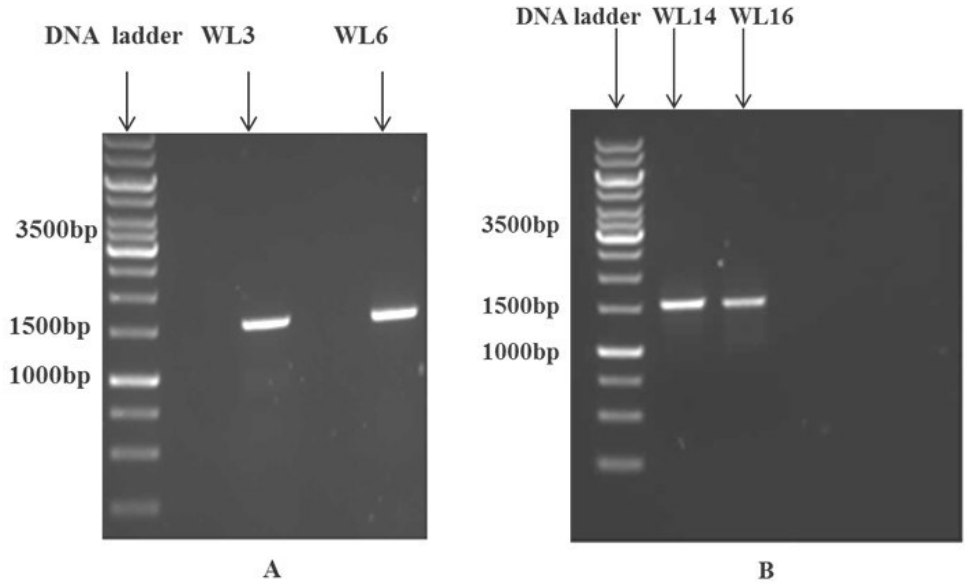


Figure 4.10 Products from PCR amplification of the 16S rRNA gene from WL strains.
For WL3, WL6 and WL14 the Phusion PCR mixture contained 30 µl of nH_2O, 10 µl of HF buffer, 1 µl of 10 mM dNTPs, 1 µl of primer 27F (10 pmol/µl), 1 µl of primer 1492R (10 pmol/µl), 5 µl of template, 1.5 µl of DMSO (3%) and 0.5 µl of Phusion hot start DNA polymerase (2 U/µl). For WL16 the concentration of the template was adjusted by adding 27 µl nH_2O to 8 µl of template. The Phusion PCR products were analysed by 0.7% agarose gel electrophoresis, and the results were as shown in (A) WL3 and WL6, (B) WL14 and WL16.

The WL3, WL6, WL14 and WL16 DNA Phusion PCR products were purified using a Qiagen gel extraction kit; the concentration of WL3, WL6 and WL14 products was 140 ng/µl, and that of WL16 was 120 ng/µl.

The Fermentas CloneJET (pJET1.2/blunt) vector was used for molecular cloning of the 16S rRNA gene PCR products from the WL strains. The PCR product concentration was used to

calculate the required volume to be used for the reaction to give a 3:1 molar ratio of plasmid to insert.

The pJET1.2/blunt vector is best for high efficiency cloning of PCR products generated with any polymerase that produces blunt ended products, such as Phusion. The PCR products were ligated directly into the vector, which contains a gene encoding a lethal restriction enzyme that gets disrupted by ligation of the DNA insert into the cloning site. Therefore only bacterial cells with recombinant plasmids are able to form colonies on the LB + Ampicillin medium plates. The cloning products were used to transform *E.coli* (JM109) competent cells. The 16S rRNA gene pJET recombinants of WL3, WL6, WL14, and WL16 gave transformation efficiencies of 3.0 x 10^4, 6.0 x 10^5, 5.0 x 10^3 and 1.25 x 10^5 CFU/µgDNA, respectively.

4.7.3 Colony PCR.

Colony PCR was carried out in order to confirm whether the transformants contained an insert of the correct size in the pJET1.2 cloning site.

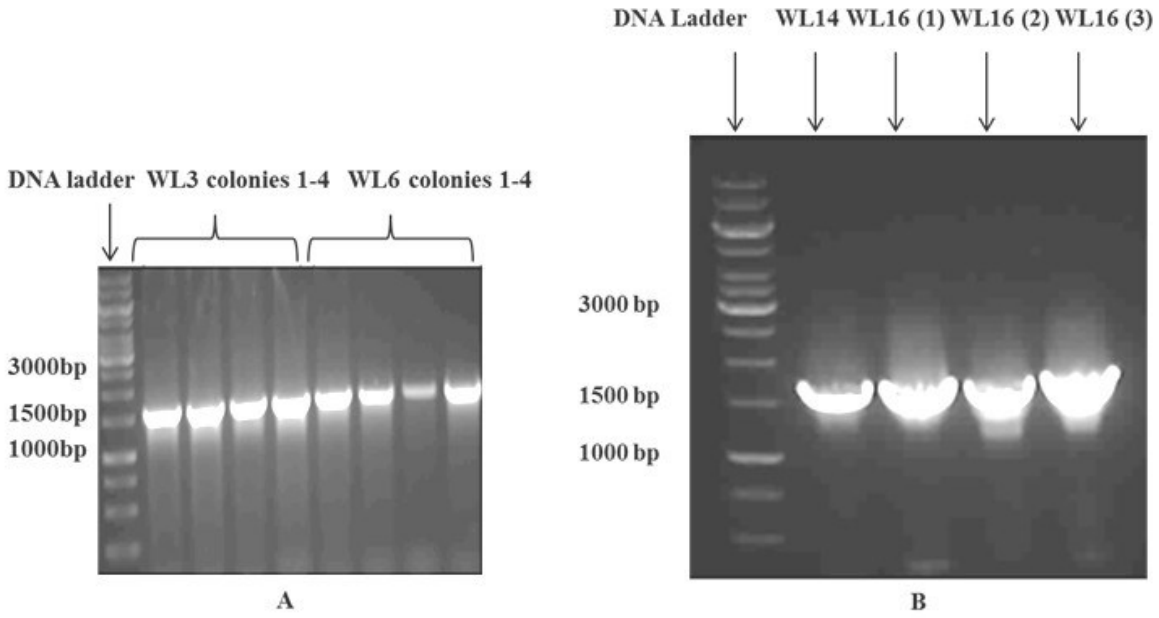


Figure 4.11 Products from colony PCR of JM109 pJET1.2 transformants believed to contain 16S rRNA genes. The PCR was run using an annealing temperature of 52°C, for 30 cycles. The PCR reaction mixture for each reaction contained H_2O (8 µl), Primer (10 pmol/µl) pJET1.2 F (1 µl), Primer (10 pmol/µl) pJET1.2 R (1 µl) and 10 µl of Biomix Red (2x). The selected transformant colony was touched with a pipette tip and introduced into the PCR reaction mixture. The PCR products were analysed on a 0.7% agarose gel. The results were as presented in (A) WL3 and WL6, and (B) WL14 and WL16.

Products of the expected size were obtained for clones derived from all four WL strains (Fig. 4.11A and B).

4.7.4 Plasmid isolation and digestion.

To isolate the plasmids, the selected transformants were grown by inoculating from single colonies of cells into prewarmed 10 ml LB + ampicillin medium and incubating overnight at 37°C with shaking at 250 rpm. Plasmids were extracted from the cells using the QIA prep spin Miniprep kit. To confirm that an insert of the expected size had been cloned into the vector, it was digested with Bgl II restriction enzyme (New England Biolabs, Hertfordshire, UK).

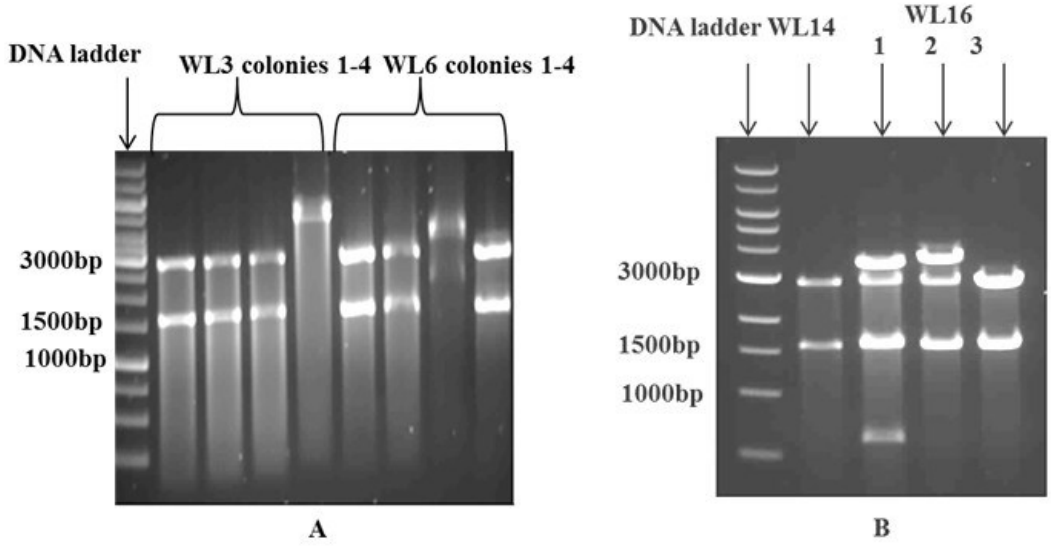


Figure 4.12 Bgl II digestion of recombinant pJET1.2 believed to contain 16S rRNA gene inserts from WL strains. The reaction mixtures for plasmid contained 1 µl DNA 1 µl 10x Buffer H, 0.2 µl BSA 50x (15 µl BSA + 15 µl neutral water), 7.3 µl (nH_2O), and 0.5 µl Bgl II except for WL14 which contained 4 µl DNA and 4.3 µl water. The digestion mixtures were incubated at 37°C overnight and analysed on a 0.7% agarose gel. The obtained results were as shown in (A) WL3 and WL6, and (B) WL14 and WL16.

For most plasmids, a product of approximately 1.5kb, consistent with the size of the 16S rRNA gene PCR product previously amplified, was observed (Fig. 4.12). This suggests that the cloning had been successful for most of the transformants analysed. However, sample 4 of WL3 and sample 3 of WL6 were not fully digested.

4.7.5 Sequencing and analysis of the plasmid inserts.

Samples of plasmid from 2 each of the WL3, WL6, and WL16 16S rRNA gene transformants and the sole WL14 representative were diluted to give 1.2 µg in a final volume of 15 µl of

nH_2O + 1.5 µl of sequencing primer (10 µM/µl) pJET1.2 F(5'-CGACTCACTATAGGGAGAGCGGC-3') /(10 µM/µl) pJET1.2 R(5'-AAGAACATCGATTTTCCATGGCAG-3'), (from Fermentas) and sent for DNA sequencing to Eurofins Genomics.

Analysis of the sequences obtained confirmed that they corresponded to 16S rRNA genes. A simple BLAST (N) was carried out, which confirmed that all of the strains were closely related to *Geobacillus* spp., and the information was used to construct a phylogenetic tree. The three isolates, WL3, WL6 and WL16 appeared to be closely related, and showed 99% 16S rRNA gene sequence identity to many *Geobacillus* spp., the closest to them all being *Geobacillus* sp. GHH01, while the 16S rRNA gene of WL14 also showed 99% identity to that of many *Geobacillus* spp.with *G. thermoleovorans* strain BGSC 96A1 as the closest strain (Fig. 4.13). WL3, WL6 and WL16 strains showed 99% identity of their 16S rRNA gene with that of *Geobacillus* sp. C56-T3, which is a *Geobacillus thermoglucosidasius* (Fig. 4.13). The most closely related enzymes to those detected in section 4.5.2 were all from *Geobacillus* spp., consistent with these 16S rRNA gene analysis results.

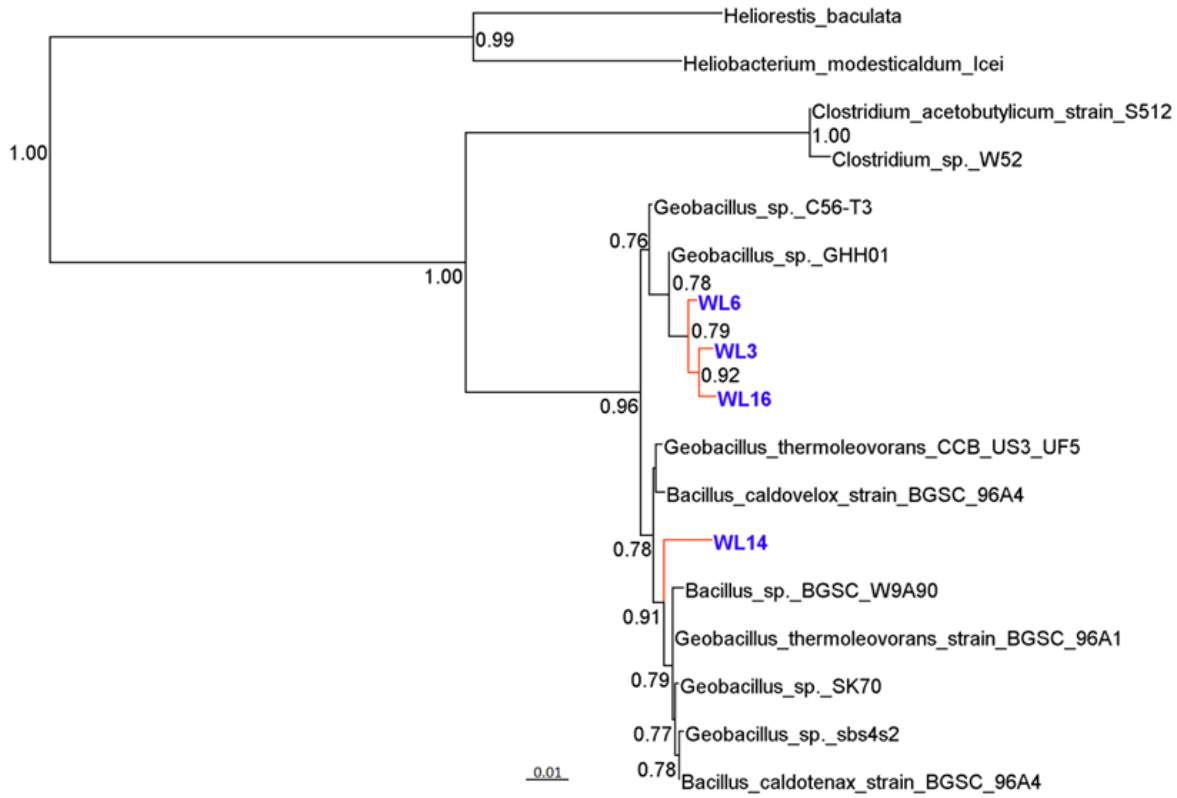


Figure 4.13 Un-rooted phylogenetic tree based on 16S rRNA gene sequences amplified from the WL strains and their closest relatives.
The WL strains are highlighted with red branches and blue labels. The trees shows where the WL strains sit. The out group is represented by *Heliorestis baculata*, *Heliobacterium modesticaldum Icei*, *Clostridium acetobutylicum* strain S512 and *Clostridium* sp. W52. The tree was constructed by estimation of the Maximum Likely-hood Phylogenies by using PhyML 3.0 as described by Guindon et al. (2010). The numbers on the nodes represent the comparisons of bootstrap and aLRT SH-like supports for the branches, while the number of changes per nucleotide is indicated by the *scale*. The higher the signal the higher is the agreement. Values with frequency above 0.5 are displayed on the tree.

4.8 Discussion.

The ability to revive strains of *Geobacillus* spp. previously stored at -80ºC by streaking onto agar plates is highly variable, and this problem was particularly prevalent with the WL strains. As a result of the studies carried out here a simple protocol has been devised that improves colony numbers obtained on agar plates, considerably. Although *Geobacillus* spp. grow rapidly under aerobic conditions it is clear that the rate of revival far exceeded the growth rate and that the revival process must be occurring at the level of individual cells.

A study of the effects of adding filtered media from revived cultures to freshly inoculated cells should have revealed evidence for any soluble "revival factors". However, in a study with WL14 the time course of revival in the filtered media was not significantly different from that in fresh media, suggesting that soluble "revival factors" were not involved. Therefore, the rapid

increase in cell counts observed by incubation in liquid media must reflect processes occurring within individual cells.

The only obvious differences between conditions in liquid and solid media are that the cells would be completely submerged in liquid media and the efficiency of aeration might be higher due to the shaking of the cultures during incubation. However, the underlying processes were not investigated further. The newly isolated strains from compost, described in subsequent chapters, were successfully revived following the procedure described in this section.

The growth rates of the WL strains on 2TY were all high, and better than that of *Escherichia coli*, on 2TY medium, which has been reported to be around 0.8/h (Gombert and Kilikian, 1997). This includes WL3, which was the slowest among the four WL strains that were used. It is worth noting that, under these growth conditions, exponential growth could be maintained over an extended period.

All of the WL strains examined were able to produce clear zones on plates containing avicel, cellobiose and CMC, using the Congo red indicator for detection of β-glucans (Teather and Wood, 1982), an indication that these WL strains were able to produce cellulase and either take up cellobiose or produce an extracellular β-glucosidase activity. The halos on the ASM + CMC plate were larger than those on the ASM + cellobiose or avicel plate for all the tested WL strains after the 24 h incubation. These findings are supported by the results of an earlier study, in which thermophilic bacteria were reported to have a higher CMC degradation rate than that of xylan, cellobiose and avicel (Ibrahim and El-diwany, 2007). However, after 8 day incubation the cellobiose clear zones were the largest, almost the whole plate was cleared of cellobiose (Fig. 4.3C). There is also a possibility that CMCase could have been degrading the cellobiose, while the diffusion of cellobiose to the cells could have also contributed to the size of the clear zones.

The WL strains were able to grow on different carbon substrates, giving the best growth on xylan and the least growth on avicel. The similar growth observed in the cultures on CMC + modified ASM + 0.1% YE and that on ASM + 0.1% YE (control) showed that the strains were not able to use CMC for growth, but instead used the easily available yeast extract in the medium. Apart from WL3, all the WL strains had cellulase activity, which was at least partially constitutive. WL14 had the highest activity recording a value of 1.68 μmoles/min/mg protein for CMCase specific activity (Fig. 4.5A). This value is lower than that of wild type and mutants

of *Pseudomonas fluorescens,* where WT, CRRmt$_4$ and CRRmt$_{24}$ CMCase specific activity was 6.77, 5.92 and 6.88 IU/mg protein, respectively (Bakare et al., 2005).

All four strains appeared to show avicelase activity, although the time course of expression was atypical. The strains were able to secrete avicelase which is able to hydrolyse the avicel to cellobiose (Kim & Kim, 1995). The avicelase activities reached by these strains during this experiment were higher than the 0.17 IU/mg reported for a *Bacillus* sp., though this activity was reported to have been consistent over several weeks (Kim & Kim, 1995). The low activities in the 0.1%YE + ASM control medium, together with the zymograms gave an indication that these strains have a constitutive cellulase activity.

Native-PAGE gels analysis of supernatants from cultures grown for an extended period (8 and 18 days) showed a number of bands some of which appeared only in the avicel cultures and not in the controls, and vice versa. All the strains, except for WL3, showed two bands on the Coomassie brilliant blue stained gel in the locations corresponding to the locations of the clear bands on the zymogram. Although the lower band was not evident in the samples from the WL3 cultures, the position of the ubiquitous upper band seemed to coincide more accurately with the cellulase activity on the zymogram (Fig 4.6C and D). As these bands were also present in the controls this demonstrates that activity must be at least partially constitutive.

However, results from the mass spectrometry analysis showed that no cellulase was detected in the clear bands. Two enzymes were detected in the sample from the clear bands in the corresponding location on the Congo red CMC gel (Fig. 4.6D) by mass spectrometry as xylan-1,4-β-xylosidase from *Geobacillus* sp. (closest strain being *stearothemophilus*), Δ-1-pyrroline-5-carboxylate dehydrogenase from *Geobacillus kaustophillus* (closest strain being HTA426), and other dehydrogenases. This may be an indication that some enzymes might have activity on other substrates other than their known substrates, or some proteins could have more than one function (moonlighting proteins). It has been demonstrated in a previous study that dehydrogenase enzymes [cellobiodehydrogenases (CDHs)] can act synergistically with polysaccharide monooxygenases (PMOs) in cellulose hydrolysis (Wilson, 2012). This could be an indication that the detected dehydrogenase enzymes may participate in similar reactions.

The CMCase activity detected on the CMC native-PAGE gels was also consistent with evidence from the CMC plates (Figures 4.2D and 4.3D) and the CMCase specific activities for WL14 (Fig. 4.5A), obtained during this study, in which WL14 showed higher CMCase activity

than WL3, WL6 and WL16. These results indicate that WL14 could be a good source of endo-glucanases.

Growth of WL strains on untreated and treated *M. x giganteus* biomass indicated that all four WL strains were able to grow slowly on both the pretreated and untreated miscanthus. Better growth on the untreated biomass was probably based on soluble material which leached out, which would not be present in the pretreated material. WL14 gave the best growth on pretreated miscanthus, showing high xylanase activity and lower avicelase and CMase activity (Fig. 4.8). However, none of the WL strains degraded pretreated *M. x giganteus* extensively, suggesting that growth was probably due to degradation of xylan oligomers generated during pretreatment.

Phylogenetic analysis based on the 16S rRNA gene sequence showed that all the four WL strains were associated with the genus *Geobacillus,* with WL3, 6 and 16 being closely related to *Geobacillus* sp. GHH01, while WL14 was closely related to *G. thermoleovorans* strain BGSC 96A1 (Fig. 4.13).

5. New microbial isolations from domestic compost using pretreated *M. x giganteus* as carbon source.

5.1 Introduction.

The degradation of lignocellulose in natural environments is mostly achieved by the action of mixed microbial communities (Perez et al., 2002). These mixed microbial communities produce enzymes which degrade the plant biomass (Eichorst et al., 2013). The hydrolysis of plant biomass by microbial enzymes is very important for carbon recycling as well as production of biofuels and other important products used in industrial processes. The microbial enzymes need to overcome the plant cell wall recalcitrance for successful degradation of lignocellulosic biomass, which is a key process to the successful development of consolidated bioprocessing.

Microorganisms capable of carrying out fermentation at high temperatures are potentially very useful for the economical production of biofuels. Fermentation at high temperatures will not require expensive cooling processes, and the cost of recovering the produced ethanol will be greatly reduced (Balakumar and Arasaratnam, 2012). Micro-organisms capable of growing at very high temperatures have high growth and metabolic rates (Skinner and Leathers, 2004). Thermophilic microorganisms and thermostable enzymes are better for lignocellulosic biomass conversion because enzymatic hydrolysis processes carried out at low temperatures are said to require high dosages of enzymes which still result in low enzymatic hydrolysis rate, with only low sugar yields which are often partially hydrolysed (Bhalla et al., 2013a). The problem of contamination during the fermentation process is also greatly reduced at high temperatures.

The characterisation of thermophilic glycoside hydrolases (and their genes), and other enzymes involved in the degradation of pre-treated *M. x giganteus* was a primary objective of this project. Domestic compost collected from West London Composting Company was used as inoculum to provide a natural mixed microbial community of thermophilic/thermotolerant microorganisms specialised in the degradation of plant biomass. Pretreated *M. x giganteus*, a promising renewable source of plant biomass for production of chemicals or fuels, was used as the only carbon source in the growth medium.

5.2 Growth of compost cultures on pretreated *M. x giganteus.*

5.2.1 Monitoring growth of cultures.

Cultures were started by using inoculum from domestic compost from West London Compost Company as described in chapter 2. These domestic compost samples mostly contained garden compost, which was mostly grass and garden plants. It was hoped that the thermophilic bacterial population present in these compost samples were adapted to degradation of grass substrates and would therefore be able to grow on *M. x giganteus*. These isolations were done using *M. x giganteus* (1% w/v) in Rastogi Isolation Medium (RIM), as a primary carbon substrate to find consortia of organisms that were able to degrade it.

Two aerobic cultures were set up initially, namely, TR1A and TR2A (TR refers to treated *Miscanthus x giganteus* as the only carbon source in the growth medium, and culture 1A and culture 2A as labels for the two starting cultures) and a control which was not inoculated, but contained 1 g of pretreated *M. x giganteus*, all in unbuffered Rastogi isolation medium (RIM). Another set of two aerobic cultures were set up the same way as TR1A and TR2A, except that untreated *M. x giganteus* was used in place of the pretreated *M. x giganteus*. These cultures were called UT1A and UT2A (UT refers to untreated *M. x giganteus*, and 1A and 2A were culture labels). A control (without inoculation) was also set up for the untreated cultures.

The inoculated cultures and controls were incubated for one week at 55^{o}C with shaking at 250 rpm, then each culture was subcultured into fresh 1 g pretreated *M. x giganteus* in unbuffered RIM. The UT1A and UT2A cultures were also subcultured into 1g untreated *M. x giganteus* in unbuffered RIM. Controls were set up for the pretreated and untreated *M. x giganteus* without addition of inoculum. Growth of the cultures was monitored by recording the suspended protein concentration over a period of 17 days (Fig. 5.2A and B), as an indirect measure of growth, as described by (Ishida et al., 1997), as measurements of cell density were likely to be inaccurate due to the presence of suspended solids and microbial growth directly on the solid biomass (which was allowed to settle before taking samples for protein measurement). After six weeks of incubation the remaining miscanthus and cells were centrifuged, the pellets were put into small weighing boats and dried as shown in Fig. 5.2C-i and ii.

Both TR1A and TR2A initial subcultures grew, but TR1A subculture 1 grew better (Figures 5.1A and B, and 5.2 A) and degraded more pretreated *M. x giganteus* than TR2A subculture 1 (Fig. 5.2C-i and ii).

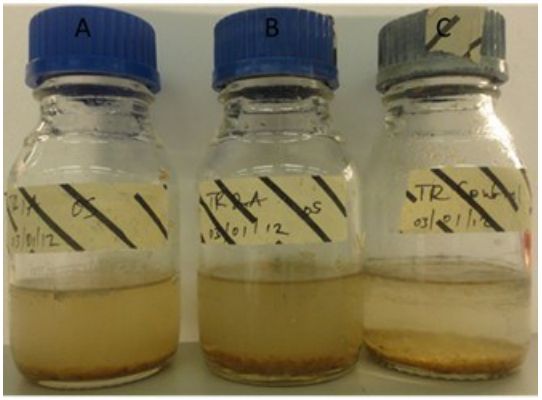
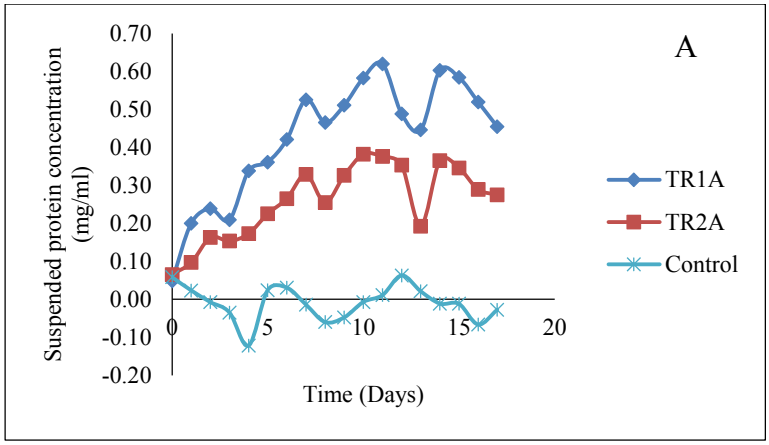
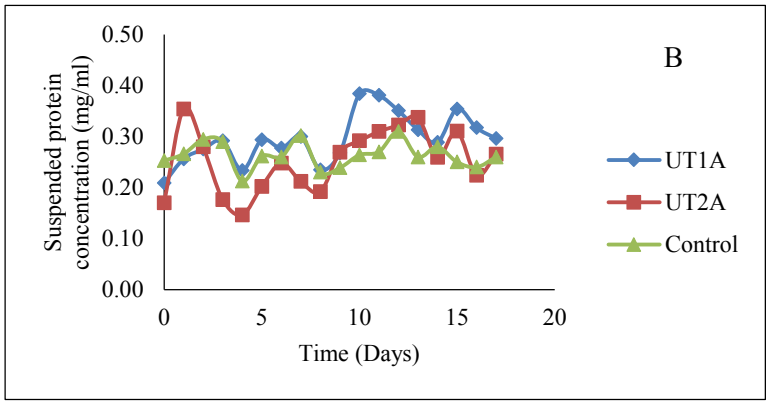


Figure 5.1 Two week old initial subcultures started by using inoculum from the original TR1A and TR2A cultures. The subcultures, TR1A (A) and TR2A (B), were grown on 1% pretreated *M. x giganteus* in RIM at 55°C with shaking at 250 rpm. The control (C) was not inoculated but incubated in the same medium under the same conditions.

Neither UT1A nor UT2A subcultures grew on untreated *M. x giganteus*, as shown by figure 5.2B. Both UT1A and UT2A cultures looked clear just like the control for pretreated *M. x giganteus* (Fig. 5.2C-i). Thus, UT1A and UT2A subcultures appeared to be unable to degrade untreated *M. x giganteus* over the incubation period (Fig. 5.2C-i and ii). However, the untretreated miscanthus cultures gave a high initial protein concentration, presumably arising from the miscanthus itself. Although the untreated miscanthus subcultures did not show any increase in protein concentration it is possible that cells may have been growing on the released protein components, giving no net increase in protein concentration (Fig. 5.2B).

Culture TR1A was selected and used for further subcultures, as it showed better growth (Figures 5.1A and B, and 5.2A) and degradation of the pretreated *M. x giganteus* than TR2A (Fig. 5.2C). Only pretreated *M. x giganteus* was used for the subsequent experiments.





C

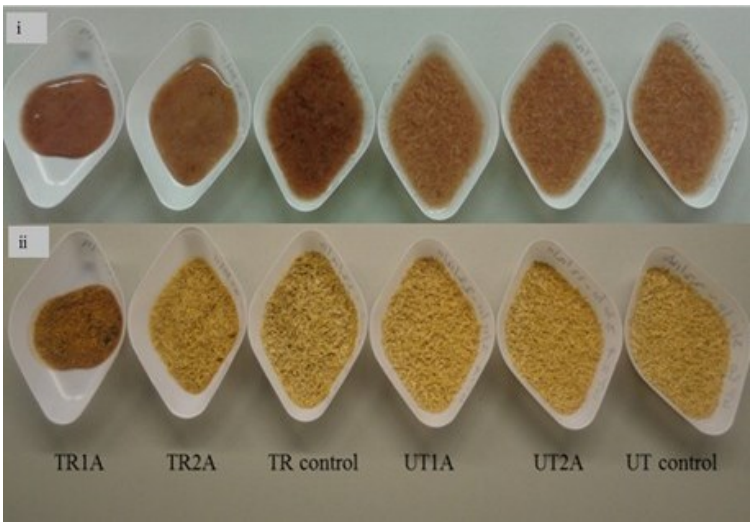


Figure 5.2 Comparison of growth of the intial TR1A and TR2A subcultures on pretreated and untretreated *M. x giganteus*.
(A) Comparison of growth of the TR1A and TR2A first compost subcultures shown in Fig. 5.1. grown on 1% w/v pretreated *M. x giganteus* in unbuffered Rastogi isolation medium (RIM) and the control. (B) UT1A and UT2A grown on 1% w/v untreated *M. x giganteus* and the control in unbuffered RIM. (C) Remaining pellets of the cultures of TR1A, TR2A, UT1A and UT2A and their controls on pretreated (TR) and untreated (UT) *M. x giganteus* in unbuffered RIM after six weeks of incubation at 55°C with shaking at 250 rpm. The cultures were centrifuged in 50 ml eppendorf tubes at 4 000 x g at 20°C for 20 min using an Eppendorf 5810-R; after centrifugation the pellet from each culture was collected on weighing boats as shown in (C-i). The pellets after drying are shown in (C-ii).

5.2.2 Microbial growth on varying amounts of pretreated *M. x giganteus*.

Initial isolations were done using 1% (w/v) miscanthus. Because of concerns that the isolates may be growing solely on the more easily accessible hemicellulose fraction, an experiment was set up to investigate growth on different amounts of miscanthus. This was done by setting up TR1A subculture 2 using inoculum from the TR1A subculture 1.

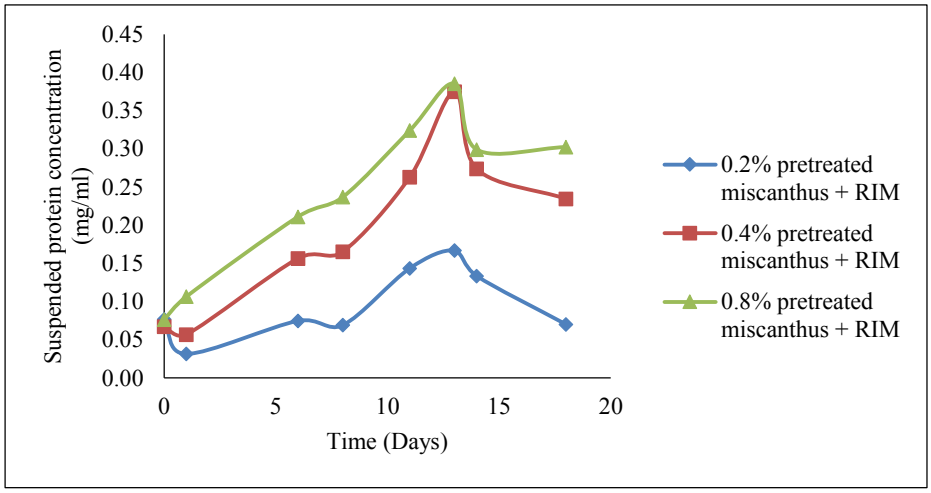


Figure 5.3 Comparing growth of TR1A mixed culture on varying amounts of pretreated *M. x giganteus*. Prepared from the first subculture by taking 0.5 ml of the TR1A subculture 1 (taken after 2 weeks) and subculturing into each of 0.2%, 0.4% and 0.8% pretreated miscanthus + unbuffered RIM. Microbial growth was monitored by measuring the suspended protein concentration. The plotted results are the averages of two subcultures for each amount of miscanthus.

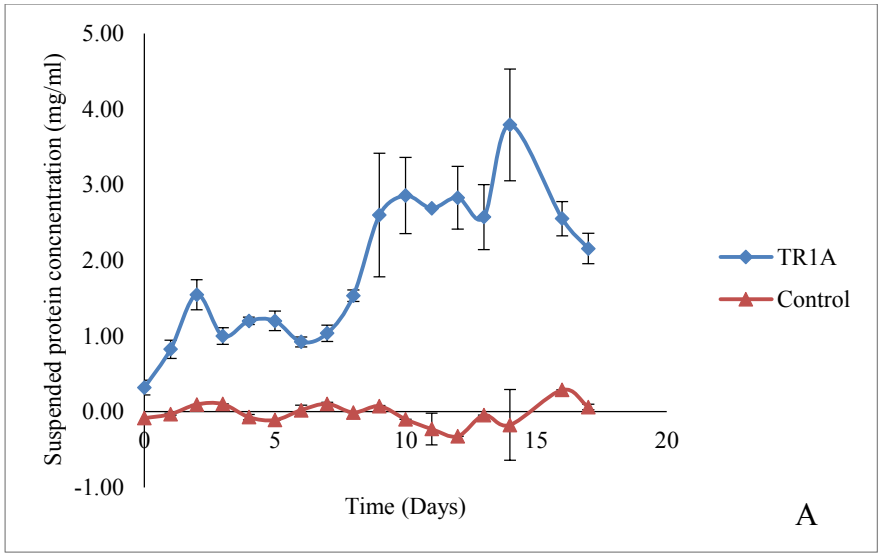
The results in Fig. 5.3 showed that microbial growth rate was higher with a higher content of pretreated miscanthus than with lower amounts. Also, the 0.8% pretreated miscanthus gave the suspended protein concentration slightly higher than that of 0.4% pretreated miscanthus on most of the days during the incubation period. The 0.2% miscanthus subculture gave the lowest suspended protein concentration throughout the incubation period.

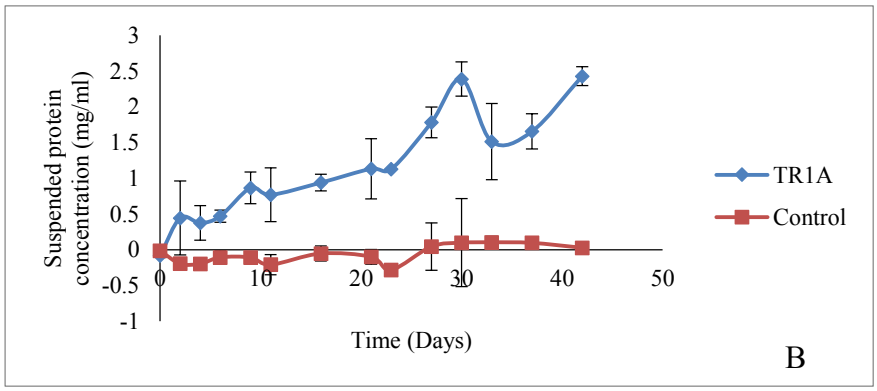
These results could be an indication that the microbes were growing on the easily accessible components of the pretreated miscanthus whose amounts could increase with more miscanthus. However, it could also be the case that the microbial community were able to degrade more pretreated miscanthus with increase in miscanthus concentration as the degrading enzymes were presented with more substrate when supplied with more pretreated miscanthus. Therefore, 1% w/v concentration of pretreated miscanthus in RIM was chosen and used throughout this study.

5.2.3 Growth in buffered RIM.

The Rastogi isolation medium (RIM) was buffered for subsequent experiments as cultures in buffered RIM (Fig. 5.4) were found to grow to higher cell densities than in unbuffered medium (Fig. 5.2A). The TR1A culture was repeatedly subcultured five times, with six week intervening growth cycles, to obtain a stable microbial community capable of consistently degrading pretreated *M. x giganteus*. Three subcultures of this stable community incubated under the same conditions for about 2 weeks (Fig. 5.4A), 6 weeks (Fig. 5.4B) and 8 weeks (Fig. 5.4C) were used to monitor growth of the mixed microbial community by analysing the cell protein accumulating in the liquid fraction of the culture.

Growth of the TR1A subcultures improved with the addition of the buffers to the RIM. The unbuffered TR1A subculture in Fig. 5.2A gave suspended protein concentration below 1mg/ml after a two week incubation period, whereas the buffered subculture in Fig. 5.4 A gave a protein concentration of 1 mg/ml or more from day 2 onwards. The growth of cultures could be reproduced after storage at -80^{o}C in either glycerol or without glycerol. Reproduction of cultures from non-glycerol stocks from -80^{o}C could be an indication that some members of the mixed microbial group in the TR1A culture were spore formers.





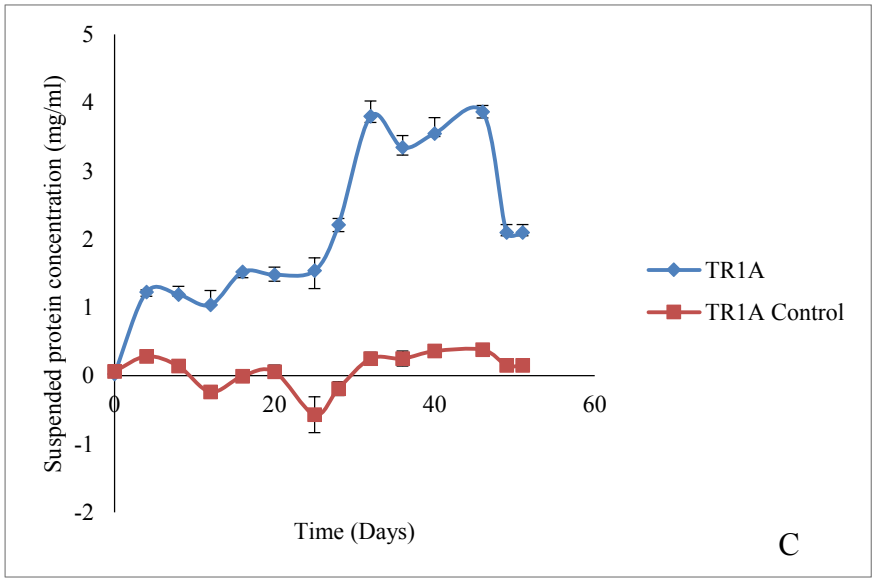


Figure 5.4 Growth of TR1A subcultures 6, 7 and 8.
The TR1A subcultures 6 (A), 7 (B) and 8 (C) were grown on pretreated miscanthus in buffered RIM. Growth of the subcultures was monitored for about 2 weeks in (A), 6 weeks in (B) and about 8 weeks in (C). Growth was monitored by measuring suspended protein concentration according to the method described by Ishida et al., (1997). The error bars represent analytical standard deviation.

5.2.4 The effect of increased aeration on growth of the TR1A subculture 6.

The apparent growth rate and final cell density was much higher when TR1A subcultures were incubated with shaking in 250 or 500 ml conical-flasks stoppered with cotton wool (Fig. 5.5), as the cultures were more vigorously aerated due to the shape of the flasks. Shake flasks had not been used previously due to evaporation of the culture liquid over the long incubation periods.

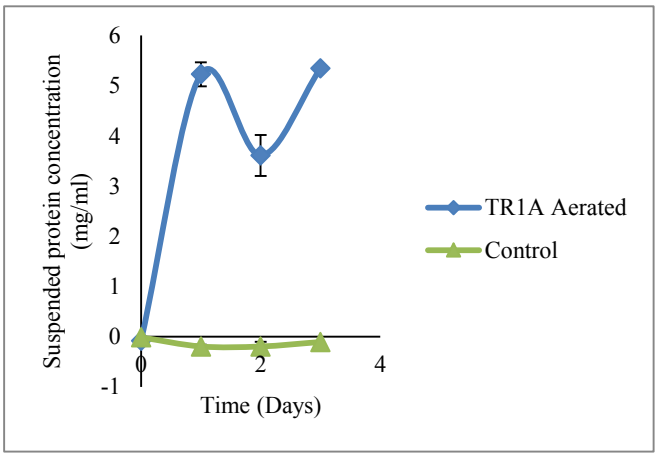


Figure 5.5 The effect of increased aeration on the growth of TR1A subculture 6.
The TR1A subculture 6 and the control were grown in a flask stoppered with cotton wool shaken under the same conditions as the previous experiments. The error bars refer to standard deviation of three analytical replicates.

Provision of improved aeration in the flask clearly favoured growth of the aerobic members of the community, which were subsequently shown to dominate the culture, at least in terms of variety of strains (Figures 6.1 and 6.2). However, the Duran incubation bottles were still preferred for the experiments in this study because evaporation of the culture liquid was minimised. Some of the investigations required long periods of incubation and cultures incubated in flasks dried out before the biomass biodegradation was complete. While this could have been compensated by addition of water to the cultures, this would have confused the sequential growth rate measurements. Furthermore, despite the presence of air, a lower rate of aeration could potentially favour mixed aerobic and anaerobic communities.

5.2.5 Microbial degradation of the pretreated *M. x giganteus* by the enriched culture.

An investigation was carried out to find out which part of the TR1A culture contained the microorganisms involved in the degradation of the pretreated *M. x giganteus*. The effect of

storing the TR1A culture without glycerol at -80ºC on the ability of the microbial community to degrade the pretreated miscanthus was also assessed. Fig. 5.6 shows the centrifuged TR1A subcultures after 8 weeks of incubation. The culture in Fig. 5.6A was started by using inoculum from an ongoing TR1A subculture 6 with inclusion of some undegraded pretreated *M. x giganteus* solids in the inoculum; B was started using a non-glycerol -80ºC stock of TR1A subculture 6 which also included undegraded pretreated *M. x giganteus* solids in the inoculum, while the liquid fraction only of the ongoing TR1A subculture 6 was used to start culture C. Fig 5.6D-i and D-ii shows dried pellets of B1 and 2, respectively. All these cultures were grown for approximately 8 weeks.

The pellets of residual solids from the inoculated cultures were much smaller than in the controls. The remaining pellets from the cultures (Fig. 5.6A-C, tubes labelled 1) show that the pretreated *M. x giganteus* was extensively degraded compared to the controls (Fig. 5.6A-C, tubes labelled 2). Also, the remaining pellets from the cultures (Fig. 5.6D-i) looked much finer than the pellets from the controls (Fig. 5.6D-ii).

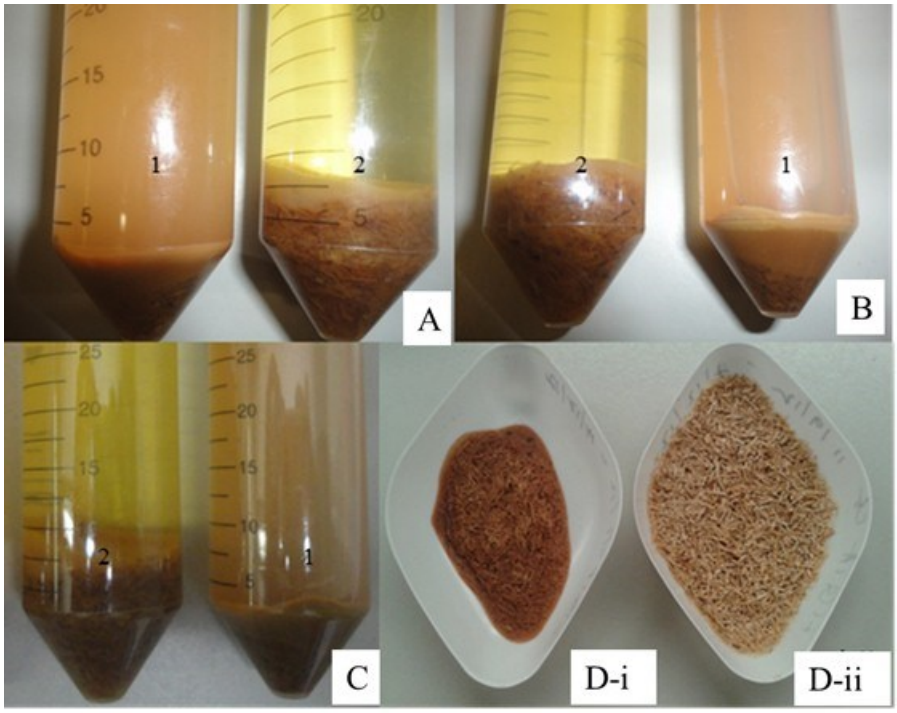


Figure 5.6 Growth of TR1A subculture using different sources of inoculum.
(A) Using inoculum from an ongoing subculture 6 with inclusion of the undegraded particles of pretreated *M. x giganteus* in the inoculum, (B) using stock culture of TR1A subculture 6 stored without glycerol at -80^{o}C, with inclusion of the undegraded particles of pretreated *M. x giganteus* as inoculum, (C) using only the liquid fraction of the ongoing TR1A subculture 6 as inoculum. The tubes labelled 1 are from inoculated cultures, those labelled 2 are from the uninoculated controls. The experimental and control cultures were incubated at 55^{o}C with shaking at 250 rpm and grown for about 8 weeks. The remaining contents of both the control and the inoculated cultures were centrifuged at 20^{o}C for 20 min in 50 ml centrifuge tubes at 4 000 x g in an Eppendorf 5810-R and dried. Fig. 5.6D-i and D-ii shows the dried pellets for TR1A subculture, and the control, respectively, of Fig. 5.6B.

5.2.6 Production of pigments during growth of TR1A subcultures.

After about two weeks of growth the TR1A cultures always gained a reddish pigmentation which increased with incubation time (Fig. 5.7A–C). After centrifugation of the liquid fraction of the cultures, the pellet from the TR1A culture looked reddish, while that of the control had the colour of pretreated miscanthus (Fig. 5.8A and B). The pigmentation was also observed in the dried sample pellets of the TR1A subcultures, but not in the pellets from the controls (Fig. 5.6D-i and D-ii). The red colouration could have resulting from pigments formed by some of the microbes (eg. *Miothermus* spp. [Yarger et al., 2006], *B. thermoruber* and *B. lichenformis* detected during this study [Figures 6.5 and 6.6, respectively]).

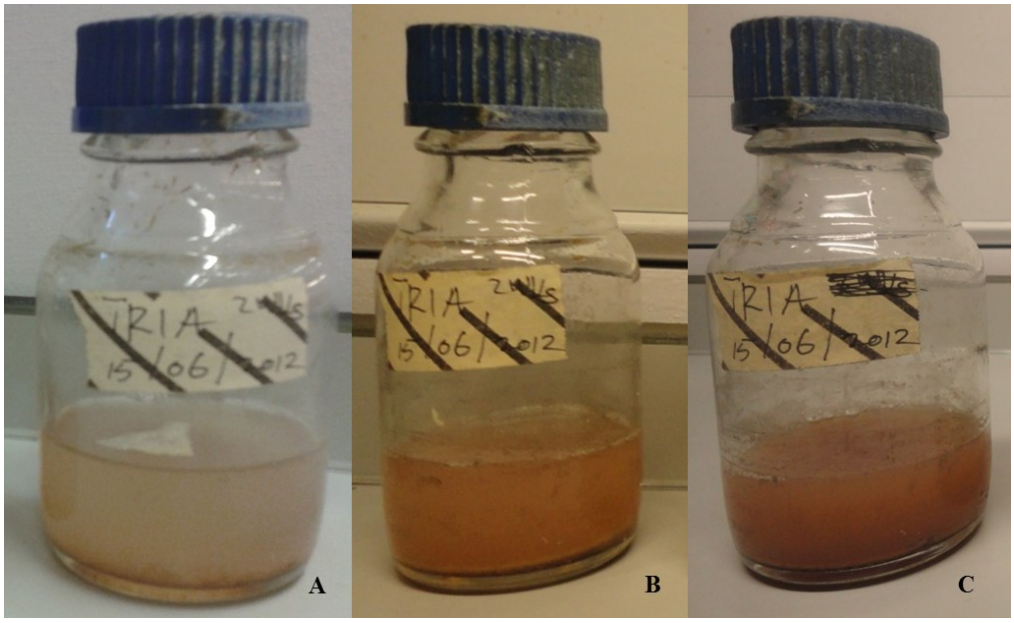


Figure 5.7 Pigmentation in TR1A cultures.
Two week (A), 1 month (B), and two month and three week old (C), TR1A subculture 4.



Figure 5.8 Colour of the pellets from TR1A subculture and control.
The pellets from subculture 4 (A) and the control (B) were prepared by centrifuging 0.5 ml of the liquid fraction of the subculture.

5.2.7 Degradation of pretreated miscanthus in TR1A subcultures.

Fig. 5.9 shows the weights of the pellets recovered by centrifuging both the remaining solids and liquid fractions of TR1A subcultures 6, 7 and 8, and their controls after 6 weeks of incubation. Each subculture was grown for six weeks before being used as inoculum for the next subculture. The oven dry weights of the harvested pellets are shown in Fig. 5.9. The results confirm that degradation of pretreated *M. x giganteus* had occurred in all of the cultures, with just over 50% of the pretreated *M. x giganteus* being degraded in the TR1A subcultures

over a six week period of incubation (ignoring the contribution of the microbial cells to this dry weight).

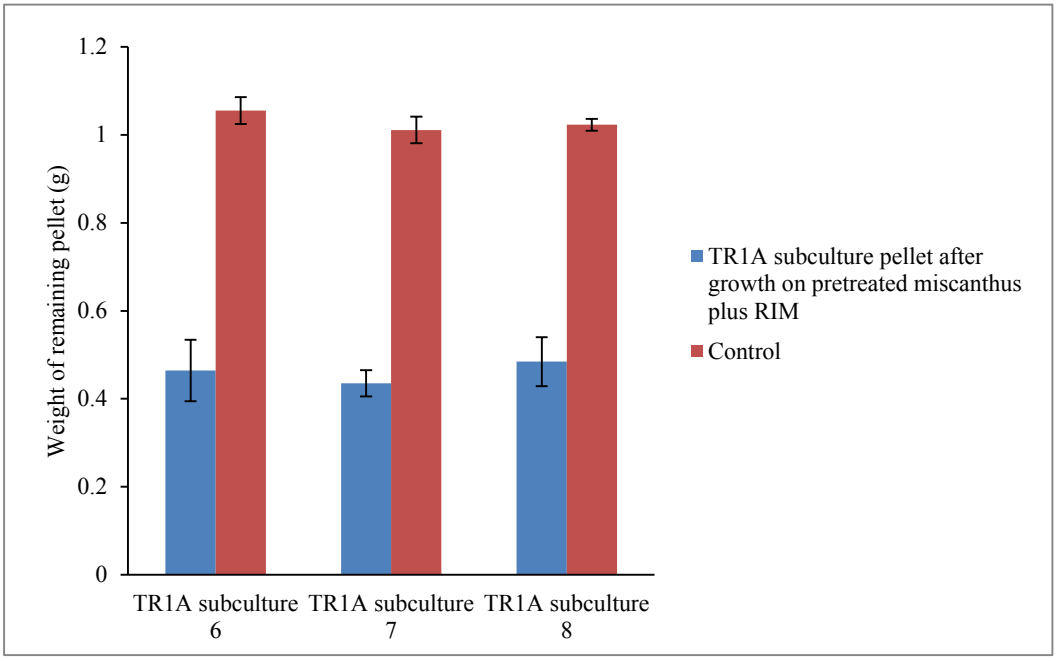


Figure 5.9 Weight of the remaining pellets from TR1A subculture 6, 7, 8, and their controls.
The subcultutres were grown on 1 g pretreated *M. x giganteus* in RIM at 55ºC with shaking at 250 rpm for six weeks. The recorded results are oven dry weights of the samples. The error bars represent standard deviation of three analytical replicates.

5.3 Compositional analysis of lignocellulose residues from the TR1A subcultures.

Although some of the original miscanthus had clearly been degraded, it was not clear whether all fractions were being degraded equally or whether some components were being preferentially utilised. Compositional analysis was carried out on the remaining pretreated miscanthus after 6 weeks growth of TR1A subculture 6 to examine the change in carbohydrate and lignin content due to microbial degradation. The compositional analysis of the TR1A subculture 6 pellet obtained after 6 weeks growth was carried out using the NREL-LAP method as outlined by Sluiter et al., (2008). The resulting composition was compared with the composition of the undegraded pretreated miscanthus from the 6 week control to determine the carbohydrates that had been degraded.

Over 50% of the mass of pretreated *M. x giganteus* was degraded in the TR1A subculture 6 during the 6 week incubation period (Fig. 5.10). This was made up of close to 56 % of the glucose, just over 50 % of xylose and 47 % of the arabinose. No mannose was detected in the

undegraded miscanthus pellet, an indication that mannose was easily degraded. These results indicate that the mixed microbial community in the TR1A subcultures was using all four types of carbohydrates present in the pretreated *M. x giganteus* with only marginal selectivity for glucose.

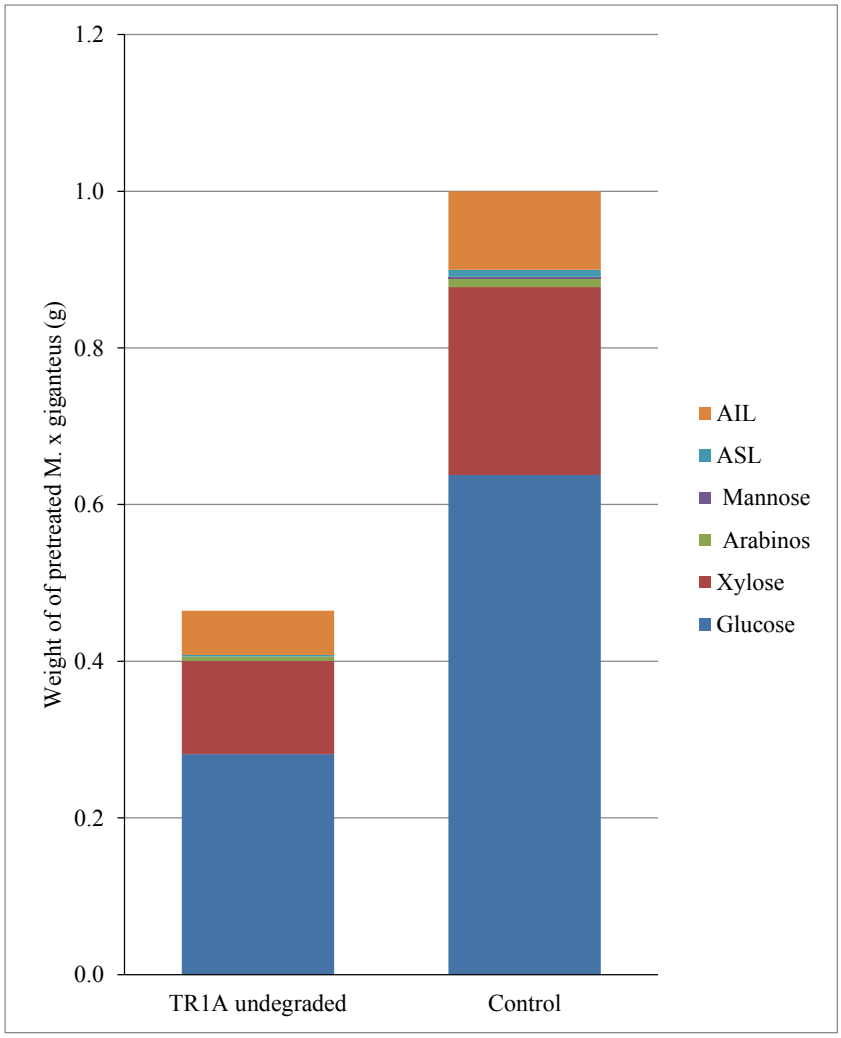


Figure 5.10 Relative compositional analysis of residues from the TR1A subculture 6 and control.
The subculture and control were grown for six weeks and were represented as the proportion of the residual dry weight.

Fig. 5.10 also shows that some of both the Acid Soluble Lignin (ASL) and the Acid Insoluble Lignin (AIL) appeared to have been degraded in the TR1A subculture 6 during the incubation period. Most of the ASL was degraded, while just below 50 % of the AIL had degraded during the 6 week incubation period. This suggests that some ligninase activity must have been present in the TR1A subculture.

Microorganisms have been reported to produce ligninolytic enzymes. Bacterial species such as *Bacillus* sp. LD003, *Pandoraea norimbergensis* LD001 and *Pseudomonas* sp. LD002 have been reported to produce ligninolytic enzymes, with *Bacillus* sp. LD003 as the best ligninolytic enzyme producing group (Bandounas et al., 2011). In another study, another *Bacillus* sp., SHC1, was reported to produce higher manganese peroxidase and lignin peroxidase than *Ochrobacterium* sp. SHC2 and *Leucobacter* sp. SHC3 (Rahman et al., 2013).

It was observed during a previous study that expression of ligninolytic enzymes is triggered by limitation of nitrogen and carbon (Dosoretz and Grethlein, 1991), and also due to exposure of cultures to atmospheric oxygen (Dosoretz et al., 1990). The latter leads to accumulation of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide (H_2O_2), as by-products of aerobic respiration in the culture (Belinky et al., 2003). The lignin-degrading peroxidases and the Fenton-type chemistry that degrades lignin non-enzymatically may be supported by the produced H_2O_2 (Sweeney and Xu, 2012).

Superoxide dismutase enzymes protect cells from toxicity due to presence of ROS by catalysing the dismutation of superoxide anion radicals to oxygen and hydrogen (Belinky et al., 2003). The enzyme superoxide dismutase was detected in the supernatants from the TR1A subcultures (Table 5.1), an indication that ROS were present in the cultures. The TR1A subcultures were grown under aerobic conditions, with pretreated *M. x giganteus* as the only carbon source, which was not easily accessible. Exposing the cultures to atmospheric oxygen during transfers could have resulted into formation of superoxides (Bar-Lev and Kirk, 1981; Dosoretz et al., 1990; Leisola et al., 1984).

The presence of superoxides could have triggered the mixed microbial community to express ligninolytic enzymes such as lignin peroxidases. In the presence of hydrogen peroxide the peroxidases catalyse the reactions that lead to lignin degradation (Passardi et al., 2007a; Passardi et al., 2007b). These reactions involve the oxidation of the lignin side chains of non-phenolic lignin units due to removal of one electron in the presence of H_2O_2, due to generation of reactive radicals. This results in the breaking of the C-C and ether linkages, as well as the aromatic rings in the lignin (Coelho-Moreira et al., 2013). This could possibly explain the partial degradation of lignin observed in Fig. 5.10. The general reaction is given below as illustrated by Coelho-Moreira et al., (2013).

1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H_2O_2 ⟶ 3,4-dimethoxybenzaldehyde + 1-(3,4-dimethoxyphenyl)ethane-1,2-diol + H_2O

5.4 HPLC analysis of the TR1A subculture supernatants.

It was observed that when the TR1A subculture inoculation bottles were opened, an aroma similar to that of sweet fermentation products was detectable. An attempt was therefore made to analyse these supernatants for the production of alcohols and other fermentation products. Supernatant samples were collected from the TR1A subculture 8 after two weeks and six weeks of incubation, and also from the six week control. These, as well as the RIM medium were analysed by HPLC (Figures 5.11 and 5.12).

Products with the same retention times as pyruvate (9.6 min) and lactate (12.8 min) were detected by UV analysis in the supernatants of TR1A subcultures (Fig. 5.11A and B). Both increased in concentration with time of incubation, as observed by comparison of the two week (Fig. 5.11A) and six week culture results (Fig. 5.11B). Smaller peaks were also detected at about the same retention times in the control supernatant and the RIM medium, an indication that the observed peaks could be mixed with other components from the RIM medium. Formate, with a retention time of 14 min, was detected in the supernatant from the six week old culture (Fig. 11B).

No monomeric carbohydrates were detected, indicating that they were being used directly by the microbial community immediately upon release, or that the cells may have been consuming oligomeric carbohydrates directly. A previous study reported a similar finding; it was observed that after growing *Anaerocellum thermophilum* DSM 6725 on crystalline cellulose or on xylan, glucose and cellobiose, or xylose and xylobiose, respectively, accumulated in the culture medium, but did not accumulate in the culture medium during growth on plant biomass.(Yang et al., 2009) Similar observations were made during another study in which the produced sugars were immediately converted to hydrogen during bacterial fermentation (Lo et al., 2011). However, it has also been suggested that the reduction in the reducing sugars could be due to the oxidation of cleaved oligosaccharides to their lactones (Turbe-Doan et al., 2013), or the oxidation of glucose to non-fermentable glucuronic acid (Cannella et al., 2012).

Peaks detected by UV at 6.9, 16.2 and 18.6 minutes in both 2 and 6 week samples (Fig. 5.11A and B), and a peak at 16.9 minutes in the 6 week sample (Fig. 5.11B), were not present in the control or the medium, so must represent metabolites produced by the culture. However, they could not be identified with any of the standards used. The first three metabolites also increased in concentration with increasing incubation time as shown in Fig. 5.11A and B.

The presence of lactate in the TR1A culture could have resulted from oxygen limitation, which is probable because the cultures were grown under thermophilic conditions. Detection of lactate and formate in the supernatant from the TR1A subcultures indicates that fermentation of glucose occurred in the TR1A subcultures. Lactate and formate are fermentation products of *Geobacillus* spp. (Cripps et al., 2009). Despite the cultures being aerated, some of the organisms that were detected in the microbial communities in TR1A subcultures turned out to be obligate or facultative anaerobes and hence, capable of fermentation, reducing the amount free sugars in the culture. These include *Thermoanaerobacterium saccharolyticum* (Shaw et al., 2008)*, Brevibacillus thermoruber*, and *Geobacillus thermoglucosidasius* strains (Bihari et al., 2010; Cripps et al., 2009).

There is a possibility that any produced ethanol could have been evaporating as these cultures were grown at 55^{o}C. This could have resulted into too low an alcohol concentration remaining in the cultures to be detected by the RI (which is a relatively insensitive detection technique), (Fig. 5.12). Analysis of the gaseous phase by headspace GC analysis has been used in a previous study (Wei et al., 2013), and could probably have detected traces of alcohol and any light volatiles that were produced in the TR1A subcultures.

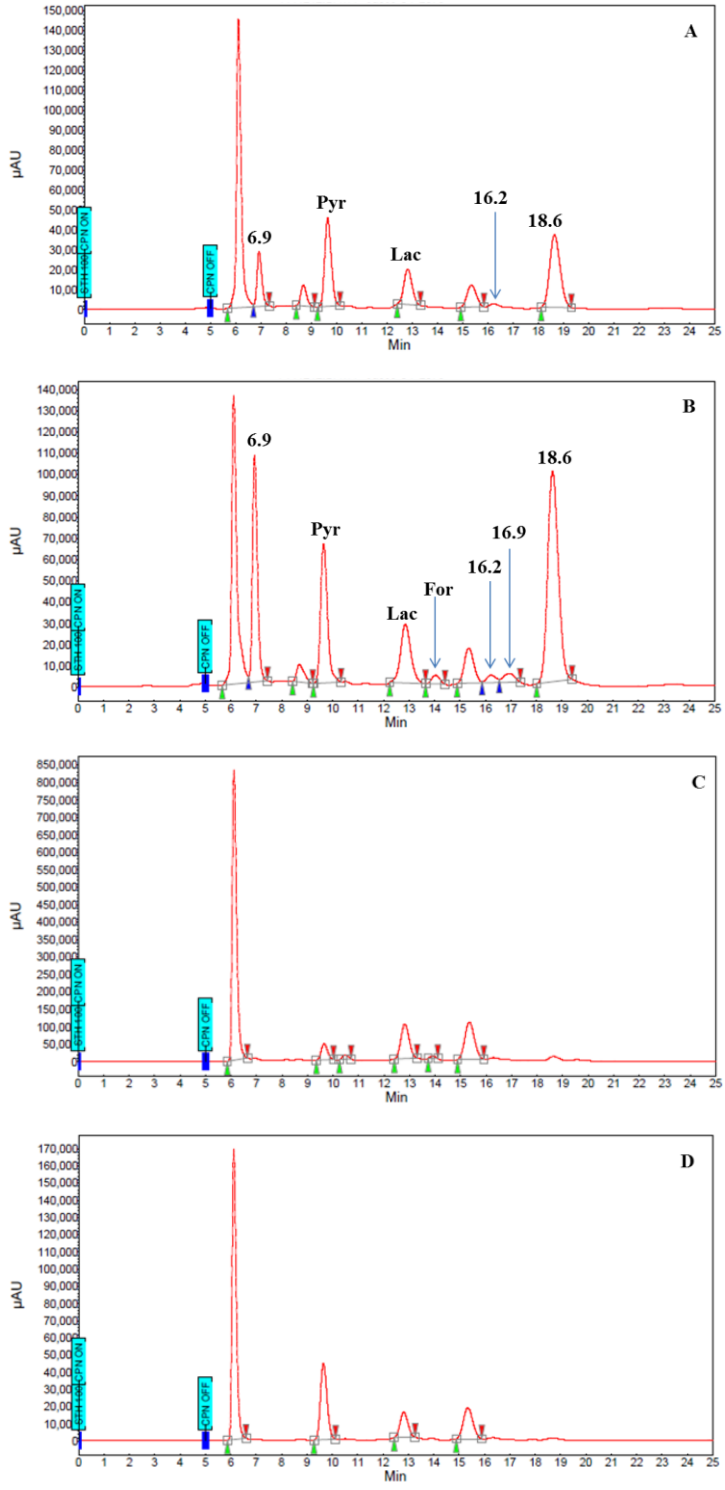


Figure 5.11 HPLC chromatograms with UV detection analysing supernatant from TR1A subculture 8.
HPLC chromatograms of supernatant from TR1A subculture 8 with UV detection using an Aminex HPX87H ion exchange column (300 x 7.8 mm) of TR1A subcultures supernatants after (A) two weeks, (B) six weeks incubation, (C) control supernatant after six weeks incubation, and (D) RIM medium alone. The TR1A subculture and the control were incubated at 55°C with shaking at 250 rpm. The peaks at 9.6 min and 12.8 min in A and B, and 14 minutes in B have retention times which correspond to pyruvate (Pyr), lactate (Lac) and formate (For), respectively. Smaller peaks were detected at these retention times in the control (C) and the medium (D). Peaks detected at 6.9, 16.2 and 18.6 min in both A and B, and the peak at 16.9 min in B could not be identified using any of the standards. The peaks under discussion are labelled with their identified products or retention times and the smaller ones are highlighted with arrows in A and B. The rest of the peaks in A and B were probably components from the RIM medium.

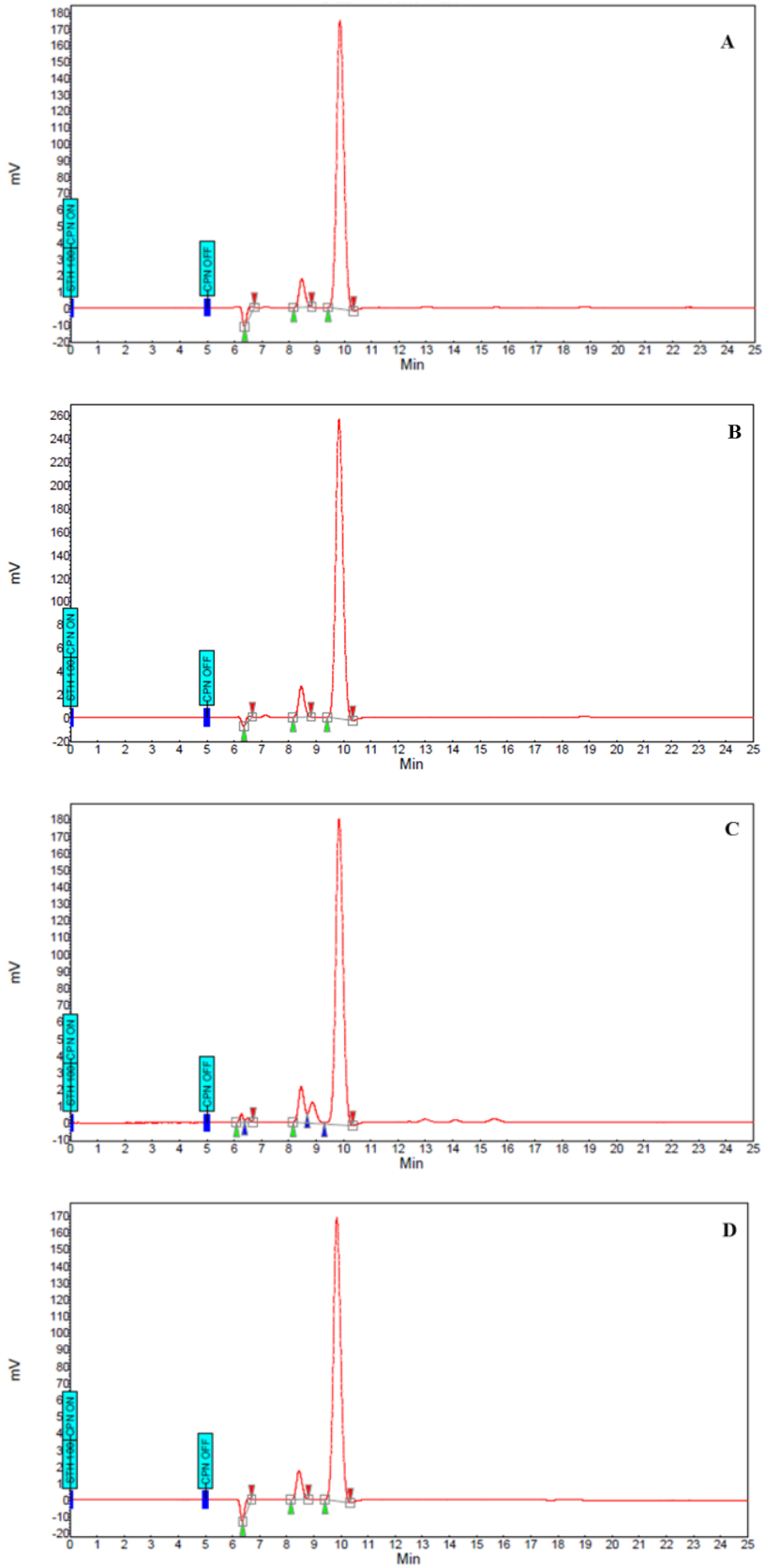


Figure 5.12 HPLC chromatograms with RI detection analysing supernatant from TR1A subculture 8.
The same samples as in Fig. 5.11 were used. Similar peaks were detected in experimental samples, A and B, as well as in the control (C) and the medium (D).

5.5 Enzyme assay of the culture fluid.

5.5.1 Determination of glycoside hydrolase activity.

Assays were carried out to screen for enzyme activities that could be responsible for the observed degradation of the pretreated *M. x giganteus* in the TR1A subcultures. The liquid fraction was collected after two and six weeks incubation from TR1A subcultures 6, 7 and 8, to represent early, middle and late stage TR1A subcultures. The detected enzyme activities for each substrate are recorded in Fig. 5.13.

The specific activity (which is presumably an aggregate of all enzymes with that activity) of avicelase and CMCase increased with both time of incubation (Fig. 5.13A and B) and further subculturing (Fig. 5.13A-C). The avicelase activity for the early and middle subcultures was 0.01 and 0.04 U/mg respectively, for the second week, and 0.02 and 0.08 U/mg respectively, for the sixth week, which, in the case of the early stage cultures, was only slightly higher than the background activity (control). One unit (IU) of enzyme activity is defined as the amount of enzyme that released 1μmole of sugar per minute from xylan, CMC, or avicel. The CMCase specific activity for the early and middle stage subcultures was 0.04 and 0.15 U/mg respectively, for the second week, and was 0.1 and 0.27 U/mg, respectively, at the sixth week.

The avicelase and CMCase activities from the late stage subculture were significantly higher than in the previous stages, after both 2 and 6 weeks (0.83 and 0.7 U/mg, respectively, for avicelase, and 2.09 and 2.00 U/mg, respectively, for CMCase) (Fig. 5.13C) and, unlike the previous cultures, did not increase from 2 to 6 weeks. The increased enzyme activities observed in the late stage TR1A subculture was consistent with the faster growth in this culture than the middle stage subculture as shown in Fig. 5.4. However, this was not true for the early stage subculture as it recorded faster growth than the middle stage subculture. This was possible as the early stage TR1A subculture might have consisted many other microorganisms which were not directly involved in the degradation of the pretreated *M. x giganteus*.

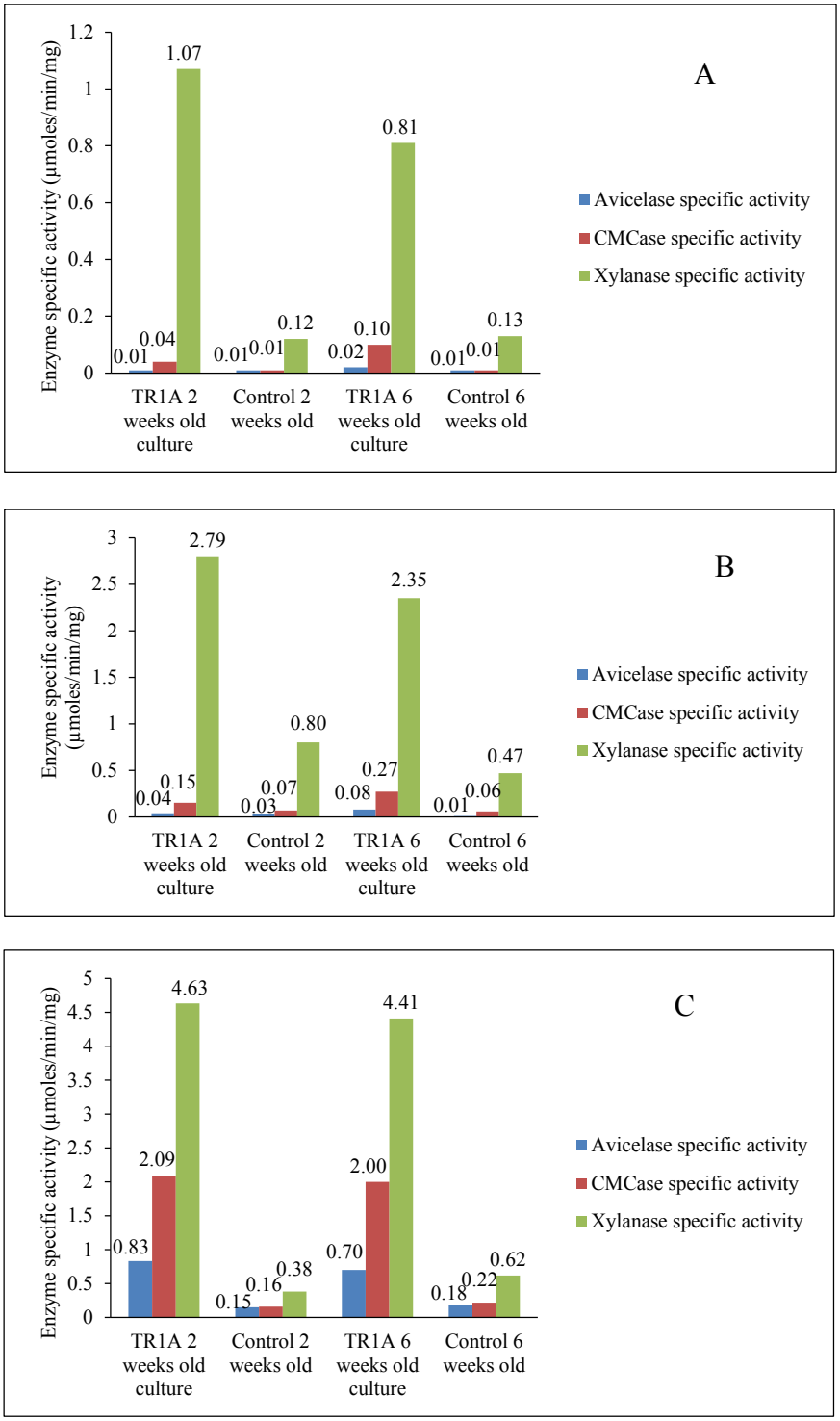


Figure 5.13 Specific enzyme activities in the TR1A subcultures 6, 7 and 8.
Specific enzyme activities of early stage subculture (TR1A subculture 6) (A), middle stage subculture (TR1A subculture 7) (B), and late stage subculture (TR1A subculture 8) (C). The culture fluids from the three different stages of the TR1A subcultures were sampled after two weeks and six weeks of incubation and assayed for avicelase, CMCase and xylanase activity. The harvested cultures were centrifuged at 4 000 x g at 20°C for 20 min in an Eppendorf 5810-R. The collected supernatant (0.5 ml) was concentrated by centrifuging in a bench top accuSpin™ Micro centrifuge (Fisher Scientific) at 10 000 x g for 15 min using a 3 000 MWCO PES vivaspin centrifugal membrane separator from Sartoriuos stedim biotech. The total protein concentration was determined by using the Biuret reagent. The DNS (3,5-dinitrosalicylic acid) method (Miller et al., 1960) was used to measure the enzyme activity. The number on the top of each bar records the actual specific activity for the assay performed.

The xylanase specific activity also increased with subculturing, but the measured activity within each subculture was slightly higher at the second week than by the sixth (Fig. 5.13A-C). The xylanase specific activity for the early subcultures was 1.07 and 0.81 U/mg, for weeks two and six respectively, 2.79 and 2.35 U/mg, for weeks two and six respectively, for the middle subcultures, and 4.63 and 4.41 U/mg for weeks two and six respectively, for the late stage subcultures.

These observations suggest that the initial microbial community in TR1A was growing primarily on hemicelluose derived xylose sugars. At longer incubation times with the early and middle stage subcultures it is clear that some cellulose was starting to be degraded (or at least expression of the relevant enzymes is induced). However, by sub-culture 8 it looks like the culture has been enriched for cellulose degraders and that this activity is induced (or may be constitutive) much earlier in the incubation (Fig. 5.13A-C).

5.5.2 PAGE gel analysis and zymograms.

It is probable that the activities detected in the culture fluids of TR1A are the cumulative activities of a number of enzymes. In order to identify the origins of at least the major activities the proteins concentrated from the culture fluids were also run on native poly-acrylamide gels so that the enzymes could be separated and activities identified. A CMC substrate (0.05%) was added to the gel preparation mixture. Two gels were run for each protein sample; one was stained with Congo red stain to detect enzyme activity on the zymogram and identify the location of the bands showing enzyme activity. The second gel was stained with Coomassie brilliant blue stain and used to establish the relative abundance of the proteins that showed activity on the zymogram. The Coomassie-stained gel and the Congo red gel were subsequently used for excising the relevant protein bands for identification.

5.5.2.1 Zymogram analysis of glycoside hydrolase activity in TR1A initial cultures and subcultures 1, 6, 7 and 8.

Culture fluids were collected from the initial TR1A culture and TR1A subculture 1 at different ages and analysed on CMC-PAGE zymograms for glycoside hydrolase activity. Supernatants from the initial TR2A culture and subculture 1 were also analysed in the same way for the presence of any cellulolytic enzyme activity (Fig. 5.14).

CMCase activity was detected in the initial cultures of TR1A and TR2A (Fig. 5.14B). All of the samples from TR1A subculture 1 (Fig. 5.14D lane TR1A1, and F lanes 2TR1A1, 3TR1A1, 6TR1A1 and 2.5TR1A1) showed clear zones, indicating the presence of CMC degrading glycoside hydrolases. Protein bands showing activity on the zymograms were excised from the Congo red and Coomassie Brilliant blue gels and analysed by mass spectometry for partial protein sequence. The protein sequences from the mass spectrometry analysis were specific for the proteins from the identified microorganisms. No sequence was obtained from gels B and D bands labelled 1 and 2, however bands labelled 3 in gels B and D were identified from their partial protein sequence as xylan 1,4-β-xylosidase from *Thermobacillus composti* (closest strain being KWC4), and glucoamylase from *Aspergillus niger,* respectively. Bacterial spp. with 99% 16S rRNA gene sequence identity to that of *Thermobacillus composti* KWC4 were detected by 16S rRNA gene amplification from the DNA isolated from TR1A subculture 6 pellet (Fig. 6.1A). *Aspergillus niger* would not have been identified by a 16S rRNA screen as amplification of the 18S rRNA gene would have required universal eukaryotic primers.

The observed CMCase activity in the initial TR2A culture (Fig. 5.14B, lane TR2A0) did not persist in TR2A subculture 1. The lanes which contained samples from TR2A subculture 1 (Fig. 5.14D lane TR2A1 and F lanes 2TR2A1 and 6TR2A1) did not show much activity in comparison with those from TR1A subculture 1 (Fig. 5.14D lane TR1A1 and F lanes 2TR1A1, 3TR1A1, 6TR1A1 and 2.5TR1A1). A low activity from TR2A subculture 1 was observed in lane 6TR2A1 of Fig. 5.14F, but this was not as strong as activities detected in TR1A subculture 1. This may explain the marginal degradation of pretreated *M. x giganteus* by the microbial community in TR2A subculture 1 (Fig. 5.2C-i and ii), relative to TR1A.

The results also showed that different enzymes were predominant in the culture at different sampling stages, shown by the different positions of the zones of clearing when analysing samples from different stages of the cultures (Fig. 5.14B, D and F). Samples from two week old cultures of TR1A subculture 1 and TR1A subculture 6, 7 and 8, (Fig. 5.14F, lane 2TR1A1, Fig. 5.15B lane 2TR1A6, Fig. 5.16B lane 2TR1A7 and Fig.5.17D, lane 2TR1A8 CR, respectively) showed different clearing zones on the zymograms, while lane 3TR1A1 of Fig. 5.14F and lane 6TR1A6 of Fig. 5.15B gave a similar pattern of clear zones for samples collected at three weeks and at six weeks. This indicates that the expression profile of different subcultures was changing during the sub-culturing stages, but similar profiles were obtained at different sampling times during any single stage.

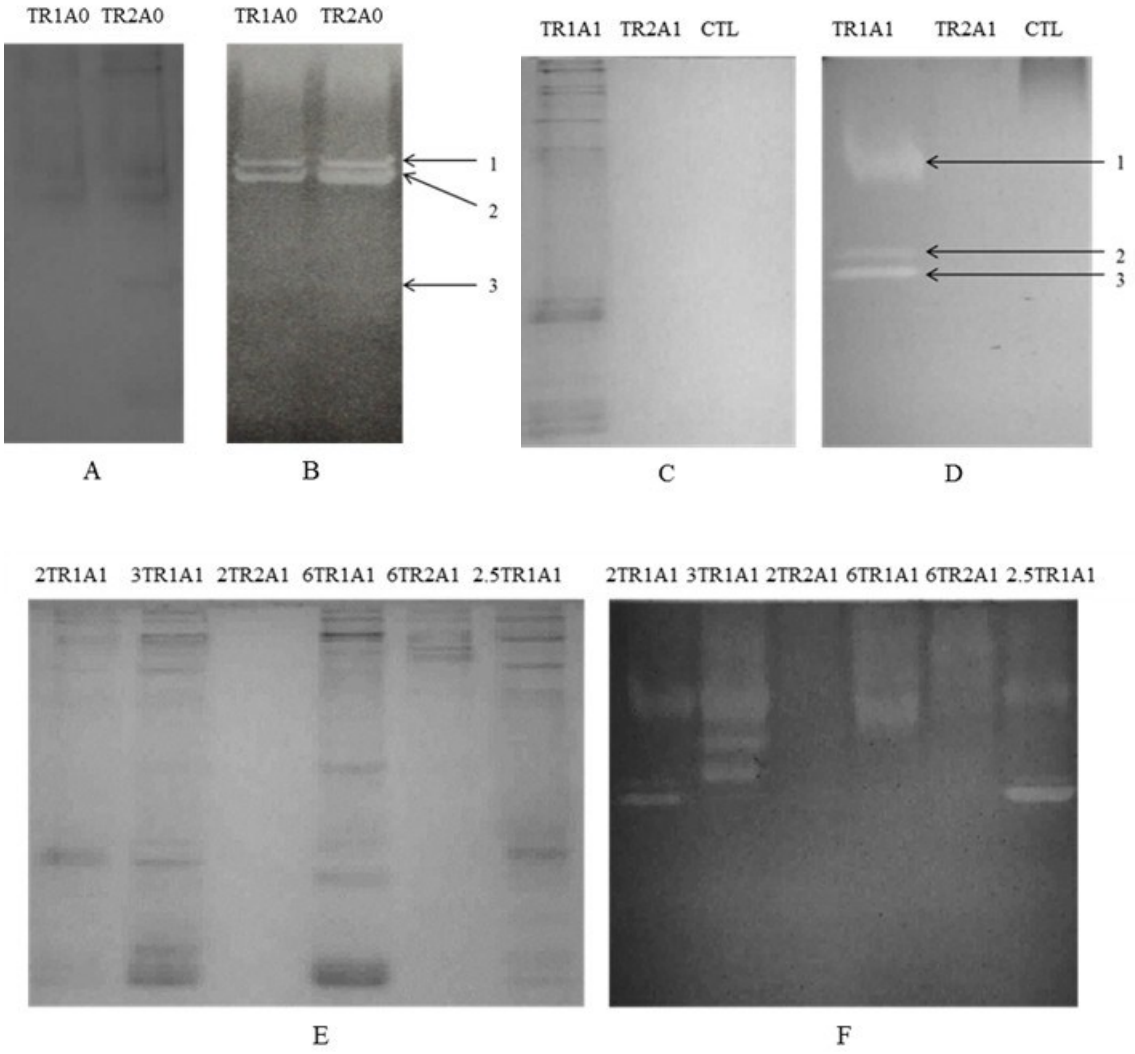


Figure 5.14 Native CMC PAGE gel (12%) of TR1A and TR2A cultures.
Coomassie brilliant blue stained 12% native PAGE gels of initial TR1A and TR2A cultures on pretreated *M. x giganteus* (A, C and E) and Congo red stained native PAGE zymograms of liquid samples from initial TR1A and TR2A cultures (B), TR1A and TR2A first subcultures (D and F), for analysis of CMCase activity at different ages of the culture. About 0.05% CMC (from Sigma) was added to the native gel preparation mixture. Samples from 5 day old initial cultures of TR1A and TR2A were analysed on gels A and B, lanes TR1A0 and TR2A0 for TR1A and TR2A, respectively. TR1A and TR2A suculture 1 were analysed on gels C to F. Two week old first subcultures of TR1A and TR2A were analysed on gels C and D, TR1A subculture 1 is in lane TR1A1, TR2A subculture 1 is in lane TR2A1, and the control is lane CTL. In gels E and F, TR1A two week old subculture 1 is in lane 2TR1A1, three week old culture in lane 3TR1A1, TR2A two week old subculture 1 in lane 2TR2A1, TR1A six week old subculture 1 in lane 6TR1A1, TR2A six week old subculture 1 in lane 6TR2A1 and two and half week old TR1A subculture 1 in lane 2.5TR1A1.

Mass spectrometry of band 1 (Fig. 5.15B) revealed that it contained multiple enzymes, including xylan 1,4-β-xylosidase from *Geobacillus* spp. (closest strains being G11MC16 and Y412MC52), and xylose isomerase from *Bacillus subtilis* and *Thermobispora bispora* (closest strains being ATCC 19993 / DSM 43833 / CBS 139.67 / JCM 10125). The more discrete bands labelled 2 to 4 were identified as β-xylosidase from *Geobacillus stearothermophilus* and *Paenibacillus* sp. (closest strain being DG-22), glucoamylase from *Aspergillus niger*, and

xylan 1,4-β-xylosidase from *Thermobacillus composti* (closest strain being KWC4), respectively. These and other enzymes identified from these bands are also shown in Table 5.1. All these bacterial genera have been reported to have CMCase activity (Rastogi et al., 2010), *T. bispora* DSM 43833 (formerly known as *Microbispora bispora*) was also reported to degrade cellulose (Waldron et al., 1986).

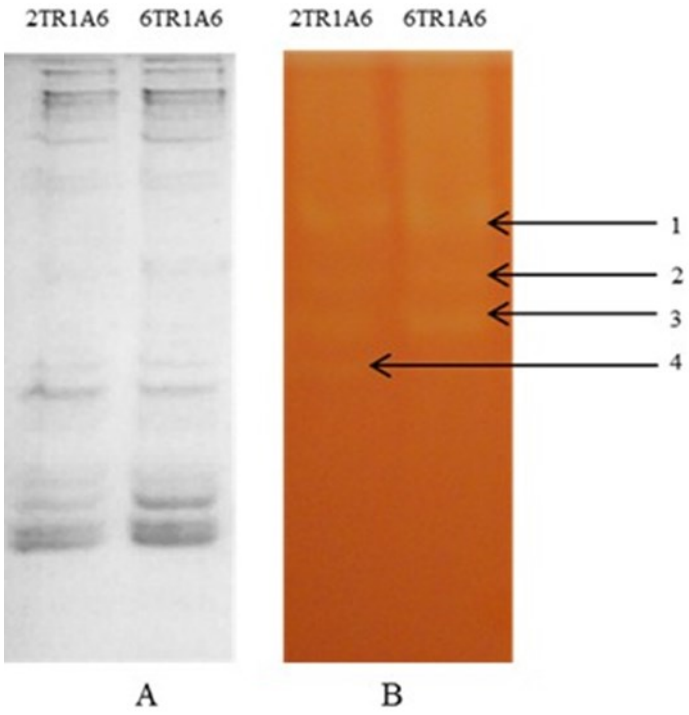


Figure 5.15 TR1A subculture 6 supernatant assayed for CMCase activity.
The samples were prepared as described in Fig. 5.13. (A) Coomassie brilliant blue stained 12% CMC-PAGE gel of two week old TR1A subculture 6 (lane 2TR1A6), six week old TR1A subculture 6 (lane 6TR1A6). (B) zymogram on 12% CMC-PAGE gel with the same samples as those loaded in 2TR1A6 and 6TR1A6 in (A). The arrows labelled 1 to 4 in (B) show clear zones due to CMCase activity on the CMC zymogram after staining with Congo red.

Endo-β-N-acetylglucosaminidase H from *Streptomyces plicatus* was detected in all three bands in gel B (Fig. 5.16). Other enzymes detected in band 1 were xylanase/ β-xylosidase and xylan 1,4-β-xylosidase from *Geobacillus sterothermophilus* and *Geobacillus* sp. (closest strain being Y412MC52), respectively.

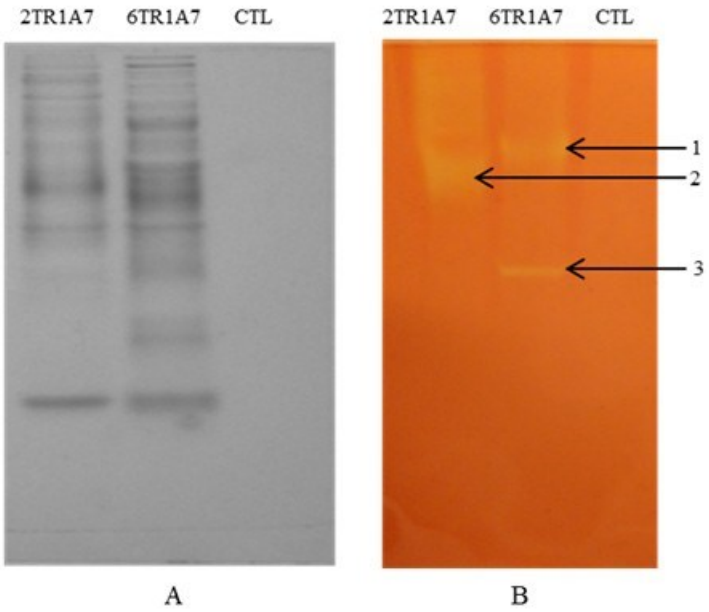


Figure 5.16 Native PAGE gel (12%) and zymogram for CMCase activity of supernatants of TR1A subculture 7 (A) CMC-PAGE gel Coomassie brilliant blue stained and (B) CMC-PAGE zymogram of two week old culture in lane 2TR1A7 and six week old culture in lane 6TR1A7, and the control in lane CTL. The bands labelled 1 to 3 are clear regions resulting from CMCase activity of the bands at these locations.

TR1A subculture 8 gave zymogram profiles which were different from those of the other TR1A subcultures, consistent with the dramatic increase in exoglucanase activity observed. Samples analysed on gels C and D (Fig. 5.17) were from a two week old TR1A subculture 8, while the rest of the gels in Fig. 5.17 were of samples from six week old cultures. The supernatant from the six week old TR1A was also analysed on 0.05% xylan-native PAGE gel (Fig. 5.17G and H) to compare enzyme activity with that on CMC. All the zymograms had a similar pattern of clear zones, whether analysed on CMC or xylan containing gels, again consistent with the similar activities observed in 2 week and 6 week samples. It could be that selection pressure has resulted in strains which constitutively produce enzymes involved in the degradation of both CMC and xylan, which are therefore present in cultures growing on any carbon source. Alternatively, some of the enzymes may be capable of degrading both CMC and xylan. Similar observations have been reported by other researchers (Todero Ritter et al., 2013).

Almost the same pattern of cleared zones was produced in gels B, D, F and H in Fig. 5.17. Most of the identified glycoside hydrolases given in Table 5.1 were β-xylosidases, these could have also been involved in the degradation of CMC to produce the clear zones observed on the CMC containing zymograms (Figures 5.14B, D and F, 5.15B, 5.16B, 5.17B, D and F).

The bands labelled 1 to 4 in Fig. 5.17B were identified by mass spectrometry. Band 1 contained transaldolase from *Meiothermus silvanus* (closest strain being DSM 9946). Band 2 contained

a manganese containing catalase from *Thermus thermophilus* or *Thermus aquaticus* (closest strain being Y51MC23), xylose isomerase from *Geobacillus* sp. (Y412MC52 as closest strain), and glyceraldehyde-3-phosphate dehydrogenase from *Meiothermus silvanus* (closest strain being DSM 9946) and *Meiothermus ruber* (closest strain being DSM 1279). Enzymes detected in band 3 were xylan 1,4-β-xylosidase from *Geobacillus* sp. (Y412MC52 as closest strain), β-xylosidase from *Geobacillus stearothermophilus,* and endo-1,4-β-glucanase A (also called glucanase A or cellulase A) from *Thermobispora bispora* (closest strain being DSM 43833). Superoxide dismutase from *Meiothermus silvanus* (closest strain being DSM9946) was detected in both bands 3 and 4. *Thermobispora bispora* (closest strain being DSM 43833) and *Meiothermus* spp. were detected in the DNA isolated from TR1A subcultures 6 and 8 (Fig. 6.1A).

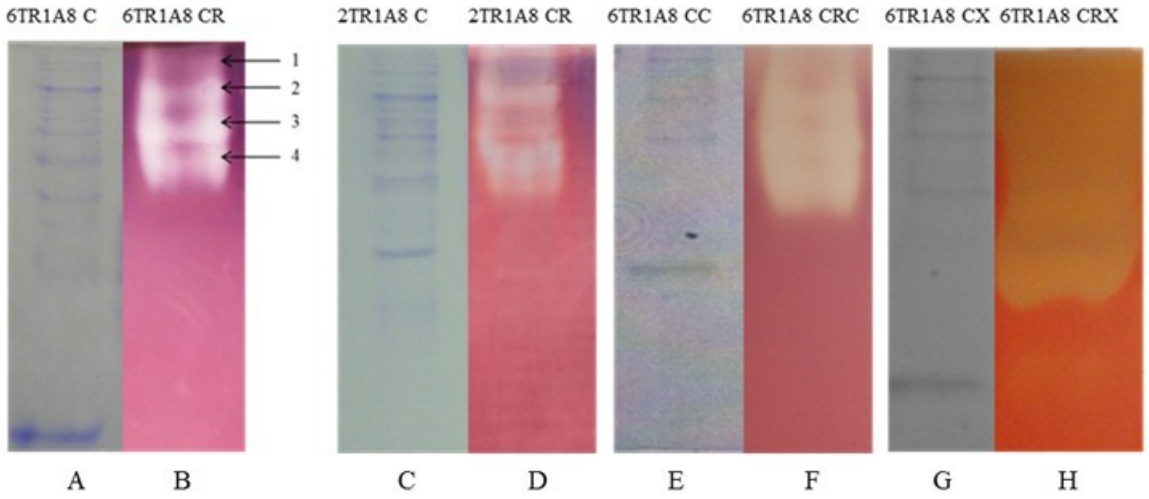


Figure 5.17 Native CMC and xylan PAGE gels (12%) of supernatant from TR1A subculture 8.
Native CMC-PAGE gel (12%) containing Coomassie brilliant blue stained gels and zymograms of TR1A subculture 8 samples from a six week old culture (A and B, loaded in lanes 6TR1A8 C and 6TR1A8 CR, for Coomassie brilliant blue and Congo red stained gels, respectively), and from a two week old culture (C and D, loaded in lanes 2TR1A8 C and 2TR1A8 CR, for Coomassie brilliant blue and Congo red stained gels, respectively). The supernatant from the six week old TR1A subculture 8 was loaded on native CMC-PAGE gel (E and F, loaded in lanes 6TR1A8 CC and 6TR1A8 CRC, for Coomassie brilliant blue and Congo red stained gels, respectively), and on native xylan-PAGE gel (G and H, loaded in lanes 6TR1A8 CX and 6TR1A8 CRX, for Coomassie brilliant blue and Congo red stained gels, respectively) to compare glycosidase activity on CMC and xylan. The CMC and xylan gels were prepared by adding 0.05% CMC or Beechwood xylan, respectively. The 0.05% CMC or xylan substrate was added to the gel preparations of both the Congo red and Coomassie brilliant blue stained gels. The bands labelled 1 to 4 in gel B and bands in gel A, were analysed by mass spectrometry for identification of the relevant proteins.

5.5.2.2 Identification of the enzymes detected in the supernatants of TR1A subcultures.

The protein bands which showed enzyme activity through visible clear zones and other bands of interest were cut out from both Coomassie stained gels and the zymograms. The cut-out bands were prepared by using the Trypsin Ingel Digest method before being sent for protein identification by mass spectrometry. The identified enzymes including the more closely related producing microorganisms are given in Table 5.1. The table also shows the enzyme producing microorganisms that were detected in the pellet DNA or from the isolated microbial colonies from the TR1A subcultures, as well as the gels and the subculture stage at which the enzymes were detected. The list of the detected peptides of the identified enzymes and the locations of the peptides in the enzyme sequence is given in the appendix. Same enzymes produced by different microorganisms are shown to have different molecular weight (Table 5.1). It is possible for different organisms to produce same enzymes but the produced enzymes can be of different molecular weight (Semenova et al., 2009).

Table 5.1 Identification of the enzymes detected in the supernatant of TR1A subcultures 6, 7 and 8.
D means that the producing microorganism was detected in the TR1A subculture pellet DNA or isolated as an individual microorganism from the TR1A subcultures. ND means that the enzyme producing microorganism was not detected. The provided molecular weight of the predicted enzymes was obtained from data base.

GEL FIGURE	IDENTIFIED ENZYME	PRODUCING ORGANISM	APPROXIMATE ENZYME MW (KDA)	DETECTION OF PRODUCING MICROORGANISM IN TR1A SUBCULTURES
Fig. 5.15	**TR1A SUBCULTURE 6**			
B	Xylan 1,4-β-xylosidase	*Geobacillus* sp. G11MC16	80	*Geobacillus* spp. isolated
B	Xylan 1,4-β-xylosidase	*Geobacillus* sp. *Y412MC52*	80	*Geobacillus* spp. isolated
B	β-xylosidase	*Geobacillus stearothermophilus*	80	D
B	β-xylosidase	*Paenibacillus* sp. *DG-22*	79	Some *Cohnella/Paenibacillus* spp. isolated
B	Glucoamylase (glucan 1,4-α-glucosidase glaA)	*Aspergillus niger*	68	ND
B	Xylan 1,4-β-xylosidase	*Thermobacillus composti KWC4*	38	D
A	Acid-α amylase	*Aspergillus niger*	55	ND
A	Xylulokinase	*Thermobacillus composti KWC4*	54	D
A	Xylulokinase	*Paenibacillus curdlanolyticus YK9*	54	Some *Cohnella/Paenibacillus* spp. isolated
A	Xylulose kinase	*Bacillus subtilis*	55	D
B	Xylose isomerase	*Thermobispora bispora (strain DSM 43833)*	43	D
A	Superoxide dismutase	*Meiothermus silvanus DSM 9946*	23	D
A	β-xylosidase	*Paenibacillus sp. DG-22*	79	Some *Cohnella/Paenibacillus* spp. isolated
A	Superoxide dismutase	*Paenibacillus* sp. *oral taxon 786 str. D14*	22	Some *Cohnella/Paenibacillus* spp. isolated
A	Superoxide dismutase [Mn]	*Geobacillus kaustophilus HTA426 / Geobacillus thermoleovorans*]	23	*Geobacillus spp.* isolated
B	Xylose isomerase	*Bacillus subtilis*	50	*Bacillus* spp. isolated
A	Xylose isomerase	*Geobacillus* sp. *Y412MC52*	50	*Geobacillus* spp. isolated
A	Xylan 1,4-β-xylosidase	*Geobacillus* sp. *(strainC56-T3)*	80	*Geobacillus* spp. isolated

GEL FIGURE	IDENTIFIED ENZYME	PRODUCING ORGANISM	APPROXIMATE ENZYME MW (KDA)	DETECTION OF PRODUCING MICROORGANISM IN TR1A SUBCULTURES
Fig. 5.16	**TR1A SUBCULTURE 7**			
B	Endo-β-N-Acetylglucosaminidase H (Mannosyl-glycoprotein endo-β-Ngi)	*Streptomyces plicatus*	33	ND
B	Xylanase	*Geobacillus stearothermophilus*	70	D
B	β-xylosidase	*Geobacillus stearothermophilus*	80	D
B	Xylan 1,4-β-xylosidase	*Geobacillus sp. Y412MC52*	80	*Geobacillus* spp. isolated
A	Enolase or 2-phosphoglycerate dehydrogenase or 2-phospho-D-glycerate hydro-lyase	*Meiothermus silvanus DSM 9946*	45	D
A	4-α-glucanotransferase	*Thermoproteus neutrophilus V24 Sta*	55	ND
A	Δ-1-pyrroline-5-carboxylate dehydrogenase	*Geobacillus thermodenitrificans NG80-2*	57	*Geobacillus* spp. isolated
A	Xylose isomerase	*Geobacillus thermodenitrificans NG80-2]*	51	*Geobacillus* spp. isolated
A	Xylose isomerase	*Bacillus* sp. *NRRL B-14911*	49	*Bacillus* spp. isolated
A	Enolase (2-phosphoglycerate dehydratase)	[*Bacillus halodurans C-125/ Geobacillus* sp. *Y4.1MC1*	46	*Bacillus* spp. isolated
A	Δ-1-pyrroline-5-carboxylate dehydrogenase	*Geobacillus* sp. *Y412MC52*	57	*Geobacillus* spp. isolated
A	Xylan 1,4-β-xylosidase	*Geobacillus thermodenitrificans NG80-2]*	62	*Geobacillus* spp. isolated
A	Xylan 1,4-β-xylosidase	*Bacillus cellulosilyticus DSM2522*	62	*Bacillus* spp. isolated
A	Xylose isomerase	*Geobacillus kaustophilus HTA426*	50	*Geobacillus* spp. isolated
A	Aldehyde dehydrogenase (NAD(+))	*Micromonospora aurantiaca ATCC27029*	54	ND
A	Aldehyde dehydrogenase (NAD(+))	*Rhodomicrobium vannielii ATCC 17100*	55	ND

GEL FIGURE	IDENTIFIED ENZYME	PRODUCING ORGANISM	APPROXIMATE ENZYME MW (KDA)	DETECTION OF PRODUCING MICROORGANISM IN TR1A SUBCULTURES
A	Aldehyde dehydrogenase	*Streptomyces clavuligerus ATCC 27064*	55	ND
A	Enolase	Staphylococcus xylosus	47	*Staphylococcus* sp. isolated
A	Dihydrolipomamide dehydrogenase component of pyruvate dehydrogenase E3	*Staphylococcus carnosus subsp. Carnosus TM300*	49	*Staphylococcus* sp. isolated
A	Superoxide dismutase	*Meiothermus silvanus DSM 9946*	23	D
A	β-xylosidase/α-L-arabinfuranosidase,	*Cellvibrio japonicus Ueda107*	38	ND
A	α-N-arabinofuranosidase	*Clostridium papyrosolvens DSM 2782*	37	ND
A	Transaldolase	*Paenibacillus* sp. *oral taxon 786 str. D14*	24	Some *Cohnella/Paenibacillus* spp. isolated
A	Xylose isomerase	*Bacillus licheniformis ATCC 14580*	50	D
A	Enolase	*Meiothermus silvanus DSM 9946*	45	D
Fig. 5.17	**TR1A SUBCULTURE 8**			
B	Transaldolase	*Meiothermus silvanus DSM 9946*	23	D
B	Mn catalase (manganese containing catalase)	*Thermus thermophiles/Thermus aquaticus*	33	*Themus* groups detected
B	Xylose isomerase	*Geobacillus* sp. *Y412MC52*	50	*Geobacillus* spp. isolated
B	Glyceraldehyde-3-phosphate dehydrogenase	*Meiothermus silvanus DSM 9946*	36	*Meiothermus* spp. detected
A	NAD-dependent aldehyde dehydrogenase	*Geobacillus kaustophilus HTA426*	56	*Geobacillus* spp. isolated
A	Glyceraldehyde-3-phosphate dehydrogenase	*Meiothermus ruber DSM 1279*	36	*Meiothermus* spp. detected
B	Xylan 1,4-β-xylosidase	*Geobacillus sp. Y412MC52*	80	*Geobacillus* spp. isolated
B	β-xylosidase	*Geobacillus stearothermophilus*	80	D
B	Endo-1,4-β-glucanase A	*Thermobispora bispora DSM 43833*	47	D

GEL FIGURE	IDENTIFIED ENZYME	PRODUCING ORGANISM	APPROXIMATE ENZYME MW (KDA)	DETECTION OF PRODUCING MICROORGANISM IN TR1A SUBCULTURES
B	Superoxide dismutase	*Meiothermus silvanus DSM 9946*	23	D
A	Catalase	*Geobacillus* sp. *Y4.1MC1*	55	*Geobacillus* spp. isolated
A	Xylose isomerase	*Bacillus subtilis*	13	*Bacillus* spp. isolated
A	Xylose isomerase	*Geobacillus kaustophilus HTA426*	50	*Geobacillus* spp. isolated
A	Xylose isomerase	*Geobacillus* sp. *Y412MC52*	50	*Geobacillus* spp. isolated
A	Transaldolase	*Paenibacillus* sp. *JDR-2*	23	Some *Cohnella/Paenibacillus* spp. detected
A	Xylose isomerase	*Thermoanaerobacterium thermosulfurigenes and Thermoanaerobacterium thermosaccharolyticum DSM571*	50	*Thermoanaerobacterium* spp. detected

5.5.2.2.1 Identified enzymes and their importance in the degradation of lignocellulosic biomass.

5.5.2.2.1.1Glucoamylase.

The glucoamylase produced by *Aspergillus niger* was detected in the initial cultures and first subcutures of TR1A and TR2A (Fig. 5.14B and D) and in the supernatant from TR1A subculture 6 (Table 5.1). This was the same microorganism from which glucoamylase general acid was first identified as Glu179 through site mutagenesis (Sierks et al., 1990). It was not clear how *Aspergillus niger* could have existed in a culture incubated at 55^{o}C as it has not been reported to grow at temperatures above 45^{o}C. Glucoamylase belongs to the glycoside hydrolase family 15, which comprises of enzymes glucoamylase (EC 3.2.1.3), alpha-glucosidase (EC 3.2.1.20) and glucodextranase (EC 3.2.1.70) (Bairoch, 1999). Glucoamylases catalyse the degradation of α-1,4-glycosidic bond (Al-Turki et al., 2008), to remove β-D-glucose from the reducing ends of starch (such as amylose and amylopectin) as well as oligo or polysaccharides (Sakaguchi et al., 1992). These enzymes are also capable of degrading α-

1,6, α-1,3 and α-1,2 bonds, at lower levels (Al-Turki et al., 2008). They follow the inverting mechanism during the hydrolysis reactions (Weil et al., 1954).

Glucoamylases are highly used in industrial processes involved in fermentation, food, textiles and paper production processes (Quang et al., 2002). They are also important in the starch hydrolysis for alcohol production, as well as the making of high glucose corn syrup (Quang et al., 2002). The glucoamylases are produced commercially from *Bacillus* spp. and *Aspergillus* spp., (Al-Turki et al., 2008). They representing 25-33% of the world enzyme market and are in the second position after proteases (Quang et al., 2002).

5.5.2.2.1.2 Xylan 1,4-β-xylosidases/β-xylosidases.

The xylan 1,4-β-xylosidases/ β-xylosidases were the most abundant group of enzymes in the TR1A subcultures (mostly produced by *Geobacillus* spp. *Paenibacillus* spp. and *Bacillus* spp.), and were isolated from all the three stages of TR1A subcultures (Table 5.1). These enzymes were detected 7 times, 5 times and 2 times, in the supernatants from TR1A subcultures 6, 7 and 8, repsectively. The xylan 1,4-β-xylosidase (code EC 3.2.1.37) belongs to the glycoside hydrolase family 43. This enzyme is also known as xylobiase, β-xylosidase, exo-1,4-β-xylosidase, β-D-xylopyranosidase, exo-1,4-β-D-xylosidase, or 1,4-β-D-xylanxylohydrolase, and has a system name called 4-β-D-xylanxylohydrolase (Chinchetru et al., 1989). The xylan 1,4-β-xylosidase catalyses the hydrolysis reaction involving the successive removal of D-xylans from the non-reducing termini by degradation of the (1->4)-β-D-xylans. β-xylosidase hydrolyses glycosidic bonds of 1,4-β-xylooligosaccharides, as well as exhibiting alpha-l-arabinofuranosidase activity on 4-nitrophenyl α-l-arabinoside (Rojas et al., 2005).

The β-xylosidases work synergistically with endoxylanases during hydrolysis of glucuroxylan as well as with α-L-arabinofuranosidase and endoxylanase during arabinoxylan hydrolysis (Semenova et al., 2009). The β-xylosidases have been reported to increase efficiency of degradation of lignocellulosic materials (maize cobs) when they were added to enzymatic preparation of *Celloviridine* G20X without its own β-xylosidase (Semenova et al., 2009).

It was also observed that degradation of xylan more than doubled when β-xylosidase and β-l-arabinofuranosidase were added to purified xylanases (Tuncer and Ball, 2003a). This could partly be the reason for the successful degradation of pretreated *M. x giganteus* observed in the TR1A subcultures, as both the β-xylosidase/α-l-arabinofuranosidase and xylanase, as well as

α-N-arabinofuranosidase enzymes were detected in the TR1A subculture 7 supernatant (Table 5.1). The deconstruction of hemicellulosic polysaccharides requires an optimized mixture of enzymes to be produced by microorganisms.

5.5.2.2.1.3 Xylanase.

Xylanase (code EC 3.2.1.8) is involved in the production of xylose. It breaks down the β-1,4-backbone of the complex polysaccharide xylan in the plant cell wall to release short xylans (Collins et al., 2005). The xylanase was detected in the supernatant from the TR1A subculture 7 (Table 5.1), with *Geobacillus stearothermophilus* identified as the producing microoganism. The xylanase mainly belongs to glycoside hydrolase families 10 and 11. Other families including 5, 7, 8 and 43 have also been identified with xylanases but they have not been extensively studied (Collins et al., 2005). Xylanase has been reported to be produced by bacteria, algae, fungi, protozoa, gastropods and anthropods (Prade, 1996). The xylanase enzymes were first reported in 1955 by Whistler and Masak, and were called pentosases. Their official name is endo-1,4-β-xylanase, but they are also called xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase (Collins et al., 2005).

5.5.2.2.1.4 Endo-β -N-Acetylglucosaminidase H.

The enzyme endo- β -N-acetylglucosaminidase H is also called Endo H. It was detected in the supernatant from TR1A subculture 7 (Table 5.1). It is an endoglycosidase secreted by *Streptomyces plicatus* (Tarentin.Al et al., 1974). The Endo H enzyme is involved in the hydrolysis of the β -1,4-glycosidic link of the N,N-diacetylchitobiose core of the high-mannose and hybrid asparagine linked oligosaccharides (GlcNAc-β(1,4)-GlcNAc) (Rao et al., 1999).

The Endo H belongs to the glycosyl hydrolase family 18, and it follows the retention of configuration mechanism during the hydrolysis process (Rao et al., 1999). This enzyme is widely used for the characterization and functional analysis of oligosaccharides and glycoproteins (Maley et al., 1989; Oneill, 1996; Tarentino and Plummer Jr, 1994).

5.5.2.2.1.5 Endo-1,4-β-glucanase A.

The endo-1,4-β-glucanase A, also simply called glucanase A, belongs to glycoside hydrolase family 6 and is denoted by the code EC 3.2.1.4. Glucanase A was detected in the supernatant from TR1A subculture 8 during this study (Table 5.1). The enzyme glucanase A is involved

in the hydrolysis of β-1,4-glycosidic bonds in cellulose, cereal β-D-glucans and lichenin by following the inversion of anomeric configuration mechanism (Damude et al., 1996). The microorganism *Thermobispora bispora* (closest strain being DSM 43833) was identified as the producing microorganism by mass spectrometry analysis, and was detected in the pellet DNA from the early and late TR1A subcultures (Table 5.1, and Figures. 6.1-A, and 6.2A and B).

5.5.2.2.1.6 Xylose isomerase.

The production of biofuel from the hemicellulose fraction of plant biomass will become economical and a realistic enterprise if the rates of conversion of pentoses and hexoses are high. This depends on the successful conversion of all the available sugars in the lignocellulosic biomass into biofuel (Brat et al., 2009). The xylose isomerase enzyme (code XI EC 5.3.1.5) catalyses the interconversion of D-xylose to D-ribulose, as well as the isomerization of D-ribose to D-ribulose, and D-glucose to D-fructose. Xylose isomerase is an intracellular enzyme and its presence could be due to cell lysis. However, it has also been reported to be secreted as an extracellular enzyme by an alkalophilic-thermophilic *Bacillus* sp. (Chauthaiwale and Rao, 1994). The results in Table 5.1 show that xylose isomerase was the second most abundant enzyme detected in the TR1A subcultures 6, 7 and 8, after the xylan 1,4-β-xylosidase/β-xylosidase group. This enzyme was produced mostly by the *Firmicutes*, especially the *Geobacillus* spp. This shows that the xylose isomerase enzyme was very important for the survival of the mixed microbial community in the TR1A subcultures due to successful xylose metabolism.

Wild-type *Saccharomyces cerevisiae* has been reported to be capable of metabolising xylulose slowly, but is unable to metabolise xylose (Kuyper et al., 2004). Bruinenberg et al., (1983) recommended that metabolic engineering for yeasts for xylose utilization should be based on the cloning of the xylose isomerase. A lot of studies have been carried to try and introduce the xylose isomerase into *S. cerevisiae* (Amore et al., 1989; Brat et al., 2009; Moes et al., 1996; Sarthy et al., 1987; Walfridsson et al., 1995). Xylose isomerase was expressed from the rumen bacterium *Prevotella ruminicola* T C2-24 into *S. cerevisiae* CEN (Hector et al., 2013). The transformed *S. cerevisiae* CEN was observed to ferment D-xylose leading to high ethanol yields, confirming the need for xylose isomerase for successful fermentation of xylose by *S. cerevisiae*.

5.5.2.2.1.7 Transaldolase.

Transaldolases produced by *Paenibacillus* spp. and *Meiothermus silvanus* DSM 9946 were detected in the supernatant from TR1A subcultures 7 and 8 (Table 5.1). Transaldolase is an important enzyme in the pentose phosphate pathway and in the ribulose monophosphate cycle of formaldehyde fixation (Levering and Dijkhuizen, 1986; Sprenger et al., 1995). It is involved in the xylose metabolism by catalysing the production of fructose-6-phosphate which later enters into the glycolytic cycle and gets degraded into ethanol. Microorganisms that do not produce transaldolase have been reported to be unable to grow on xylose (Levering and Dijkhuizen, 1986).

5.5.2.2.1.8 Superoxide dismutase and catalase.

Superoxide dismutase produced by *Meiothermus* spp*, Geobacillus* spp. and *Paenibacillus* spp. was detected in all the three TR1A subculture stages, three times, once and twice from the TR1A subcultures 6, 7 and 8, respectively (Table 5.1). *Meiothermus* spp. and *Geobacillus* spp. were detected to be some of the most abundant groups of microorganism in the TR1A subcultures (figures 6.1 and 6.2), consistent with the abundance of the superoxide dismutase in the TR1A subcultures.

Catalases produced by *Thermus thermophilus* or *Thermus aquaticus* (closest strain being Y51MC23) *and Geobacillus* sp. (Y4.1MC1 identified as the closest strain) were also detected in the supernatant from the TR1A subculture 8. Superoxide dismutase breaks down superoxide into oxygen and hydrogen peroxide (Bannister et al., 1987; Zelko et al., 2002) to protect the cells from oxidants, the hydrogen peroxide subsequently gets converted to water and oxygen by catalase (Chelikani et al., 2004; Zámocký and Koller, 1999).

5.5.2.2.1.9 Acid α-amylase.

An acid α-amylase, also called 1,4-α-D-glucan glucanohydrolase (code EC 3.2.1.1), from *A. niger* was detected in the TR1A subculture 6 (Table 5.1). This enzyme is involved in the endohydrolysis of (1->4)-α-D-glucosidic linkages in polysaccharides which contain three or more (1->4)-α-linked D-glucose units (Boel et al., 1990).

5.5.2.2.1.10 Other enzymes.

Other enzymes detected in the supernatants from the TR1A subcultures which may not be involved directly in the deconstruction of lignocellulosic biomass included enolase, glyceraldehyde 3-phosphate dehydrogenase, aldehyde dehydrogenase, Δ-1-pyrroline-5-carboxylate dehydrogenase and dihydrolipomamide dehydrogenase (Table 5.1). Aldehyde dehydrogenase might be involved in the detoxification activities by removing aldehydes and alcohols from the culture (Kwok and Weiner, 2005).

Xylulokinase was also detected in the supernatant from the TR1A subcultures. The metabolism of xylose depends on its conversion to xylulose-5-phosphate, which is produced by action of xylose isomerase followed by xylulokinase (code EC 2.7.1.17). The xylose isomerase converts xylose to xylulose which subsequently gets phosphorylated to xylulose-5-phosphate by the action of xylulokinase. The xylulose-5-phosphate then enters the glycolysis pathway through the nonoxidative pentose phosphate pathway (Jin et al., 2002), and gets degraded into ethanol. However, all these are intracellular enzymes and may naturally be abundant in the cells.

5.6 Discussion.

A mixed microbial community was successfully developed from the compost inoculum by enrichment culture using pretreated *M. x giganteus*. Microbial consortia obtained by enrichment techniques are close to those functioning in natural environments (Nichols et al., 2008), and may have the ability to utilize raw lignocellulosic biomass synergistically as in nature (Feng et al., 2011).

The microbial consortium obtained during this study was able to grow on pretreated *M. x giganteus.* The fluctuations observed in the growth of the mixed microbial cultures (Fig. 5.2 A and B) could be due to the influence of dissolved organic matter, as well as the influence of the interactions between the mixed microbial community members and the pretreated *M. x giganteus* substrate on microbial growth (Bomber et al., 1989; Grzebyk et al., 1994). The fluctuations in microbial growth could also be due to substances secreted into the culture medium by members of the mixed microbial group as observed during a previous study (Sakami et al., 1999).

The mixed TR1A microbial consortium degraded over half of the pretreated miscanthus during a six week incubation period. It was observed that the pretreated *M. x giganteus* was never

completely degraded even when the cultures were left to grow on for more than six weeks. Feng et al., (2011) also observed that there was no further degradation after filter paper was degraded to a certain extent by a microbial consortium.

The microorganisms in the mixed culture appeared to depend on the pretreated miscanthus as a carbon source, as those cultures in higher pretreated miscanthus biomass content showed more growth indicated by higher protein concentration than those cultured in lower pretreated miscanthus amount. Microbial growth was also better in buffered medium as the pH was maintained around 7 throughout the incubation period. The pH always dropped to below 6 in unbuffered medium, resulting into restricted growth of the microbial community.

Compositional analysis results show that the microorganisms in the mixed culture were able to use all the sugar components in the pretreated *M. x giganteus*, and were also able to degrade part of the lignin. The results showed that more of the ASL was degraded, whereas less than 50% of AIL was degraded. This shows that the mixed microbial community in the TR1A subcultures is very important, because lignin degradation is the key to successful deconstruction of the lignocellulosic biomass for successful development of consolidated bioprocessing.

Fermentation products such as formate and lactate were detected in the TR1A subcultures. However, no alcohol was detected in the TR1A subculture supernatant despite the detection of the fermenting groups of microorganisms (Fig. 6.1A and B) and products (Fig. 5.11). Other techniques such as head space GC analysis (Wei et al., 2013) or analysis by nuclear magnetic resonance (NMR) (Kim et al., 2013) could be used for analysis to detect the ethanol, as its concentration in the supernatant could be too low to be detected by HPLC analysis. Increasing the number of standards could also help to identify the unidentified products detected by HPLC in the supernatant from the TR1A subculture.

There was good enzyme activity detected in the supernatant from the TR1A subcultures (Fig. 5.13). The subculture 6 maximum CMCase and xylanase (0.1 and 1.0 U/mg, respectively) activities were lower than the CMCase and xylanase (0.24 and 1.71 U/mg, respectively) recorded in a previous study for the supernatant from a lignocellulose degrading consortia (Wongwilaiwalin et al., 2010). However, the observed CMCase activity for subcultures 7 and 8 (0.27 and 2.09 U/mg, respectively), and xylanase activities for the same subcultures (2.79

and 4.63 U/mg, respectively) were much higher than that recorded by Wongwilaiwalin et al., (2010).

The detected amounts of xylanase, CMCase and avicelase in the supernatant from the TR1A subcultures increased with number of subcultures carried out. This finding was also supported by the isolation of the endoglycoside hydrolase groups Endo-β-N-Acetylglucosaminidase H and Endo-1,4-β-glucanase A from the middle and late TR1A subcultures 7 and 8, respectively.This shows that the more stable microbial communities were capable of producing enzymes directly involved in the deconstruction of the crystalline cellulose.

The results in table 5.1 show that the mixed microbial community in the TR1A subcultures produced different enzymes and at different times, so as to achieve the observed deconstruction of the lignocellulose biomass. Proteins associated with the degradation of biomass are often observed in the bacterial extracelluar fractions, with saccharification of pretreated switchgrass by supernatants from enrichment cultures reported to be successful (Gladden et al., 2011). The detection of enzymes, such as superoxide dismutase and Mn-containing catalase involved in reactions with reactive oxygen species in the TR1A subcultures supernatant is an indication of oxidative deconstruction of lignocellulosic biomass as suggested by D'Haeseleer et al., (2013).

The results showed that there are various microbes that form symbiotic relationships and successfully produce the enzymes that work to co-catalyse the deconstruction of lignocellulosic biomass. The degradation of lignocellulosic biomass has been reported to be complex (Takasuka et al., 2013), involving synergism and other metabolic relationships among microorganisms in nature (Haruta et al., 2002). This synergism can not be reconstituted by simple mixtures of known organisms (Feng et al., 2011).

The detection of Endo-β-N-Acetylglucosaminidase H in the TR1A subculture could be the reason for the complete degradation of the mannose component of the pretreated *M. x giganteus* during the six week incubation of the TR1A subculture 6. Reports from a previous study suggest that this enzyme could have been involved in the degradation of cellulosic biomass during growth of an insect associated *Streptomyces* (Takasuka et al., 2013). Efforts to amplify Endo H or Endo-1,4-β-glucanase A from the TR1A subcultures during this study were unsuccessful. Low level of endoglucanase activity recovered from a switchgrass adapted community were also observed during a previous study (D'Haeseleer et al., 2013).

The abundance of the *Geobacillus* spp. from the liquid fraction of the TR1A subcultures is very important because this group of microorganisms secrete enzymes which easily diffuse through the whole culture and are more effective in the degradation of the plant biomass. The successful degradation of lignocellulosic biomass depends on availability of enzymes in their surrounding environment. The *Geobacillus* spp. were producers of most of the enzymes detected in the TR1A subcultures. A number of enzymes from *Thermus* spp. were detected in the supernatant of TR1A subcultures, mostly from TR1A subcultures 7 and 8 (Table 5.1). There could have been more proteins from the *Thermus* spp. in the suspended and fibre attached fractions as observed in a previous study (D'Haeseleer et al., 2013). Proteins from *Paenibacillus* spp. were found to be present in the supernatant, similar to the observations made by D'Haeseleer et al., (2013) in a study involving a thermophilic bacterium consortium adapted to deconstruct switchgrass.

The identified set of enzymes shown in Table 5.1 gives information about the many enzymes a natural mixed microbial community produces to achieve degradation of the plant biomass. These enzymes work synergistically to achieve the deconstruction of the lignocellulosic biomass and provide the right forms of sugars to be used by the microbial community. The similar pattern of cleared regions observed on CMC and xylan native gels could be due to synergistic associations between cellulases and xylanases as reported in previous studies (Beukes et al., 2008; Gao et al., 2011). It has been observed that a lot of xyloglucan hydrolases have minor endoglucanase activity, while many endoglucanases have minor xyloglucan-hydrolyzing activity (Karboune et al., 2009; Vlasenko et al., 2010). The same pattern of cleared zones for supernatants from the two week or the six week old TR1A subculture 8 showed the stability of the consortia in this subculture.

The results in Table 5.1 showed that the three subcultures had different profiles. This is not surprising as the enrichment process results in the selective adjustment of the structure of the community supporting only those microorganisms which adapt to the new environment (Feng et al., 2011; Gladden et al., 2012). It is not easy to assign roles to individual microbial groups in microbial communities that deconstruct biomass (Hess et al., 2011). Their microbial proportions are dynamically altered by changes in the substrate composition and temperature (Wei et al., 2012). Fluctuations in the relative ratios of the abundant populations in the switch grass adapted enrichment have been reported by Gladden et al., (2011). These fluctuations

could also result in differences in relative enzyme activity, and the detected enzymes (D'Haeseleer et al., 2013).

The TR1A mixed microbial culture produced high levels of xylanases during all the three subcultures, an observation which was also reported by D'Haeseleer et al., (2013), and another previous study on a bacterial consortium that was adapted to swicthgrass deconstruction under thermophilic conditions in liquid culture (Gladden et al., 2011). This may be an indication that degradation of the hemicellulose component of the pretreated *M. x giganteus* was the main enzyme activity in the TR1A subcultures, an observation that was also made on the switchgrass adapted community (D'Haeseleer et al., 2013).

A number of intracellular enzymes, such as transaldolase, xylose isomerase, superoxide dismutase and Mn-catalase were detected in the supernatant. It has been suggested that these detections may result from cell lysis during the incubation period (D'Haeseleer et al., 2013) . A similar observation was made by D'Haeseleer et al., (2013), where enzymes normally considered to be cytoplasmic enzymes were detected in the supernatant. However, other studies have reported the presence of an extracellular xylose isomerase in *Streptomyces* sp. (strain NCL 82-5-1) (Pawar and Deshmukh, 1994), a thermophilic *Bacillus* sp. (NCIM 59) (Chauthaiwale and Rao, 1994), and *Clostridium obsidiansis* after growth on pretreated switchgrass as discussed by D'Haeseleer et al., (2013).

Superoxide dismutase and Mn-catalase are normally involved in the cellular detoxification by degrading oxygen radicals, but have been reported to be present in the supernatant of *Thermobifida fusca* grown on lignin (Adav et al., 2010). It is proposed that oxygen radicals produced in the extracellular medium by the microbes may be involved in the degradation of lignin (D'Haeseleer et al., 2013). Enzymes known to be directly involved in lignin degradation were not detected in the TR1A subcultures' supernatants, this could be due to lack of knowledge of lignin deconstruction by bacteria as suggested by Bugg et al., (2011).

These results could be an indication that the way forward to a successful consolidated bioprocessing might be the involvement of the mixed microbial communities capable of deconstructing pretreated plant biomass in the culture of fermenting microbes. The mixed microbial community would be the source of the enzymes required to degrade the pretreated plant biomass to provide the right forms of sugars for the fermenting microbes to use for biofuel production.

6. Identification of bacteria present in the TR1A culture by 16S rRNA gene analysis.

6.1 Introduction.

Sequencing of the 16S rRNA gene has been used to study bacterial phylogeny and taxonomy (Patel, 2001). The 16S rRNA gene sequence is present in almost all bacteria and has not changed over time (Janda and Abbott, 2007; Woese et al., 1990). It is 1 500 bp and keeps enough information required in the study of evolution and classification of living things (Woo et al., 2008). It is a better method for classifying organisms than using variations in phenotypic characteristic (Woo et al., 2008), for identification of poorly described, rarely isolated, or phenotypically aberrant strains (Jill and Clarridge, 2004). This study was carried out to study the composition of the microbial communities in the TR1A culture.

6.2 Isolation of bacteria present in the TR1A culture.

DNA was isolated from pellets from the early stage (TR1A subculture 6) and the late stage (TR1A subculture 8) subcultures still growing over 1 year from the start of the initial culture. Pellets included both freely suspended and surface-attached bacteria bound to the solid particles in the flasks. Additionally, individual colonies were obtained by spreading samples from the flasks on plates of agar medium containing cellobiose, xylose and glucose (0.5% wt/v of each), phosphoric acid swollen cellulose, 2TY, as well as RIM with a fine powder of *M. x giganteus* (1% w/v), and actinomycetes isolation medium.

The inoculated plates were incubated aerobically at 55ºC as described in the materials and methods. The colonies which developed after incubation were subcultured several times until pure isolates were obtained. Colonies were selected based on their morphological differences and, after purification, were grown individually in 2TY liquid medium for 24 h at 55ºC with shaking at 250 rpm, for DNA isolation. Fourteen and 12 independent colonies were selected from TR1A subcultures 6 and 8, respectively. The 16S rRNA genes were amplified and prepared for sequencing.

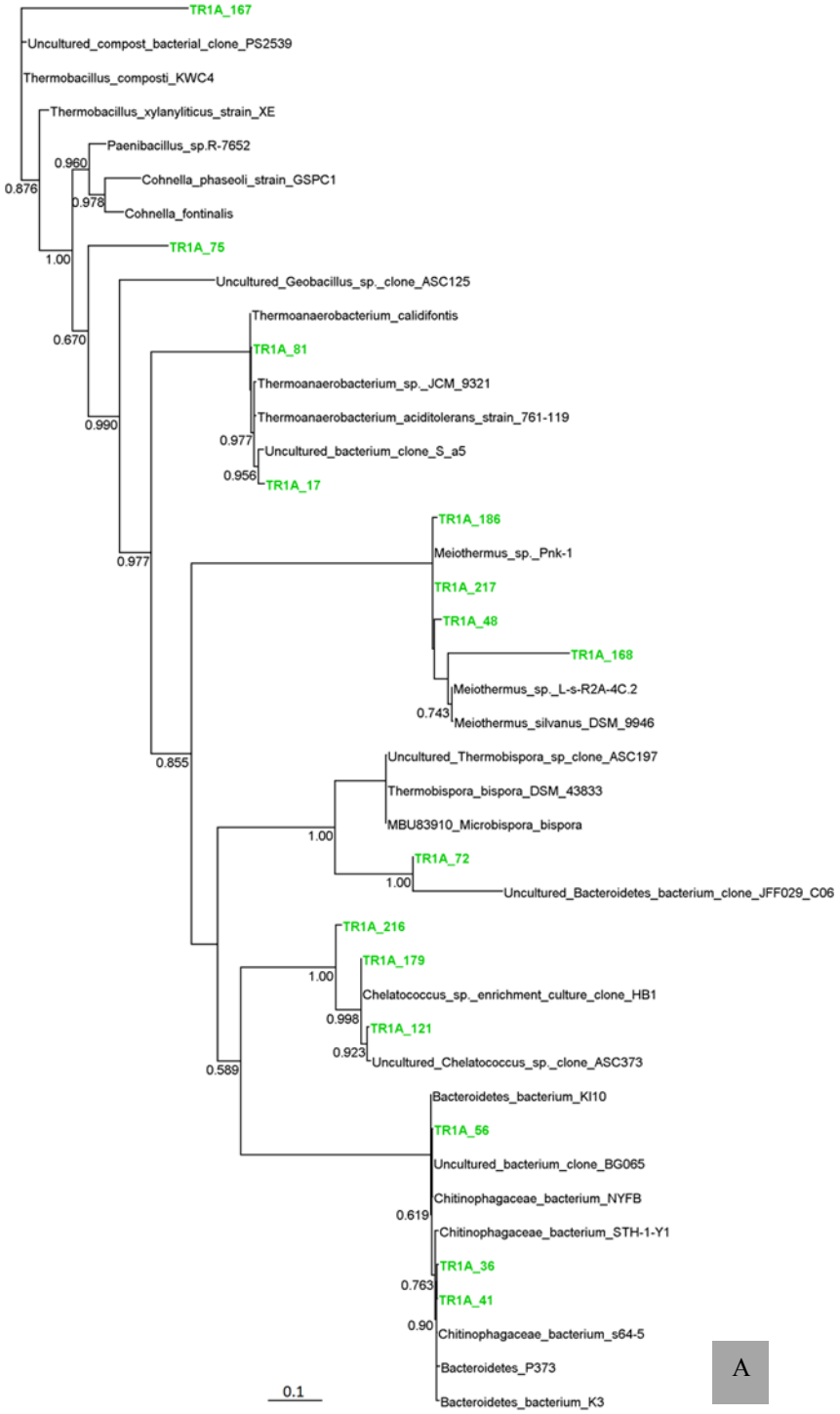
Amplification of 16S rDNA directly from the cultures should give a relatively unbiased view of the microbial populations, while colony isolation provided pure cultures for subsequent study but was potentially biased as a method for examining the population, by the need to culture the strains on a laboratory medium and due to the fact that the plates were incubated

aerobically. The focus for growth of colonies was to isolate a range of *Geobacillus* spp. which might be more catabolically versatile in consolidated bioprocessing than the TMO Renewables strain (Cripps et al., 2009) or which could supply catabolic genes which were directly transferable to the TMO strain. *Geobacillus* spp. are mostly planktonic, therefore, the liquid fraction of the cultures was used for isolations. The aim was to isolate *Geobacillus* spp. that secrete important free enzymes into the culture as these secreted enzymes were believed to be more effective in degrading lignocellulose than those that attach to the lignocellulose particles (Jorgensen et al., 2005).

16S rRNA genes present in the isolated DNA were PCR amplified using universal eubacterial primers and cloned into *E. coli* as described in the methods section. As the samples taken directly from the liquid culture contained mixed PCR products, represented by 67 and 37 PCR products prepared from subcultures 6 and 8, respectively, individual *E coli* clones were processed for DNA sequencing, in order to obtain a profile of the most abundant strains in the culture. The sequencing results were analysed using the BLAST algorithm via the National Centre for Biotechnology Information (NCBI) website and the results were then used to identify the most probable genus and species of bacteria present in the original culture. A similar analysis was done using colonies isolated on plates, except in this case only a single clone of each PCR fragment was sequenced.

6.3 Identification of the bacteria present in the TR1A culture by 16S rRNA gene analysis.

The identified species from the pellet DNA and the isolated colony 16S rRNA analysis were then used to develop phylogenetic trees using PhyML 3.0 (Guindon et al., 2010). The results are illustrated in Fig. 6.1A for the direct culture 16S rDNA identifications and Fig. 6.1B for the isolated colonies.


TR1A_167
Uncultured_compost_bacterial_clone_PS2539
Thermobacillus_composti_KWC4
Thermobacillus_xylanyliticus_strain_XE
Paenibacillus_sp.R-7652
0.960
0.876
Cohnella_phaseoli_strain_GSPC1
0.978
Cohnella_fontinalis
TR1A_75
1.00
Uncultured_Geobacillus_sp._clone_ASC125
Thermoanaerobacterium_calidifontis
0.670
TR1A_81
Thermoanaerobacterium_sp._JCM_9321
Thermoanaerobacterium_aciditolerans_strain_761-119
0.990
0.977
Uncultured_bacterium_clone_S_a5
0.956
TR1A_17
TR1A_186
0.977
Meiothermus_sp._Pnk-1
TR1A_217
TR1A_48
TR1A_168
Meiothermus_sp._L-s-R2A-4C.2
0.743
Meiothermus_silvanus_DSM_9946
0.855
Uncultured_Thermobispora_sp_clone_ASC197
Thermobispora_bispora_DSM_43833
MBU83910_Microbispora_bispora
1.00
TR1A_72
1.00
Uncultured_Bacteroidetes_bacterium_clone_JFF029_C06
TR1A_216
TR1A_179
1.00
Chelatococcus_sp._enrichment_culture_clone_HB1
0.998
TR1A_121
0.923
Uncultured_Chelatococcus_sp._clone_ASC373
0.589
Bacteroidetes_bacterium_KI10
TR1A_56
Uncultured_bacterium_clone_BG065
Chitinophagaceae_bacterium_NYFB
0.619
Chitinophagaceae_bacterium_STH-1-Y1
TR1A_36
0.763
TR1A_41
0.90
Chitinophagaceae_bacterium_s64-5
Bacteroidetes_P373
Bacteroidetes_bacterium_K3
0.1
A

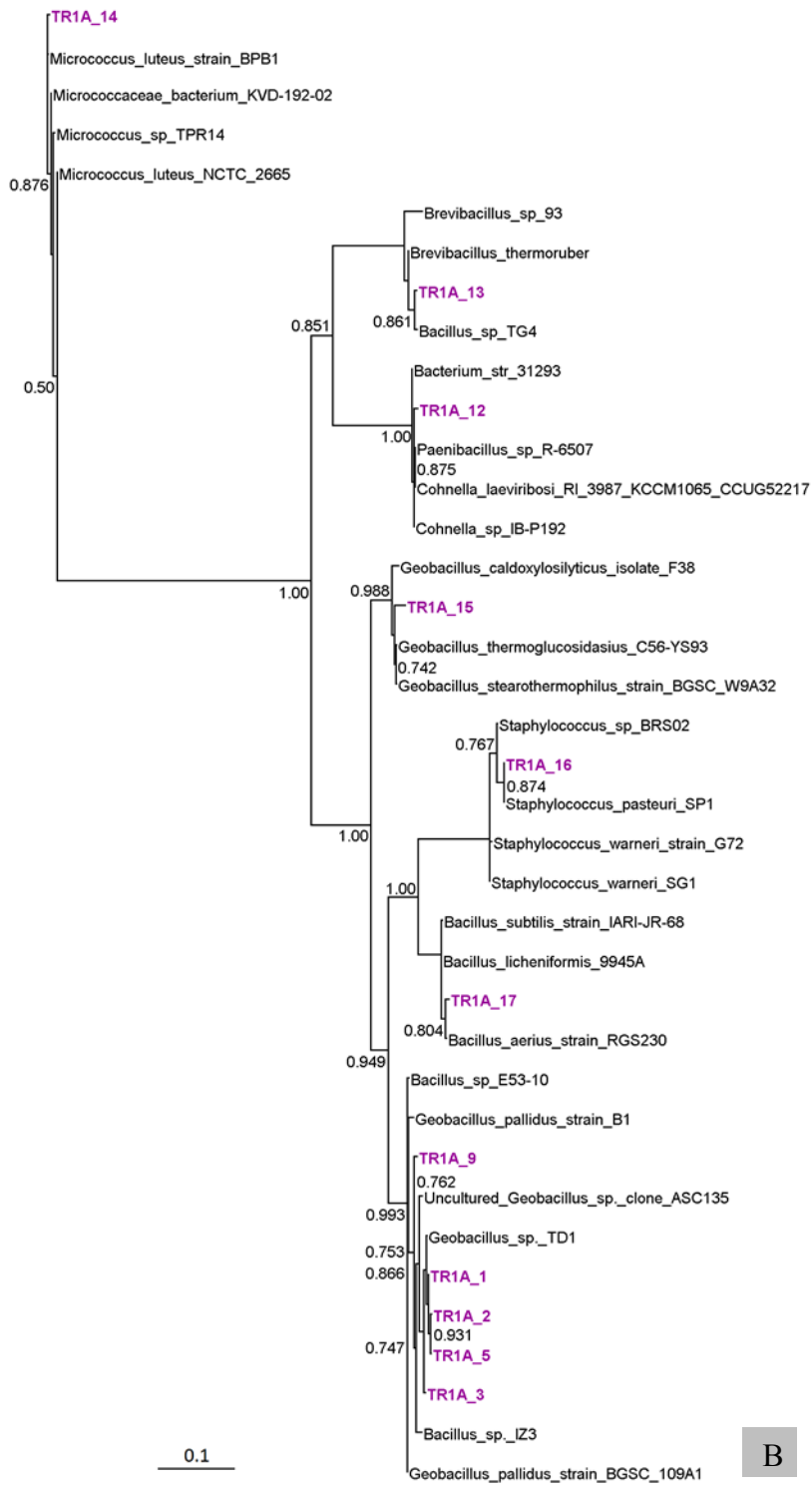


Figure 6.1 Un-rooted phylogenetic trees of microorganisms detected in the TR1A subcultures.
Un-rooted phylogenetic trees based on 16S rRNA gene sequences amplified from microorganisms detected in the TR1A subculture DNA isolated directly from culture pellets (A), and individual bacteria isolated from liquid sample from the culture (B) and their closest relatives. The trees are showing where the different isolates sit. In most cases only one of the members of the detected genera was selected and used for constructing the tree, and are highlighted in bold green in (A) and purple in (B). The trees were constructed by estimation of the Maximum Likely-hood Phylogenies by using PhyML 3.0 as described by Guindon et al. (2010). The number of changes per nucleotide is indicated by the *scale,* while the numbers on the nodes represent the comparisons of bootstrap and aLRT SH-like supports for the branches. The higher the signal the higher is the agreement. Only values with frequency above 0.5 were displayed.

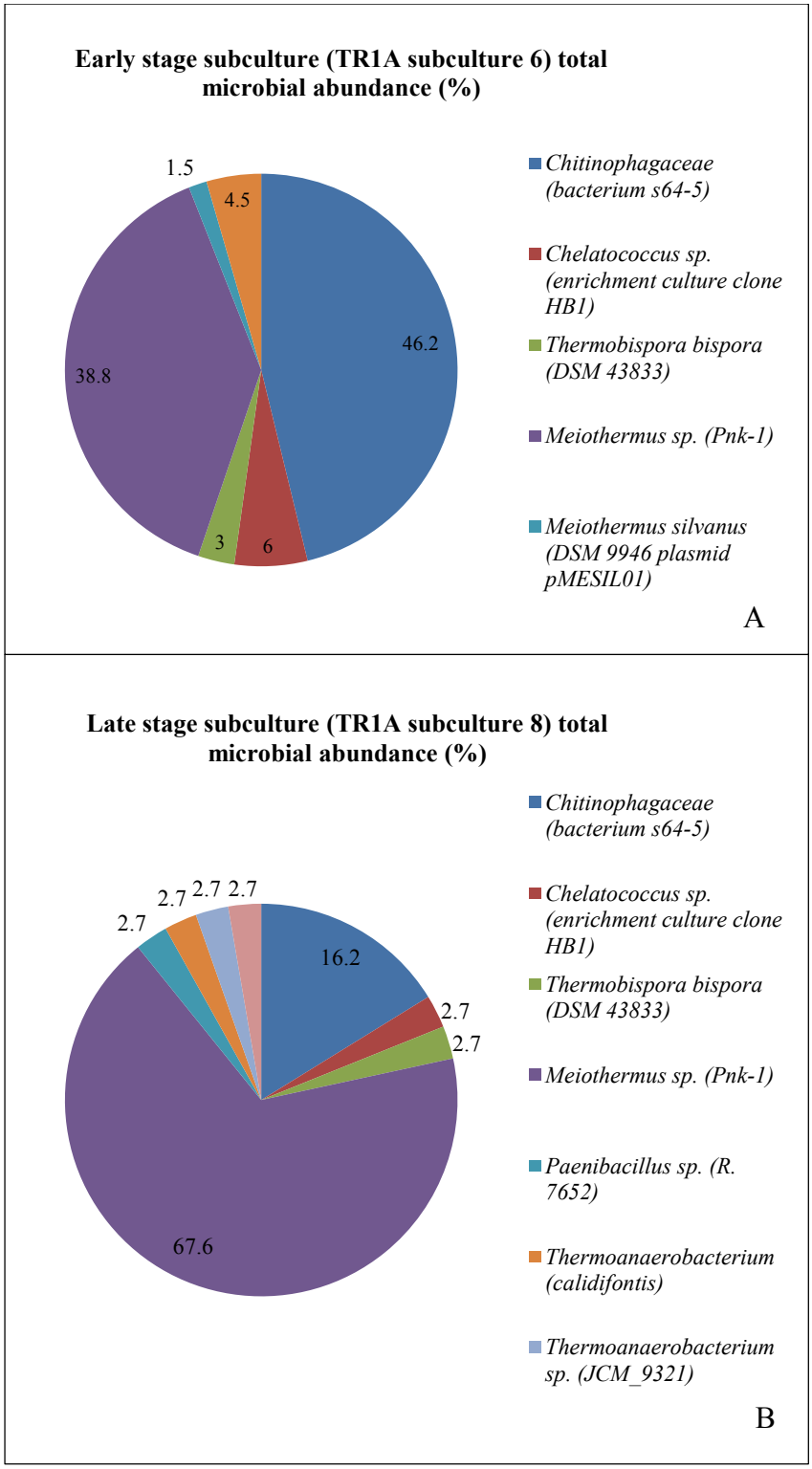


Figure 6.2 Pie charts showing the approximate abundance (%) of the microbial species identified in the TR1A subcultures 6 and 8.
The microbial species were identified by 16S rRNA gene analysis of DNA isolated directly from culture pellets from the mixed TR1A subcultures 6 and 8. Abundance was estimated from the number of times that a sequence appeared in a population of 67 and 37 clones of PCR amplified 16S rRNA genes, for the TR1A subcultures 6 and 8, respectively. The DNA isolated from TR1A subcultures 6 and 8 represented the microbial population from the early and the late TR1A enrichment subcultures, respectively. The closest species to which the clones were identified are shown in the brackets.

6.3.1 Identification of microorganisms from 16S rRNA gene analysis of total DNA isolated from the TR1A subcultures.

The phylogenetic tree in Fig. 6.1 A represent the range of microorganisms that were detected in the total DNA isolated from the TR1A subcultures.

6.3.1.1. Identification of the *Bacteroidete*s microorganisms.

The *Chitinophagaceae* family is a member of the *Bacteroidetes* phylum. TR1A_36, TR1A_41 and TR1A_56 (Fig. 6.1A) were distinct members of this family, which is poorly delineated at the sub-family level, which were present in the selected subcultures. Based on the 16S rRNA analysis, all the microoganisms in this group had 99% identity with previously identified *Chitinophagaceae* 16S rRNA gene sequences. They were the largest group of the early stage subculture microorganisms detected in the pellet DNA (Fig. 6.2A), and were second largest in the groups of microorganisms detected in the pellet DNA from the late stage subculture (Fig. 6.2B). However, comparison of the detected groups in the pellet DNA from the late stage subcultre may not be real due to the smaller number of sequences (37) analysed compared to the number of sequences (67) analysed during the early stage subculture.

The abundance of *Chitinophagaceae* in the TR1A subcultures 6 and 8 indicates that they must be one of the most important groups responsible for the degradation of the pretreated *M. x giganteus* in the TR1A culture. *Bacteroidetes* have been reported to produce high levels of glycoside hydrolases (Gladden et al., 2011), belonging to GH10, which include β-1,4-xylanase and β-1,3-xylanase (http://www.cazy.org/GH10_bacteria.html). They are also among the three major groups that were identified during a similar study carried out by Eichorst et al., (2013), in which a similar inoculum sourced from compost, mostly consisting of plant biomass, was used.

6.3.1.2 Identification of the *Thermus* microorganisms.

Meiothermus spp. were also an abundant group of microorganisms that were detected in the TR1A subcultures 6 and 8, represented by TR1A_48, TR1A_168, TR1A_186 and TR1A_217 (Fig. 6.1A). This genus belongs to the *Thermaceae* family, which includes the closely related *Meiothermus* spp. and *Thermus* spp., which can grow at extremely high temperatures. Based on the 16Sr RNA gene analysis results, most of the *Meiothermus* isolates in TR1A had 100%

identity with *Meiothermus* sp. Pnk-1, while the rest had 99% identity with *Meiothermus silvanus* or other *Meiothermus* spp.

The *Meiothermus* spp. were the second largest group of bacteria identified from the TR1A subculture 6, and were the largest group in TR1A subculture 8 (Fig. 6.2A and B). Some of the characteristics of this group may be associated with some of the observations made during enrichment of the TR1A culture. *Bacterium* S119 has 94% identity to *Meiothermus silvanus* based on the 16S rRNA gene analysis and produces a pink carotenoid pigment called Deinoxanthin, while other *Meiothermus* spp. produce yellow, or red or orange pigments (Yarger et al., 2006). Production of these pigments and the evident abundance of this genus in TR1A subcultures could be associated with the development of a reddish pigmentation observed in the TR1A subcultures (Figures 5.7 and 5.8A).

Superoxide dismutase from a *Meiothermus silvanus* DSM 9946, Mn-catalase, enolase, 2-phosphoglycerate dehydrogenase, transaldolase and glyceraldehyde 3-phosphate dehydrogenase from *Thermus* bacteria were also detected in polypeptide analysis of the TR1A subculture (Table 5.1).

The Deinococcales form a closely associated lineage to the *Thermaceae* and *Deinococcus* spp. and are also extremophiles. *Deinococcus geothermalis* and *Deinococcus murrayi* were isolated during a study of hot springs in Portugal and Italy (Makarova et al., 2001). These bacteria are said to be highly resistant to harsh environmental conditions, such as extremes of heat, and cold. *Deinococcus radiodurans*, the toughest bacterium known to man, according to the Guiness Book of World Records, is resistant to hydrogen peroxide, and can survive exposures to ionizing and ultraviolet radiation (Brim et al., 2000; White et al., 1999). Some *Deinococcus* spp. can transform nuclear waste and can survive in a vacuum (Deinos means strange or unusual in Greek). Its resistance to harsh conditions and chronic radiation is attributed to its efficient DNA repair capabilities (Cox and Battista, 2005; Englander et al., 2004; Makarova et al., 2001).

Thermus spp. (*Thermus caldophilus*) have been reported to encode endocellulases (Kim et al., 2006), and other *Thermus* spp. have been shown to degrade cellulose, CMC or ponderosa pine saw dust (Rastogi et al., 2010), an indication that *Meiothermus* spp. could have been directly involved in the degradation of the pretreated *M. x giganteus* particles in the TR1A culture. The hardiness of this group of organisms could also provide an explanation for the survival of the

microbial community in the TR1A subcultures and their successful resuscitation from -80ºC stock cultures stored without glycerol.

6.3.1.3 Other microorganisms detected at less than 10% of the microbial population in TR1A subcultures 6 and 8.

Other bacteria identified in the TR1A culture were each present at less than 10% of the microbial population (Fig. 6.2). Most of them are known to be very important for degradation of lignocellulosic biomass. These included the *Chelatococcus* spp. represented by TR1A_121, TR1A_216 and TR1A_179 (Fig. 6.1A), which were detected both in the early stage and late stage TR1A subcultures (Fig. 6.2A and B). *Chelatococcus* spp. were recently shown to hydrolyse cellulose and hemicellulose components of corn stover and switchgrass during a thermophilic high-solids fermentation of these bioenergy feedstocks (Reddy et al., 2011).

Thermobacillus spp. are represented in Fig. 6.1A by TR1A_167, which were 99% identical to *Thermobacillus composti* KWC4. *T. composti* KWC4 produces xylulokinase and xylan 1, 4-β-xylosidase, with both of these enzymes being detected in the supernatant from TR1A subculture 6 (Table 5.1). *Thermobacillus* spp. are known to be capable of carrying out thermophilic plant cell wall degradation (Rastogi et al., 2010). The 16S rRNA gene of isolate TR1A_75 detected from the pellet DNA was only 88% identical to *Thermobacillus* spp., *Paenibacillus* spp. and the uncultured *Geobacillus* sp. clone ASC125.

Other microorganisms detected at less than 10% of the population included *Thermoanaerobacterium* spp. represented in Fig. 6.1A by TR1A_17 and TR1A_81, which were only detected in the late stage subcultures (Fig. 6.2B). These bacteria are obligate anaerobes and could be surviving as spores during transfers, germinating only when the zones in the biomass became anaerobic. *Thermoanaerobacterium* spp. have been reported to be spore formers (Lee et al., 1993), *T. aotearoense* was observed to form oval terminal spores (Liu et al., 1996), another *Thermoanaerobacterium*, *T. calidifontis* is a spore forming anerobic bacterium and has been reported to degrade hemicellulose (Shang et al., 2013). The *Thermoanaerobacterium* spp. are capable of saccharifying cellulose, they have been used in thermophilic cocultures during ethanolic fermentation processes (Jiang et al., 2013).

Thermobispora bispora DSM 43833 is a strictly thermophilic actinomycete, and has been previously isolated from decaying manure and other types of manure (Liolios et al., 2010).

This species was detected in both the early stage and late stage of TR1A subcultures, both at less than 10% of the population (Fig. 6.2A and B), represented by TR1A_72 (Fig. 6.1A). The TR1A_72 16S rRNA gene sequence had 99% identity with that from *Thermobispora bispora* DSM 43833. *Thermobispora bispora* DSM 43833 produces a wide range of glycoside hydrolases, including endoglucanase A (cellulase A or endo-1,4-β-glucanase A or β-1,4-endoglucan hydrolase) (http://www.cazy.org/GH6.html). This enzyme was detected in the TR1A subculture 8 supernatant (Table 5.1), with *Thermobispora bispora* DSM 43833 as the closest producing bacterium strain, and could be one of the enzymes responsible for the observed degradation of pretreated *M. x giganteus* in the TR1A subcultures (Figures 5.2C and 5.6). *Thermobispora bispora* DSM 43833 was also identified as one of the producers of the xylose isomerase enzyme in the TR1A subculture 6 (Table 5.1).

6.3.2 Identification of microorganisms from 16Sr RNA gene analysis of the isolated microbial colonies from the TR1A subcultures.

Most of the isolated colonies belonged to the *Firmicutes* phylum, while only one group belonged to the *Actinobacteria* phylum. The phylogenetic tree of colonies isolated from the TR1A subcultures, based on 16S rRNA gene analysis is shown in Fig. 6.1B.

6.3.2.1 The *Firmicutes*.

6.3.2.1.1 *Geobacillus* spp.

The bacteria isolated solely from the liquid phase of the cultures were mostly *Geobacillus* spp. and are represented in Fig. 6.1B by TR1A_1, TR1A_2, TR1A_3, TR1A_5, TR1A_9 and TR1A_15. Enzymes detected in the TR1A subcultures produced by *Geobacillus* spp. included enolases, superoxide dismutase, aldehyde dehydrogenase, catalase, Δ-1-pyrroline-5-carboxylate dehydrogenase, xylanases (xylan 1,4-β-xylosidases, β-xylosidases, 1,4-β-D-xylan xylohydrolase) and xylose isomerases (Table 5.1). It is probable that *Geobacillus* spp. contributed to degradation of the the hemicellulose xylan component of the pretreated *M. x giganteus* in the TR1A culture.

Of the 14 colonies isolated from the early TR1A subculture 6, 12 isolates were *Geobacillus* spp. Representatives of the genus decreased in the late subcultures, with only 2 of the 12 isolated colonies being *Geobacillus* spp. Interestingly, one of the colonies isolated from the TR1A subculture 8 (Fig. 6.3A) had 99% identity to *Geobacillus thermoglucosidasius* C56-

YS93 and *Geobacillus stearothermophilus* strain BGSC W9A32, based on the 16S rRNA gene analysis results. This might be an indication that the mixed community of microorganisms in the TR1A culture could be cocultured with the TMO engineered high ethanol yielding *Geobacillus* strain (Cripps et al., 2009) for the development of consolidated bioprocessing.

To look for potentially useful enzyme activity, the isolated *Geobacillus* strains were grown on avicel, cellobiose, CMC and xylan carbon sources in modified ASM, with a control for each colony on modified ASM without any carbon source. After 3 days of incubation at 55^{o}C, the plates were stained with Congo red. No clear zones were observed around any of the colonies on the control plates, while a few of the colonies on avicel plates had tiny cleared zones in the area where the colonies developed (e.g. colonies 2 and 3 on avicel plates in Fig. 6.3C, column 2). The colonies on the control plates could have been growing on the 0.1 g yeast extract added to the modified ASM medium. The CMC plates also only showed clear zones in the locations were the colonies developed (Fig. 6.3C, column 4), while all of the colonies on the cellobiose plates showed clear zones around the colonies (Fig.6.3C, column 3). Surprisingly, the xylan plates only showed clear zones in the locations where the colonies developed (results not shown). Previous studies have shown that *Geobaacillus* spp. secrete few soluble glycoside hydrolases, while most of these enzymes are present in form of multiple enzyme complexes attached to their cell walls (Shulami et al., 2011). This could be the reason why these bacteria were unable to degrade the carbohydrates away from their colonies. This observation was also reported by another study in which *Geobacillus* spp. did not show activity on CMC plates but in the liquid medium cultures (Stathopoulou et al., 2012).

Results for 8 of the isolated *Geobacillus* strains from the early subcultures, (TR1A subculture 6) are shown in Fig. 6.3C. The *Geobacillus* strains isolated from the late subcultures, (TR1A subculture 8) gave similar results (Fig. 6.3 D, colonies labelled D1 to D4) as the strains from the TR1A subculture 6.

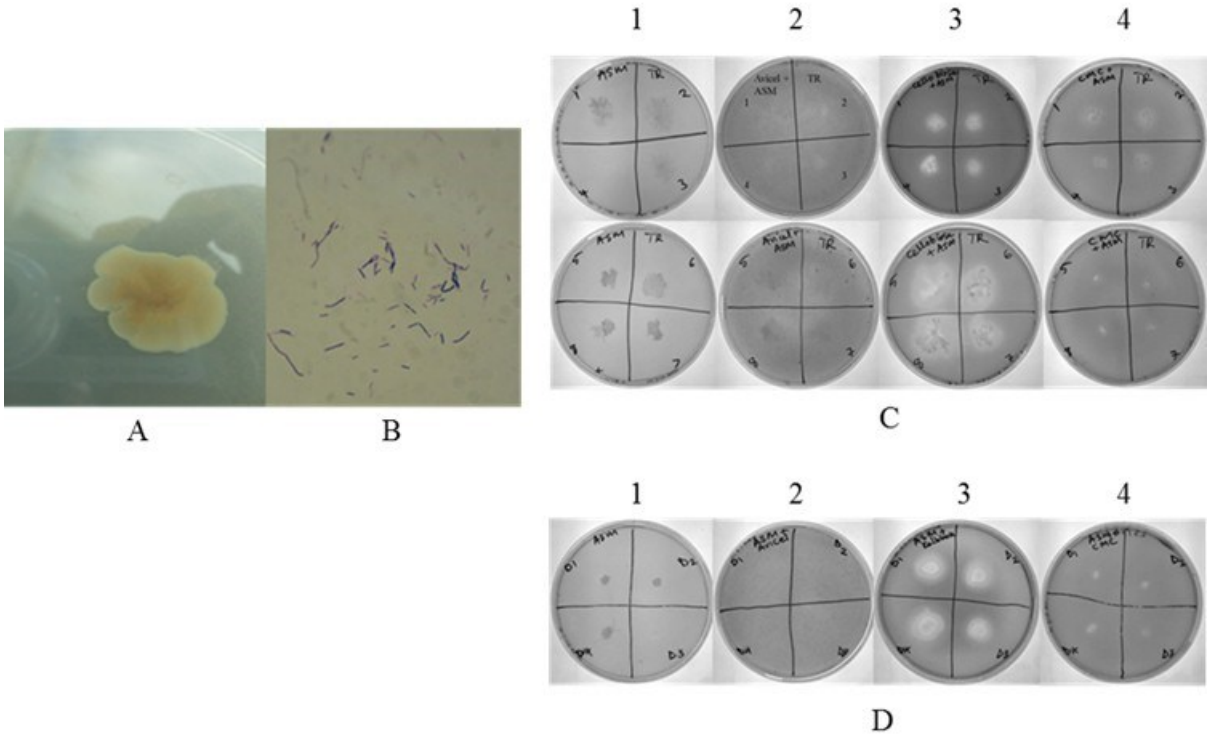


Figure 6.3 *Geobacillus* strains isolated from TR1A subcultures.
(A) Colony of *Geobacillus* strain TR1A_15 growth on a 2TY agar plate at 55°C for 24 h, (B) a Gram stain slide of the colony in A, at x1000 magnification, (C) Colonies 1 to 8 from the early subcuture (TR1A subculture 6), and (D) D1 to D4 from the late subculture (TR1A subculture 8), grown on modified ASM [(control), column 1], avicel + modified ASM (column 2), cellobiose + modified ASM (column 3) and CMC + modified ASM (column 4) agar plates incubated for 3 days at 55°C. Four isolates were inoculated on each plate. The plates were stained with Congo red stain to check for clear zones around the colonies.

6.3.2.1.2 The *Cohnella* spp.

The *Cohnella* spp. were another *Firmucutes* group isolated from both the early stage (1 colony) and late stage (3 colonies) subcultures. The colonies looked almost star shaped on 2TY plates after a 24 h incubation at 55°C (Fig. 6.4). There were no halos around the colonies after growth on avicel, cellobiose, CMC and xylan plates, with only small clear regions visible on cellobiose, CMC and xylan plates where the colonies developed (results not shown). They are also reported to have glycoside hydrolases as cellulosomes attached to their cell walls (Waeonukul et al., 2009).

The *Cohnella* spp. are represented by TR1A_12 in Fig. 6.1B. This strain was 99% identical to *Cohnella* sp. IB-P192 and *Paenibacillus* sp. R-6507 based on the 16S rRNA gene analysis comparison. Members of the *Cohnella* and *Paenibacillus* genera have been reported to be capable of producing cellulase, xylanase, β-glucanase and mannanase, and are actively involved in cellulolytic and hemicellulolytic activities during composting (Eida et al., 2012) .

Cohnella spp. have been shown to grow on switchgrass (Reddy et al., 2011). Some species, such as *Cohnella fontinalis*, are said to be xylanolytic (Rastogi et al., 2010). Enzymes characterised in the TR1A culture as being produced by *Paenibacillus* spp. included β-xylosidase, xylulokinase, transaldolase and superoxide dismutase (Table 5.1), and an extracellular solute-binding protein family 1 produced by *Paenibacillus* sp. (closest strain being JDR-2) was also detected in the TR1A subculture supernatant.

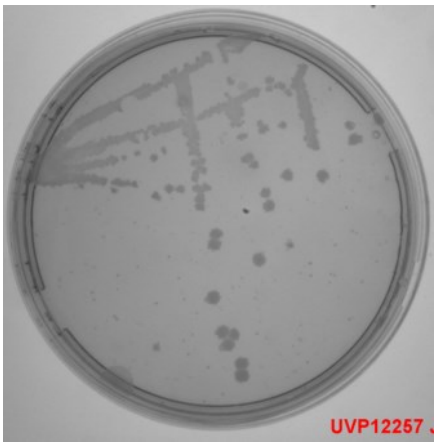


Figure 6.4 Colonies of TR1A_12 on 2TY.
Colonies of isolate TR1A_12 on 2TY plate after 24 h incubation at 55°C.

6.3.2.1.3 The *Brevibacillus* spp.

The *Brevibacillus thermoruber* was isolated from both the early stage (1 colony) and late stage (4 colonies) subcultures. This group is represented by TR1A_13 in Fig. 6.1B, which showed 99% identity to *Brevibacillus thermoruber* based on 16S rRNA gene sequence comparison. This is another member of the *Firmicutes* group reported to produce thermo stable cellulose degrading enzymes (Liang et al., 2009; Rastogi et al., 2009).

B. thermoruber can live under harsh conditions and very high temperatures. This group has previously been isolated from a hot spring (Wang et al., 2012). *Brevibacillus* spp. are endospore formers, and they have also been reported to tolerate high concentrations of ethanol (Thomas, 2006), and have successfully been used in experiments involving fermentation processes (Bihari et al., 2010). *Brevibacillus* spp. have also been reported to have carboxylmethylcellulase activity (Rastogi et al., 2009).

The isolated *B. thermoruber* formed the largest colonies with a light pink pigmentation on nutrient plates containing avicel, and showed a tint of the pink pigmentation on

carboxymethylcellulose and xylan plates after a 24 h incubation period at 55ºC (Fig. 6.5, pictures C to E). This microbial group could, therefore, also be contributing to the reddish pigmentation observed in the TR1A culture (Figures 5.6 to 5.8). No halos were visible around the colonies except in the zone where the colonies had grown on avicel, cellobiose, CMC and xylan plates after staining with Congo red (results not shown). Another study reported that colonies of cellulolytic *Cytophaga* spp. did not show any clearing zones (Schlegel, 1986).

Many *Bacillus* spp. have been observed to have cellulosomes attached to their cell walls (Bagudo et al., 2014), and may not produce enough soluble glycoside hydrolases to degrade the carbohydrates in the areas away from where the colony developed. The isolated *Brevibacillus* strain was rod shaped and Gram positive, but it was also observed that a 24 h old colony was more reactive to Gram staining than an older colony (1 week) after growth on modified ammonium salts medium (ASM) plus 2% avicel (Fig. 6.5A and B).

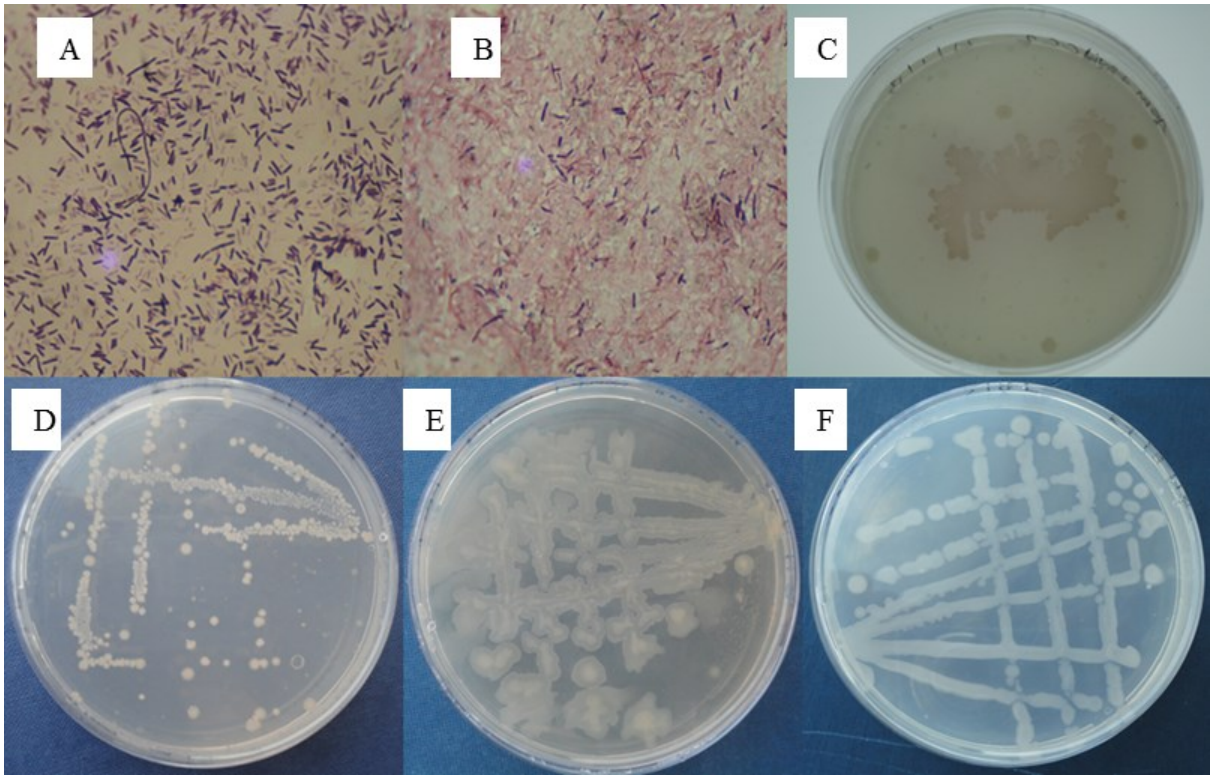


Figure 6.5 *B. thermoruber* TR1A_13 grown on carbon sources.
A colony of *Brevibacillus thermoruber* isolated from TR1A subculture 8 after 24 h growth at 55ºC on modified ammonium salts medium (ASM) plus 2% avicel (C), carboxymethylcellulose (D), xylan (E), and cellobiose (F) agar plates. Pictures in A and B show the Gram stain results of a 1 day old and 1 week old *B. thermoruber* colony, respectively, after growth on 2% avicel and modified ASM agar plate at 55ºC, (magnification-x1000).

6.3.2.1.4 *Bacillus aerius* or *Bacillus lichenformis* sp.

The 16S rRNA gene of isolate TR1A_17 had 99% identity with that of *Bacillus aerius* RGS230 and *Bacillus licheniformis* 9945A (Fig. 6.1B). As these two "species" have 98-99% identity with each other (Shivaji et al., 2006) there is clearly a classification anomaly that need resolving. The *B. lichenformis* 9945A genome has been fully sequenced (Rachinger et al., 2013) and found to encode a wide range of glycoside hydrolases (Table 6.1).

The *Bacillus lichenformis* group was not isolated from early stage subcultures, suggesting that its abundance was low, but possibly increased as the miscanthus became more extensively degraded with further subculturing and population enrichment. The isolated *B. lichenformis* strain was seen to produce a reddish pigment on some of the plates when grown on medium containing cellobiose or xylan as the only carbon source (Fig. 6.6C and D, respectively), making it another organism which may have contributed to the pigmentation observed in the TR1A culture (Figures 5.6 to 5.8).

After 24 h growth on 2TY at 55ºC, mucoidal colonies of the isolated *B. lichenformis* strain appeared on the plate (Fig. 6.6A), possibly due to production of levan as previously observed in another study (Ghaly et al., 2007). The Gram stain revealed rod shaped Gram positive cells (Fig.6.6B). The isolated *B. lichenformis* strain formed flat spreading lichen-like colonies on avicel, cellobiose, CMC or xylan plates (Fig. 6.6C to J). This strain was able to grow on avicel, cellobiose, CMC or xylan plates when incubated at 26ºC or 37ºC. The strain was only able to grow at 55ºC on 2TY plates and on avicel plates, forming larger colonies on the later, with light pink pigmentation, than the colonies formed on the avicel plate incubated at 37ºC after 24 h incubation (Fig.6.6I and J). *B. lichenformis* spp. form spores (Reuter et al., 2011).

When grown on 2% avicel or CMC, this strain had halos surrounding the colony as shown in Fig. 6.6G, K, and L, after staining with Congo red stain, and on xylan (Fig. 6.6E) even without staining with the Congo red stain (Fig. 6.6H).

The colony on the cellobiose plate did not produce a halo except for the zone where the colony had developed (Fig. 6.6F). The presence of the halos around the colony showed that this *B. lichenformis* strain could secrete glycoside hydrolases, including an exo-glucanase which degraded the carbohydrates in the surrounding area. The enzyme xylose isomerase produced by *B. lichenformis* was detected in the supernatant from the TR1A subculture 7 (Table 5.1).

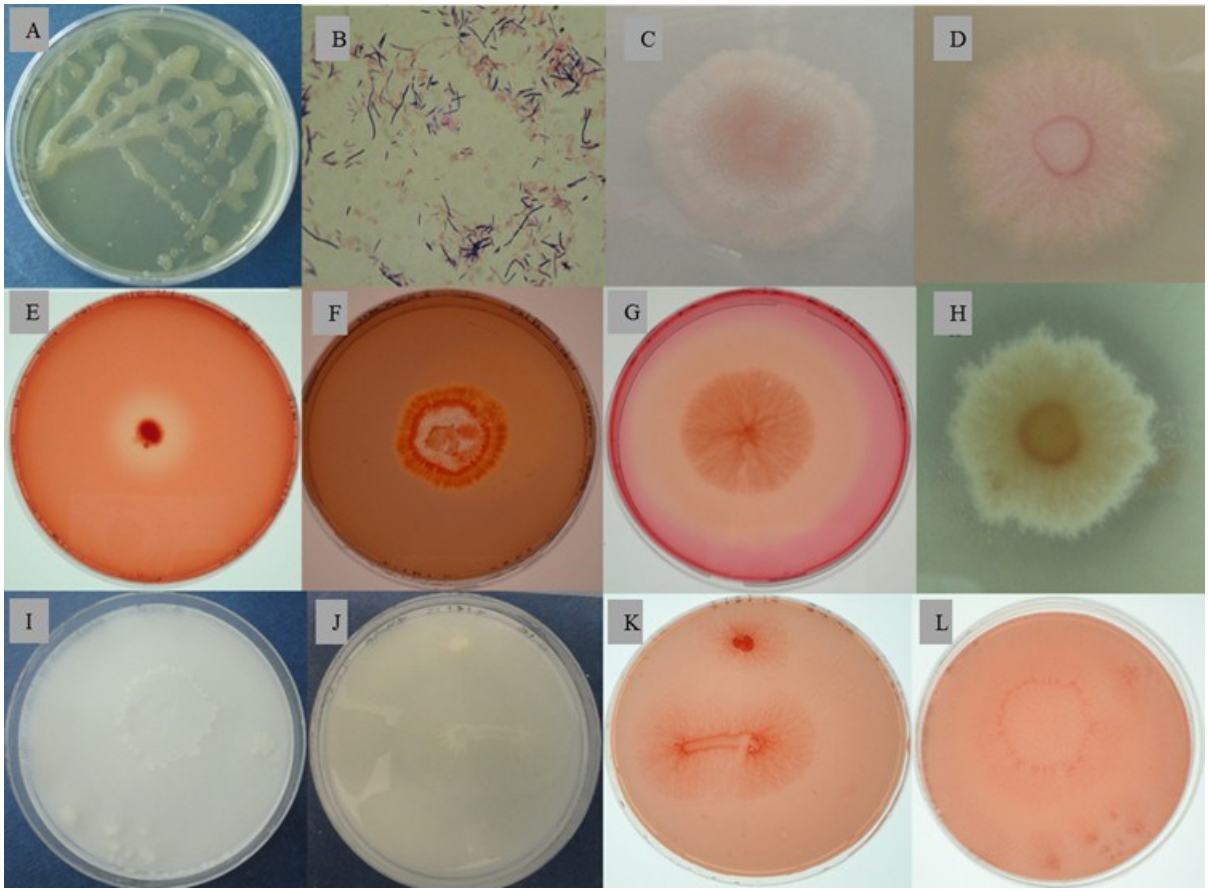


Figure 6.6 *B. lichenformis* grown on carbon sources.
(A) Colonies of the isolated *Bacillus lichenformis* after 24 h growth on 2TY at 55ºC; (B) a slide of Gram stained cells of a colony from (A), magnification-x1000; (C and F) a colony of the isolate after 3 days growth at 37ºC in modified ASM plus 2% cellobiose; (D and H) as C and F but with xylan; (G) with CMC; (E) 3 day old colony on xylan at 26ºC. Colonies on plates E, F, G, K and L were stained with Congo red. (I) Three day old colony of TR1A_17 after incubation at 37ºC on 2% avicel in modified ASM; (J) colony of TR1A_17 after 24 h incubation at 55ºC on 2% avicel in modified ASM; (L and K, respectively) the colonies in I and J after staining with Congo red.

Table 6.1 Glycoside Hydrolases and other important enzymes for lignocelluloses degradation coded for in the *Bacillus lichenformis* 9945A genome.
The genome was sequenced by (Rachinger et al., 2013). Information was sourced from NCBI.

Locus_Tag	Enzyme
BaLi_c02790	GH
BaLi_c02080	GH YbbD (GH3)
BaLi_c01720	putative GH lipoprotein
BaLi_c02790	GH
BaLi_c03540	6-phospho-β-glucosidase LicH
BaLi_c03570	putative GH
BaLi_c03580	putative GH
BaLi_c04670	β-galactosidase
BaLi_c05180	putative GH/deacetylase YcsF
BaLi_c05970	endo-1,4-β-xylanase XynA (GH11)
BaLi_c07060	putative phage GH family protein
BaLi_c07530	oligo-1,4-1,6-α-glucosidase
BaLi_c08230	exported mannan endo-1,4-β-mannosidase GmuG" (GH26)
BaLi_c09670	glycoside hydrolase family protein GH43 (β-D-xylosidase)
BaLi_c09680	β-1,4-xylosidase XynB (GH430
BaLi_c12190	putative endoglucanase YhfE (cellulase M)
BaLi_c12680	α-D-galactoside galactohydrolase MelA GH4
BaLi_c13740	glycoside hydrolase family protein (GH8)
BaLi_c14320	arabinan-endo 1,5-α-L-arabinase (GH43)
BaLi_c15160	putative glucanase YesU
BaLi_c15230	β-galacturonidase YesZ
BaLi_c19140	endoglucanase EglA (GH9)
BaLi_c19150	endoglucanase CelA (GH48)
BaLi_c19160	putative endoglucanase CelB (GH5)
BaLi_c19170	putative glycoside hydrolase CelD (GH5)
BaLi_c21530	endo-1,4-β-glucanase
BaLi_c23550	extracellular endoglucanase precursor

Table 6.1 continued. Glycoside Hydrolases and other important enzymes for lignocelluloses degradation coded for in the *Bacillus lichenformis* 9945A genome sequenced by (Rachinger et al., 2013). Information was sourced from NCBI.

Locus_Tag	Enzyme
BaLi_c26460	mannoside-phospho-β-d-glucosidase GmuD
BaLi_c27670	superoxide dismutase
BaLi_c28580	β-glucosidase (GH1)
BaLi_c29190	xylan β-1,4-xylosidase (GH43)
BaLi_c29200	putative H+-xyloside symporter XynP
BaLi_c31020	α-L-arabinofuranosidase AbfA
BaLi_c31100	arabinan endo-1,5-α-L-arabinosidase AbnA"
BaLi_c31110	putative endo-1,4-β-glucanase (cellulase M)
BaLi_c33850	glycoside hydrolase family protein (GH12)
BaLi_c35760	glycoside hydrolase family protein
BaLi_c36060	putative glycoside hydrolase family protein
BaLi_c36070	putative glycoside hydrolase family protein (GH11)
BaLi_c36230	putative xylose isomerase
BaLi_c36430	glucuronoxylanase XynC (GH30)
BaLi_c36440	arabinoxylan arabinofuranohydrolase XynD (GH43)
BaLi_c38330	β-N-acetylglucosaminidase LytD
BaLi_c40600	xylose isomerase XylA
BaLi_c40950	6-phospho-β-glucosidase LicH (GH4)
BaLi_c42060	phospho-β-glucosidase BglH
BaLi_c42100	endo-1,5-β-L-arabinosidase YxiA (β-xylosidase) GH43
BaLi_c42670	arabinogalactan endo-1,4-β-galactosidase GanB"
BaLi_c42680	β-galactosidase GanA

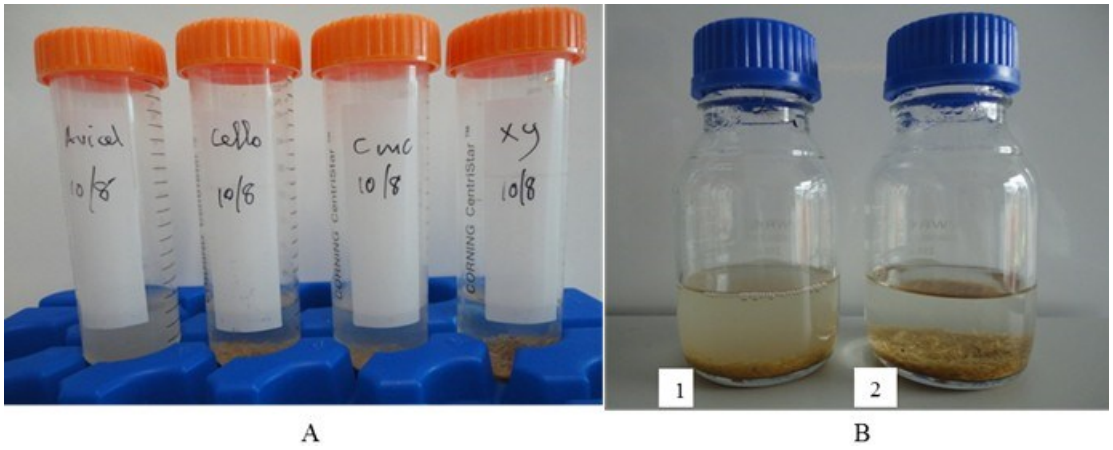
6.3.2.1.4.1 Growth and enzyme activity of *Bacillus lichenformis* TR1A_17 on 1% pretreated *M. x giganteus* in RIM.

B. lichenformis TR1A_17 had previously shown good degradation results on avicel, CMC and xylan plates (Fig. 6.6).

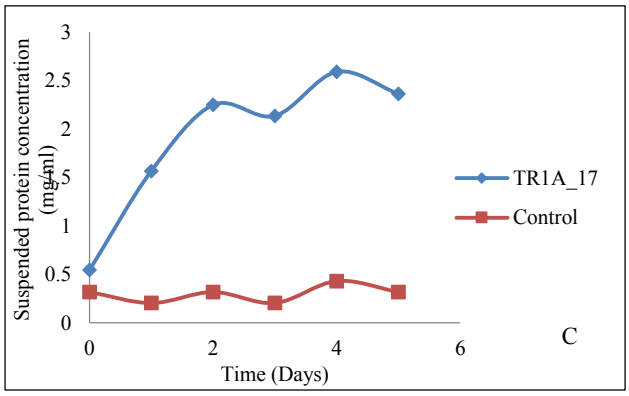
In an attempt to grow this strain on 1% pretreated *M. x giganteus* in RIM it was observed that cultures grew better when started by using inoculum from a colony from an avicel plate grown overnight at 55^{o}C. The culture did not start from overnight colonies started by using inoculum from cellobiose or xylan plates, and only started weakly when the inoculum was from a CMC overnight plate (Fig. 6.7A).

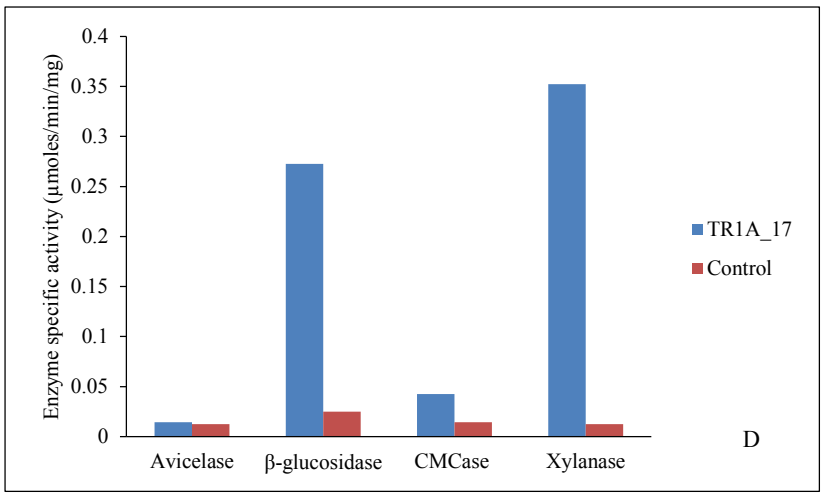
B. lichenformis TR1A_17 was the only isolate from TR1A able to grow well on 1% pretreated *M. x giganteus* in RIM as an individual culture at 55^{o}C (Fig. 6.7B) based on the suspended protein concentration recorded during the first 5 days of incubation (Fig. 6.7C). The supernatant from the 2 week old culture showed high β-glucosidase and xylanase activity (Fig. 6.7D, and F lane TR1A_17), close to that observed in the lane containing the TR1A subculture 8 supernatant (Fig. 5.17H), but no CMCase activity was detected on the CMC zymogram (results not shown), and only a low activity was detected in the supernatant (Fig. 6.7D).

However, by the sixth week of incubation, no visible degradation of the pretreated miscanthus particles was evident with the *B. lichenformis* TR1A_17 culture, although the particles in the TR1A_17 culture (Fig. 6.7B-1) appeared much finer and paler than the particles in the control (Fig. 6.7B-2).



A B





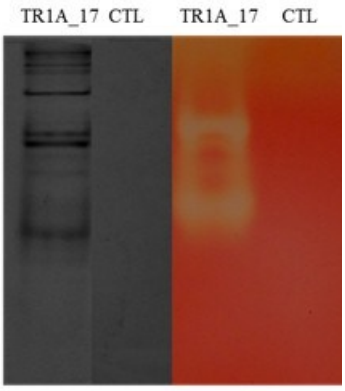


Figure 6.7 Enzyme activity of *B. lichenformis* after growth on 1% pretreated *M. x giganteus* in RIM. Overnight cultures of *B. lichenformis* TR1A_17 grown on 1% pretreated *M. x giganteus* in RIM (A). Different starter cultures of TR1A_17 in A were set up by using overnight colonies grown on 2TY plates at 55°C using inoculum from 24 h old cultures from a plate containing avicel grown at 55°C, and from plates containing cellobiose, CMC or xylan after growth at 37°C. The avicel start culture in A was used to start the TR1A_17 culture in B-1, grown on 1% pretreated *M. x giganteus* in RIM. The control contained 1% pretreated *M. x giganteus* and the same medium, but was not inoculated (B-2). The culture was incubated at 55°C with shaking at 250 rpm. Growth was monitored by measuring the suspended protein concentration (Ishida et al., 1997), and recorded as shown in (C). Enzyme specific activity of the supernatant from a 2 week old culture measured by the DNS (3,5-dinitrosalicylic acid) method according to the protocol described by Miller et al., (1960) is recorded in D. Xylanase activity was also analysed on 12% native PAGE gel containing 0.05% xylan from beechwood (Sigma), (E and F). Supernatant from *B. lichenformis* TR1A_17 was loaded in lane TR1A_17, and the control was loaded in lane CTL. The supernatant was prepared as described in Fig. 5.13.

6.3.2.2 *Staphylococcus warneri*.

TR1A_16 was another member of the *Firmicutes* phylum that was only isolated from the late stage subcultures. The isolated strain had 100% identity with *Staphylococcus warneri* SG1 based on the 16S rRNA gene sequence analysis (Fig. 6.1B). This bacterium was isolated on the actinomycetes plate that was incubated at 26^{o}C for actinomycetes isolation. It developed light yellow colonies on 2TY agar plates (Fig. 6.8A) and on avicel plus modified ASM agar plates. Initally, after 24-48 h incubation, the colonies were white/grey looking, then they became yellow, and later became granulated or rough on top with a brown base by 2 weeks of incubation (Fig. 6.8A-C).

The strain grew better at 26^{o}C than at 37^{o}C, and did not grow at 55^{o}C, on 2TY agar and 2% avicel plus modified ASM. It has cocci shaped Gram positive cells (Fig. 6.8D). A halo was visible around the colony on the avicel plate after staining with Congo red (Fig. 6.8E), an indication that it was able to secrete enzymes capable of degrading avicel. It is a skin saprophyte and could have been a possible contaminant in the TR1A subcultures. However, it was able to grow in a coculture with TR1A_13 on 1% pretreated *M. x giganteus* in RIM at 26^{o}C, with visible degradation of pretreated *M. x giganteus* particles in the remaining pellet after 6 week incubation (Fig. 6.8F).

The genome of this microbial strain codes for a number of important glycoside hydrolases, some of them are shown in Table 6.2. *Staphylococcus warneri* SG1 produces a β- N-acetylglucosaminidase enzyme which is related to another endoglycosidase enzyme (Endo-β-N-acetylglucosaminidase H (produced by *Streptomyces plicatus*) that was detected in the TR1A subculture 7 supernatant (Table 5.1). β- N-acetylglucosaminidase has been reported to have β-glucosidase activities (Ferrara et al., 2014). Enzymes detected in the supernatant from TR1A subculture 7 as produced by *Staphylococcus* spp. were enolase and dihydrolipomamide dehydrogenase component of pyruvate dehydrogenase E3 (Table 5.1), indicating that *Staphylococcus* spp. could have been present in the TR1A subcultures growing at 55^{o}C.

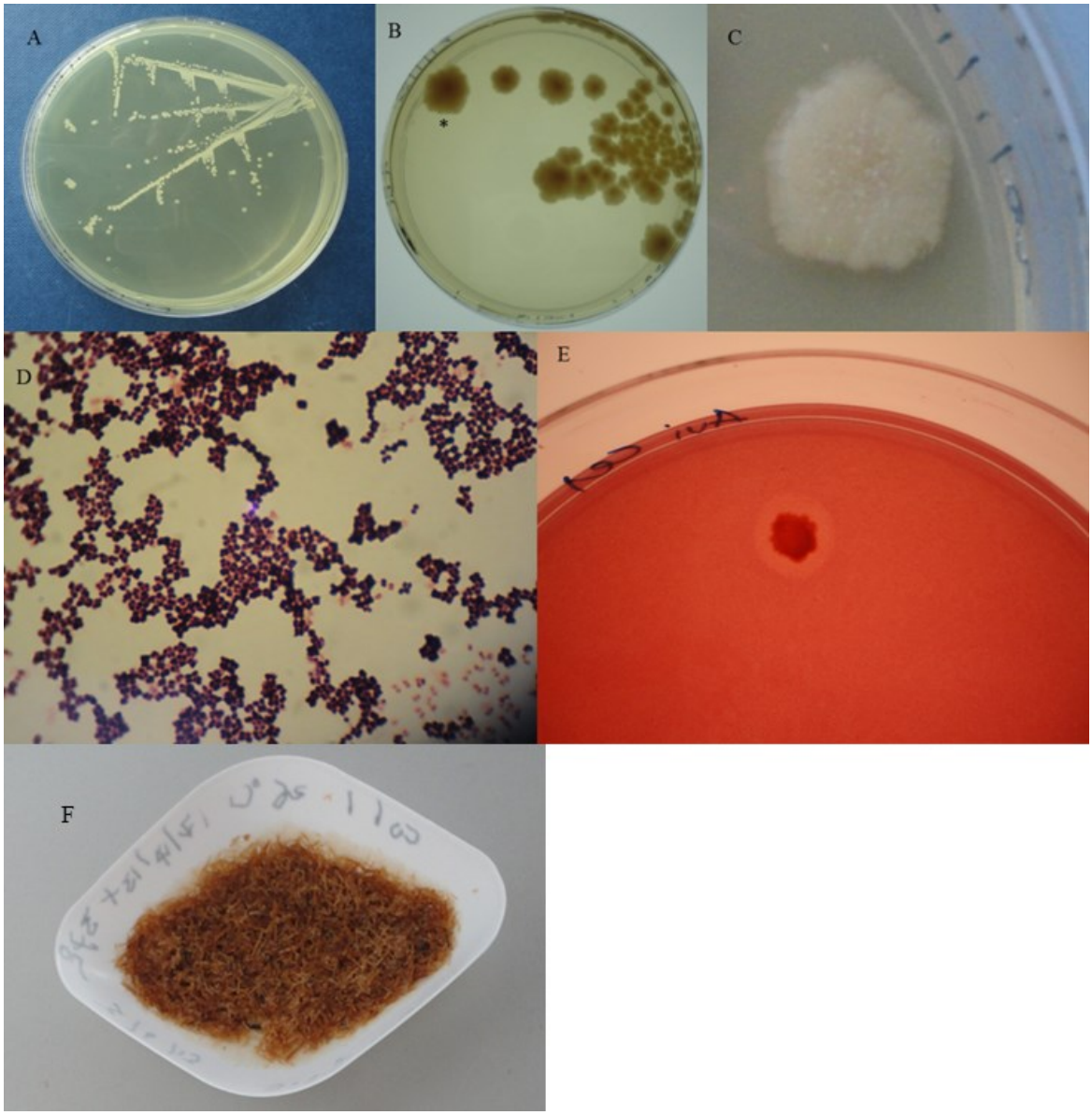


Figure 6.8 The isolated *Staphylococcus* sp. strain TR1A_16.
The isolated *Staphylococcus* sp. grown on 2TY agar plate at 26ºC after 72 h (A), base of a 2 week incubation plate grown on actinomycetes isolation medium (B), top of the colony marked with a star in B (C), a Gram stained slide from a colony on plate in A, at x1000 magnification (D), clear zone around a colony grown on 2% avicel in modified ASM at 26º C for 3 days, after staining with Congo red stain (E). Remaining pellet of pretreated *M. x giganteus* from a coculture of *S. warneri* strain TR1A_16 and *B. thermoruber* strain TR1A_13 after 6 week incubation on 1% pretreated *M. x giganteus* in RIM at 26ºC (F).

Table 6.*2* Glycoside hydrolases coded for in the *Staphylococcus warneri SG1* genome. Information was obtained from NCBI.

Locus_Tag	Glycoside hydrolase
A284_01425	6-phospho-β-glucosidase (GH1)
A284_01840	gluconokinase
A284_02020	gluconokinase
A284_02140	endo-1,4-β-glucanase (cellulose M)
A284_03015	putative N-acetylmuramoyl-L-alanine amidase (β- N-acetylglucosaminidase)
A284_03535	6-phospho-β-galactosidase
A284_04925	N-acetylmuramoyl-L-alanine amidase (β- N-acetylglucosaminidase)
A284_05055	putative aminopeptidase (cellulose M)
A284_06180	α-D-1,4-glucosidase
A284_10980	α-glucosidase
A284_11475	β-D-glucuronidase (GH2)
A284_11490	Glucuronate isomerase

6.3.3 The *Actinobacteria phylum*.

This group was also only isolated from the late stage TR1A subculture 8. The group is represented by TR1A_14 in Fig. 6.1B, and is shown branching out from the the *Firmicutes* isolates. The TR1A_14 has 99% identity with *Micrococcus luteus* strain BPB1 based on the 16Sr RNA gene analysis results.

This isolate grew better at 26^{o}C than at 37^{o}C. It produced yellow colonies on 2TY agar plates (Fig. 6.9A). It was not able to grow at 55^{o}C, and could be a contaminat on the plate cultures as it is also a skin saprophyte. The isolate was Gram positive with cocci shaped cells (Fig. 6.9B). Microorganisms in this genus have been reported to produce an insoluble yellow pigment on most solid media (Kocur et al., 1972).

They do not form spores, but have a tendency to go into dormance during harsh conditions, from which they easily get resuscitated in liquid medium (Kaprelyantst and Kell, 1993). They are able to emerge from dormancy because their genome encodes for the *Rpf* gene

(Resuscitation Promoting Factor) (Young et al., 2010). Therefore, *M. luteus* strain TR1A_14 could have been present in the TR1A subculture in the dormancy state, and was able to be isolated at 26ºC as this is close to the optimum growth temperature (28ºC) for actinomycetes (Kokare et al., 2004).

Micrococcus spp. can tolerate and use highly toxic carbon compounds, and are a very important microbial group for bioremediation activities in the environment (Sandrin and Maier, 2003). *Micrococcus luteus* has been reported to be capable of deconstructing various types of celluloses by producing endogenous and exogenous cellulases (Sarkar and Varma, 1988). The isolated strain was able to grow on avicel, with a small halo visible around a three day old colony after staining with Congo red (Fig. 6.9C).

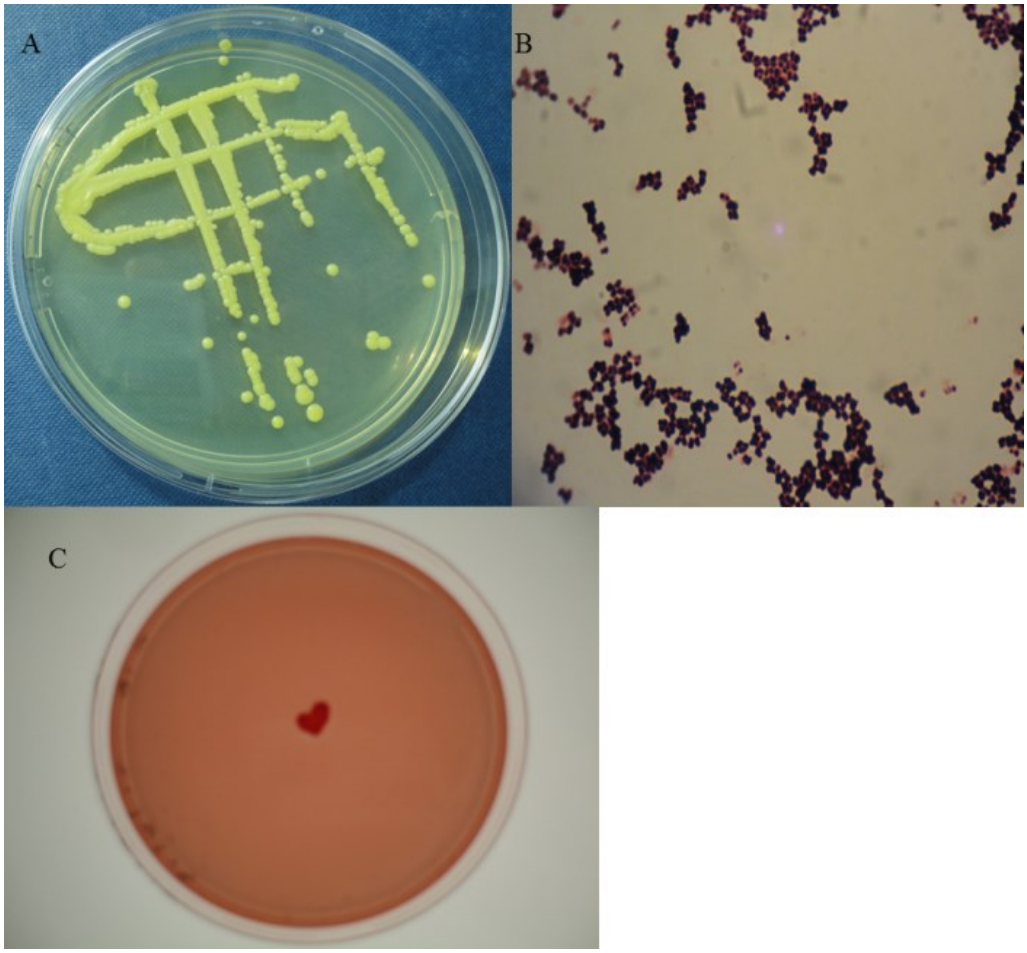


Figure 6.9 The isolated *Micrococcus* sp. strain TR1A_14.
(A) The isolated *Micrococcus* sp. strain TR1A_14 grown on 2TY at 26ºC after 3 days incubation. (B) A Gram stain slide of the culture on plate in (A), magnification–x1000. (C) A colony of the isolated *Micrococcus* sp. grown on a 2% avicel in modified ASM agar plate at 26ºC for 3 days, after staining with Congo red.

6.4 Discussion.

The results show a representation of the microorganisms in the TR1A culture. These results are similar in some way, but also different from the findings of other researchers who carried out a study using plant compost as an inoculum to study the community dynamics of cellulose degrading microbial communities (Eichorst et al., 2013). The same microbial phyla reported by Eichorst et al., (2013) were also found to be the dominant microbial groups present in the TR1A culture mixed microbial population.

However, in addition to the *Firmicutes, Bacteroidetes* and the *Thermus*, reported by Eichorst et al., (2013), members of the *Actinomycetes* were also found to be present. The microbial groups representing the *Actinomycetes* were *Thermobispora bispora* detected in the DNA isolated from the TR1A subcultures' pellets, and the *Micrococcus luteus* strain TR1A_14 isolated as an individual microorganism from the liquid fraction of the TR1A subcultures. The *Bacteroidetes* and *Thermus* spp. were the main groups present in the mixed microbial community with *Thermus* spp. making up the second largest component of the early stage and largest component of the late stage subcultures (Fig. 6.2A and B). The TR1A mixed microbial composition changed with the number of subcultures, in contrast to observations in a previous study in which the microbial composition was constant over 2 years (Haruta et al., 2002).

Identification of the mixed microbial community in the TR1A culture from the DNA isolated from the culture pellet as well as isolating the individual colonies from the culture gave a better representation of the mixed microbial community composition of the TR1A culture. The microorganisms isolated as individual colonies were all *Fermicutes* except one group which represented the *Actinobacteria* phylum. The fermicutes included *Geobacillus* spp*., Cohnella or Paenibacillus* spp*., Brevibacillus* spp*., Bacillus* spp. and *Staphylococcus* sp. *Micrococcus* sp. was the only isolated colony which represented the *Actinobacteria* phylum. The isolated colonies from the liquid fraction of the subcultures were mostly *Geobacillus* spp.

The microorganisms detected in the culture pellet DNA were different from those isolated from the liquid fraction of the culture. The isolate TR1A_75 was detected in the DNA pellet, and had 88% identity with some of the groups such as *Paenibacillus*, and *Geobacillus* spp*.,* isolated from the liquid fraction. Using only a liquid sample might have excluded or reduced the population of organisms attached to the pretreated *M. x giganteus* particles in the inoculum,

potentially including the main lignocellulose degraders as reported by another study (Edwards et al., 2007).

It was observed during previous studies that cultivable microbial groups isolated from the planktonic fraction were frequently not detected in the adherent fractions and vice versa (Koike et al., 2003; Larue et al., 2005; Morrison et al., 2009). Microorganisms directly involved in the degradation of lignocellulosic biomass have a tendency to adhere to the biomass (Edwards et al., 2007). This might be part of the reason for the difference observed in the microbial community composition detected in the pellet DNA and those isolated from the liquid fraction of the TR1A subcultures.

It is possible that some of the microbes in the TR1A mixed microbial group might aid in the fermenting processes in the cocultures as some of the members have been reported to be able to carry out fermentation. Examples include *Thermoanaerobacterium* spp. (Jiang et al., 2013), *Brevibacillus thermoruber*, (Bihari et al., 2010), and *Geobacillus thermoglucosidasius* strains (Cripps et al., 2009), all of these microbial groups were detected in the TR1A subcultures. Co-existence of both aerobic and anaerobic bacteria in mixed microbial communities has been reported (Haruta et al., 2002). No fungal colonies were isolated, presumably due to the high incubation temperature at which the TR1A culture was grown, an observation that was also reported by Haruta et al. (2002).

7. Expression of Csac_0137, an endoglucanase enzyme encoding gene from *Caldicellulosiruptor saccharolyticus*, in *Geobacillus thermoglucosidasius* strains, NCIMB 11955 and DL33.

7.1 Introduction.

Some *Geobacillus* species, such as *Geobacillus* sp. R7 (Zambare et al., 2011) and *Geobacillus* sp. T1 (Assareh et al., 2012) have been shown to express extracellular endo/exoglucanases. The genome of *Geobacillus* sp. strain WSUCF1 has been reported to contain 13 open reading frames for xylan degrading enzymes and another 3 open reading frames for cellulases (Bhalla et al., 2013b). However, the ability of these *Geobacillus* spp. to degrade lignocellulosic biomass has not been assessed under industrial conditions.

Creation of a strain that can both degrade and ferment pre-treated lignocellulose into ethanol would only be possible through successful metabolic engineering of *Geobacillus* species (Argyros et al., 2011), by introducing into *Geobacillus* strains cellulases capable of degrading cellulosic biomass. The purpose of carrying out this study was to improve the ability of two *Geobacillus thermoglucosidasius* strains, NCIMB 11955 and DL33, to degrade lignocellulosic biomass.

Metabolic engineering of two strains of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 has been carried out to improve their glycoside hydrolase activity on lignocellulosic materials. This was carried out by introducing a glycoside hydrolase family 5 endoglucanase gene, Csac_0137, from an extremely thermophilic gram positive anaerobe called *Caldicellulosiruptor saccharolyticus* (van de Werken et al., 2008). The formed mutants were characterised by growing on a mixture of cellobiose and CMC, pretreated *M. x giganteus,* or cellobiose alone used as a sole carbon source.

7.2 Construction of plasmid pUCG4.81.1 and insertion of Csac_0137 into plasmid pUCG4.81.1.

The construction of the plasmid pUCG4.81.1 and insertion of the gene Csac_0137 (39 KDa) into this plasmid was carried out by Dr. Bartosiak-Jentys and Ali Hussain, based at Bath University. The primers Csac0137FWD and Csac0137RVR, containing a 5' 40 bp region of homology to the xylanase signal peptide and vector system, respectively, were used to amplify

Csac_0137 gene. A previously assembled construct pUCG4.81.1.PheB was digested with StuI and SacI, and the 4 845 bp vector fragment was purified. The amplified Csac0137 gene and vector fragment were both added to a 15 µl Gibson Assembly reaction (Gibson et al., 2009), and transformed into *E. coli* JM109 cells.

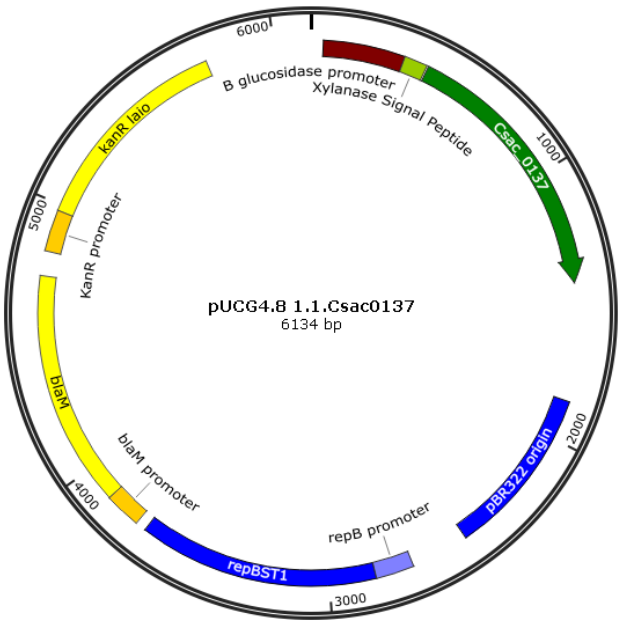


Figure 7.1 Plasmid pUCG4.81.1.
Shuttle vector containing Csac0137 gene from *C. saccharolyticus* cloned downstream of the β-glucosidase promoter.

7.3 Growth of wild-type strains *G. thermoglucosidasius* strains NCIMB 11955 and *G. thermoglucosidasius* DL33 on xylan and other carbon sources.

G. thermoglucosidasius NCIMB 11955 and *G. thermoglucosidasius* DL33 were grown on avicel, CMC and xylan to assess their ability to degrade these carbon sources. Neither strain was able to grow on avicel or on CMC (results not shown), but both were able to grow on xylan (Fig. 7.2).

Thus, both strains were good candidates for expressing the glycoside hydrolase gene for development of consolidated bioprocessing (CBP), because both strains showed the ability to degrade xylan. This is an advantage because degradation of the hemicelluloses could assist the cellulolytic degrading enzymes by providing access to the cellulose microfibrils that are

embedded in the lignocellulose biomass (VanFossen et al., 2011). Therefore, both strains were used for expressing the Csac_0137 glycoside hydrolase gene.

Both *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 were not able to grow on avicel or CMC. Like other *Bacillus* spp., previously reported (Robson and Chambliss, 1984), *Geobacillus* spp. typically lack a complete cellulase system, but may express endoglucanase or CMCase activity which does not hydrolyze crystalline cellulose. For microorganisms to be efficient in their cellulolytic activities, they need to produce one or more from the three microcrystalline degrading enzyme classes for successful deconstruction of the cellulose into glucose (VanFossen et al., 2011). However, some *Bacillus* endoglucanases (Aa et al., 1994) have shown detectable activity on microcrystalline cellulose (Kim and Kim,. 1995), but their ability to degrade real lignocellulosic biomass under industrial conditions has not been assessed.

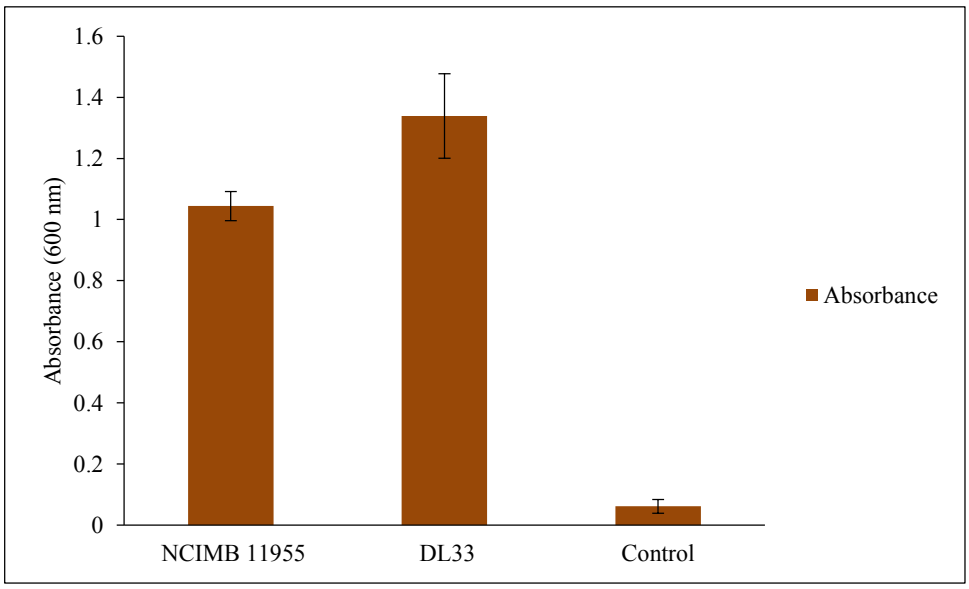


Figure 7.2 Growth of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on 2% xylan in modified ASM.
The cultures were grown at 55ºC with shaking for 24 h. Growth was monitored by measuring optical density at 600 nm. The error bars represent standard deviation of three analytical replicates.

7.4 Transformation of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 with Csac_0137.

The provided plasmid pUCG4.81.1 containing a Csac_0137 gene was first transformed into *E. coli* JM109 electrocompetent cells by heat shock to propagate the plasmid. Selection was carried out on 2TY plates containing 100 µg/ml ampicillin. Digestion of the plasmid pUCG4.81.1Csac0137 extracted from the transformed *E. coli* cells using SacI and StuI gave the products shown in Fig. 7.3 lane 2.

The plasmid pUCG4.81.1 (200 ng/µl) containing the Csac_0137 gene was then used to transform *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33, following the protocol described by (Cripps et al., 2009) modified by the use of 2TY medium. Transformants were selected on 2TY plates containing 12 µg/ml kanamycin. Both strains were successfully transformed, with the numbers of kanamycin resistance colonies giving transformation efficiencies of 7.0 x 10^4 CFU/µg DNA and 2.2 x 10^5 CFU/µg DNA, for *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33, respectively.

Digestion of the extracted plasmids gave the expected products for *G. thermoglucosidasius* DL33 and *G. thermoglucosidasius* NCIMB 11955 as shown in Fig.7.3 lanes 3 and 4, respectively.

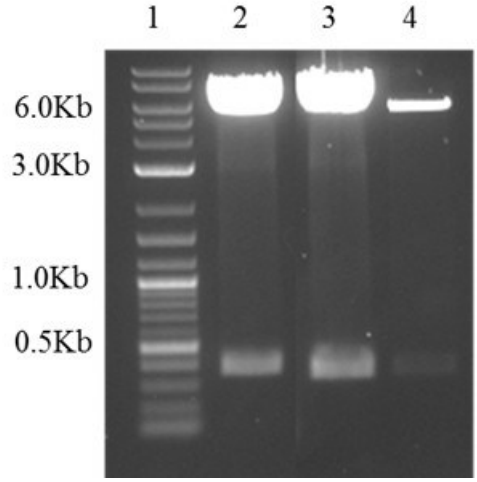


Figure 7.3 Digested plasmids of pUCG4.81.1Csac0137 and *G. thermoglucosidasius* strains, NCIMB 11955 and DL33.
Digested plasmids of pUCG4.81.1Csac0137 (lane 2), *G. thermoglucosidasius* DL33 (lane 3), and *G. thermoglucosidasius* NCIMB 11955 (lane 4). Lane 1 is the New England Biolabs 10kb DNA ladder.

7.5 Growth of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on 0.2% CMC and 0.1% cellobiose in modified ASM.

The transformed and wild strains of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 were grown on 0.2% CMC and 0.1% cellobiose in modified ASM. The 0.1% cellobiose was added to induce the expression of the inserted Csac_0137 endoglucanase in the transformed strains. The results of 24 h cultures of the transformed and wild-type strains of both NCIMB 11955 and DL33 showed that all the strains grew to about OD 0.6 measured at 600 nm (Fig. 7.4).

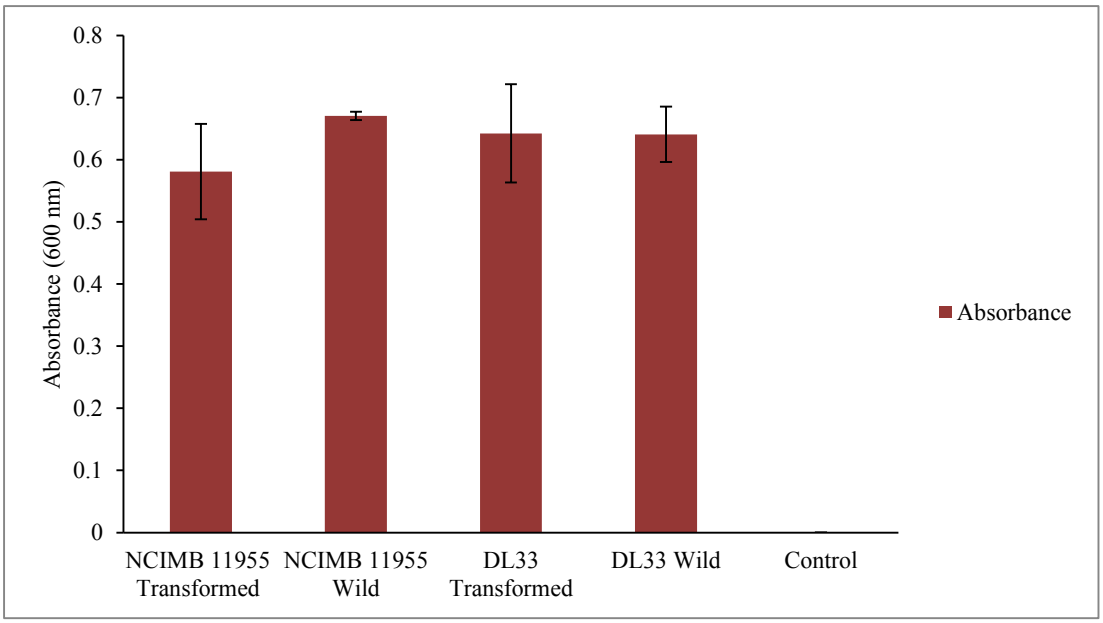


Figure 7.4 Growth of transformed and wild strains of *G. thermoglucosidasius*, NCIMB 11955 and DL33 on 0.2% CMC and 0.1% cellobiose in modified ASM.
The cultures were incubated at 55°C with shaking at 250 rpm for 24 h. The error bars represent standard deviation of three biological replicates.

7.5.1 Assessment of the CMCase specific activity of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 after growth on 0.2% CMC and 0.1% cellobiose in modified ASM.

The supernatants of the cultures and the control used in section 7.5 were assessed for CMCase specific activity. It was observed (Fig. 7.5) that the CMCase activity was almost the same for the transformed and wild-type strains of both *G. thermoglucosidasius* NCIMB 11955 and *G.*

thermoglucosidasius DL33, although it differed between the two wild-type strains. This suggests that the recombinant Csac_0137 gene was not being expressed at any significant level, in either strain, after growing on 0.2% CMC and 0.1% cellobiose in modified ASM. Interestingly, both the transformed and wild *G. thermoglucosidasius* DL33 strains showed a higher CMCase specific activity of about 0.16 µmoles/min/mg, than the transformed and the wild strains of *G. thermoglucosidasius* NCIMB 11955, each of which had a CMCase specific activity of about 0.04 µmoles/min/mg (Fig. 7.5).

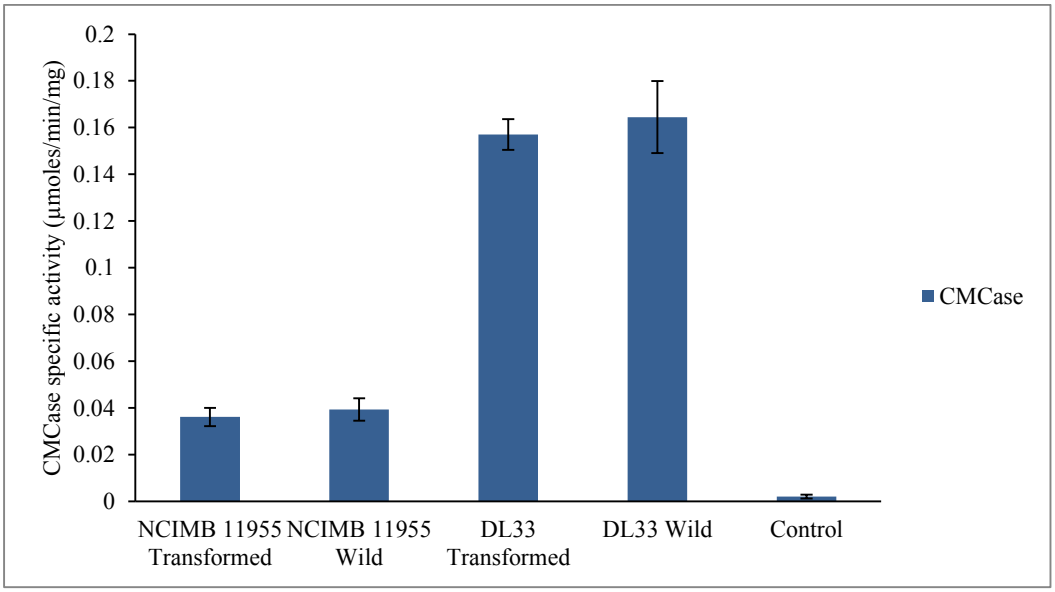


Figure 7.5 CMCase specific activity of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 after growth on 0.2% CMC and 0.1% cellobiose in modified ASM.
The cultures were incubated at 55ºC with shaking at 250 rpm. The cultures were centrifuged at 4 000 x g at 20ºC for 20 minutes in an Eppendorf 5810-R. The collected supernatant was concentrated by centrifuging 0.5 ml in a bench top accuSpin™ Micro centrifuge (Fisher Scientific) at 10 000 x g for 15 min using a 3 000 MWCO PES vivaspin centrifugal membrane separator from Sartoriuos stedim biotech. The DNS (3,5-dinitrosalicylic acid) method (Miller et al., 1960) was used to measure the enzyme activity. The error bars represent the standard deviation of three biological replicates.

The supernatants from the same cultures of the transformed and wild-type strains were analysed on 12% native PAGE zymograms containing 0.05% CMC. No significant activity was observed as there were no convincing clear zones produced on the zymograms after staining with Congo red stain. There were very faint clear zones observed in the supernatant from the transformed DL33 replicates, three zones in one of the replicates (results not shown, the bands were too faint to show on a picture).

Protein bands were visible on the native PAGE gel stained with Coomassie brilliant blue stain, but no clear differences were identified between the protein banding pattern of the transformed

and wild-type strains for both *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33.

These results showed that there was no significant expression of the Csac_0137 by the transformed strains after growth on cellobiose + CMC. It was not clear why the inserted Csac_0137 was not well expressed. Possibly, the cellobiose promoter was too weak for proper expression of the inserted endoglucanase, or it could be that the Csac_0137 gene product has poor endoglucanase activity, or that it might have very low substrate specificity for CMC, as observed for another endoglucanase, EglA (Bauer et al., 1999) .

7.6 Growth of transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on 2% cellobiose.

The growth of transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 strains on cellobiose were compared.

The results showed that both the transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 were able to grow on cellobiose, whereas no growth was observed in the control (Fig. 7.6). However, the transformed strains grew better than the wild-type strains for both *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 (Fig. 7.6). Growth of the wild-type strains on cellobiose validated the use of the cellobiose promoter for heterologous gene expression.

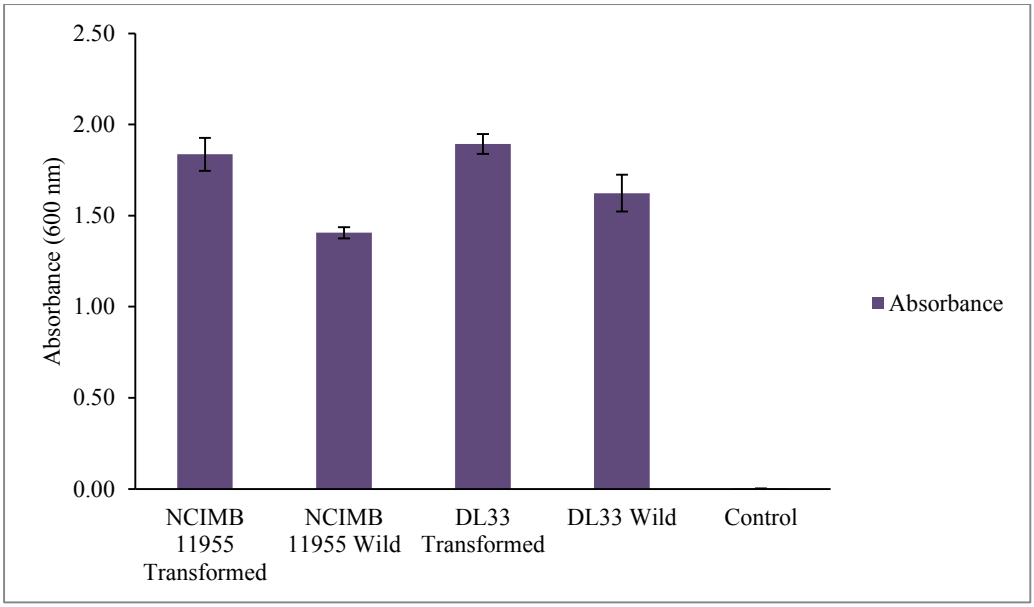


Figure 7.6 Growth of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 on 2% cellobiose in modified ASM.
The cultures and the control (uninoculated medium) were incubated at 55ºC with shaking at 250 rpm for 4 days. Growth was monitored by measuring absorbance at 600 nm. The error bars represent standard deviation of three analytical replicates.

7.6.1 Assessment of β-glucosidase activity of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 after growth on 2% cellobiose.

As both transformed strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 grew better than their respective wild-types on cellobiose it was suspected that the recombinant Csac_0137 endoglucanase could have improved the degradation of cellobiose in the culture. It was observed in a previous study that the endoglucanase, EglA, produced by *Pyrococcus furiosus* was able to degrade the β-1,4-glycosidic linkages in cellobiose (Bauer et al., 1999), so it was possible that the Csac_0137 endoglucanase was doing the same.

Improved β-glucosidase affinity to cellobiose, in the presence of endogucanase and β-glucosidase enzymes has been reported by a previous study (Lee et al,. 2012). The supernatant from the cultures was assessed for β-glucosidase activity. β-glucosidase secretion into the growth medium by *Bacillus* spp. has been reported by previous researchers (Lee et al,. 2002; Rehman et al,. 2009; Zhou et al,. 2012; Tiwari et al,. 2013; Bagudo et al,. 2014). *Trichoderma reesi* has also been reported to secrete low quantities of β-glucosidase in the medium (Nakazawa et al,. 2011;), while another study has also used the culture supernatant to measure β-glucosidase activity (Chang et al., 2012).

However, the native β-glucosidase is not expected to be secreted into the medium as it does not have a secretion signal and the cells have a cellobiose transporter. Any β-glucosidase activity in the medium could therefore be either due to cell lysis or the secreted Csac_0137 endoglucanase. The culture supernatant from the cellobiose cultures of the transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 was therefore used to assess the β-glucosidase activity of the cultures' supernatant.

The transformed strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 showed more β-glucosidase specific activity than their respective wild-types (Fig. 7.7), consistent with the better growth (Fig. 7.6). As transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 were cultured under the same conditions it was probable that the extent of cell lysis would be similar for wild-type and recombinant strains. Thus, the higher β-glucosidase activity probably reflects the activity of Csac_0137 endoglucanase.

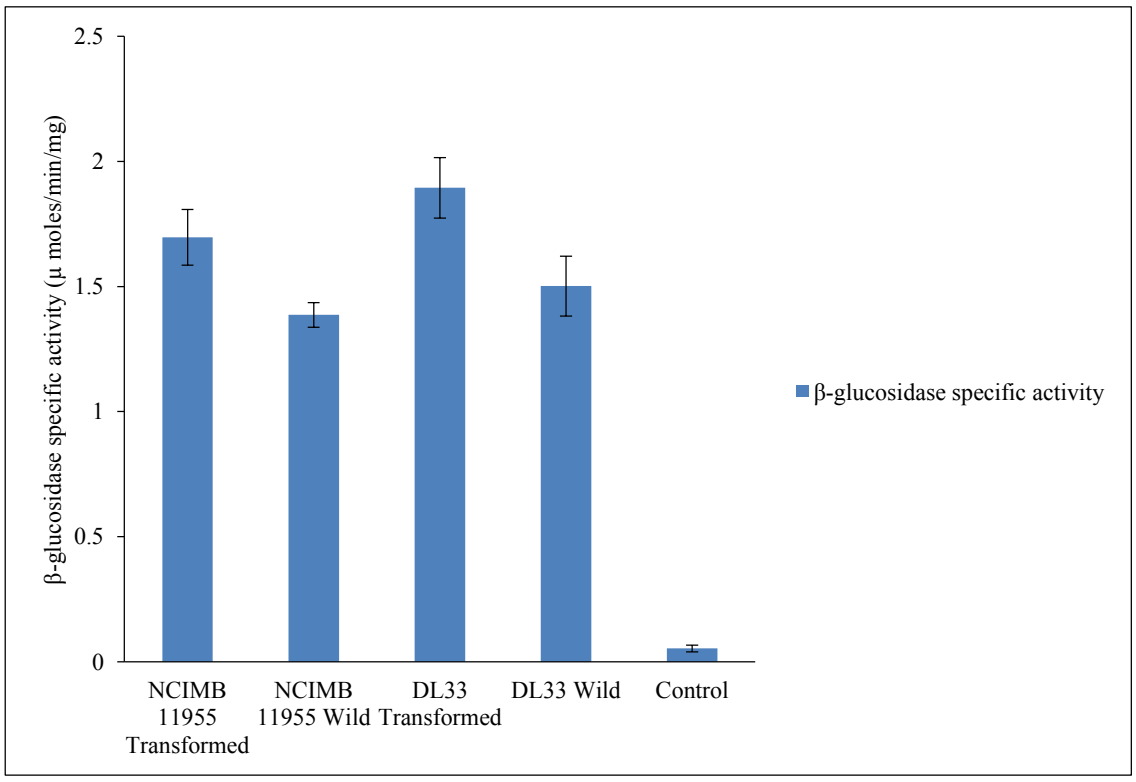


Figure 7.7 β-glucosidase specific activity of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33.
The supernatant from the 4 day old cultures after incubation at 55°C with shaking at 250 rpm was used for the assay. The supernatant preparation and CMCase activity analysis were carried out as described in Fig.7.5. The supernatant protein concentration was determined using the Bradford method. DNS (3,5-dinitrosalicylic acid) was used to measure the enzyme activity following the protocol described by Miller et al., (1960). The error bars represent standard deviation of three analytical replicates for each experiment.

7.7 Growth of transformed and the wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on pretreated *M. x giganteus*.

The transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 were grown on 1% pretreated *M. x giganteus* in RIM to assess their ability to grow on real lignocellulosic biomass. The pretreated *M. x giganteus* was used as a complex carbon source to assess whether expression of the cloned endoglucanase by the transformed *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 had an evident benefit. It has been observed during previous studies that cellulase production by microorganisms depends on the nature of the carbon source as different fermentable sugars have shown to either induce or inhibit cellulase production by different species (Sangrila et al., 2013; VanFossen et al., 2011).

No growth was observed during the first 12 days of incubation, after which the transformed strains of both *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 showed a slight increase in growth (Fig. 7.8). Transformed and wild *G. thermoglucosidasius* NCIMB 11955 strains and transformed *G. thermoglucosidasius* DL33 strains showed an increase in growth by day 16, with suspended protein concentrations of 0.31, 0.12, and 0.96 mg/ml, respectively, recorded on day 24. The transformed DL33 strain had the highest suspended protein concentration.

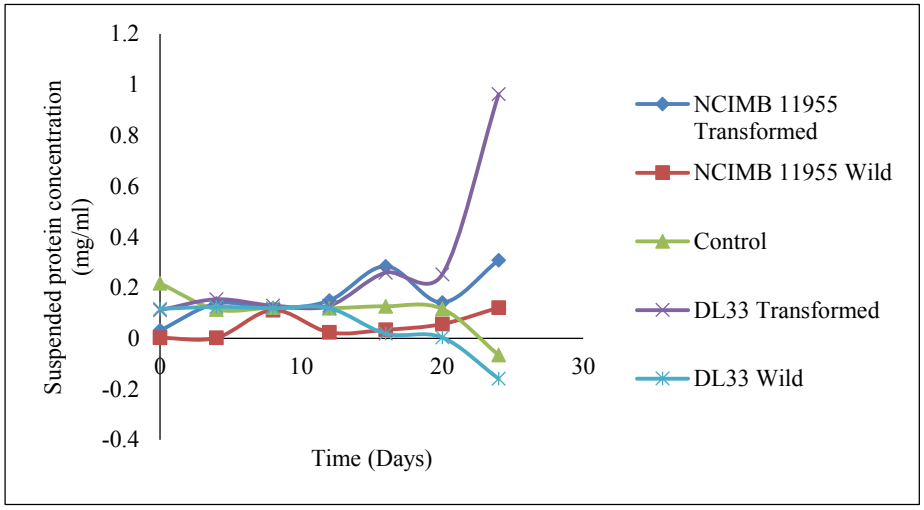


Figure 7.8 Growth of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 on 1% pretreated *M. x giganteus* in RIM.
The cultures were incubated at 55°C with shaking at 250 rpm. Growth was monitored by measuring the suspended cell protein concentration according to the method described by Ishida et al., (1997).

7.7.1 Assessment of the avicelase and CMCase specific activity of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 after growth on 1% pretreated *M. x giganteus* in RIM.

The supernatants from 24 day old cultures used in section 7.7 were analysed for avicelase and CMCase activity. After 24 days, a significant difference between the growth of wild-type and recombinant DL33 had been observed. Generally, similar avicelase (less than 0.002 μmoles/min/mg) and CMCase (less than 0.01 μmoles/min/mg) specific activity was recorded for transformed *G. thermoglucosidasius* NCIMB 11955, wild-type *G. thermoglucosidasius* NCIMB 11955 and wild-type *G. thermoglucosidasius* DL33 strains (Fig. 7.9). However, transformed *G. thermoglucosidasius* DL33 had the highest avicelase and CMCase specific activity of 0.008 and 0.03 μmoles/min/mg, respectively (Fig. 7.9). This high activity was consistent with the growth for *G. thermoglucosidasius* DL33 on 1% pretreated *M. x giganteus* in RIM recorded on day 24 as shown in Fig. 7.8.

The observed CMCase specific activity was much lower than that detected in the supernatant from the transformed and wild-type *G. thermoglucosidasius* DL33 cultures grown on 0.2% CMC + 0.1% cellobiose (Fig. 7.5), which is not surprising because pretreated miscanthus is partly crystalline and also surrounded by hemicellulose making it more difficult to degrade. The results suggested that transformed DL33 had more avicelase and CMase activity when grown on the pretreated *M. x giganteus* than its wild-type strain, resulting into higher growth than the wild-type as observed in Fig. 7.8, because more sugars were made available due to the increased avicelase and CMCase enzyme activities.

The supernatant from the same 24 day old cultures for both the transformed and the wild strains was analysed on 0.2% CMC native PAGE gels, but no activity was observed, as no clear regions were seen on the gels after staining with Congo red (results not shown). This showed that the inserted Csac_0137 endoglucanase was not expressed very well by the constructs under these conditions

The recorded avicelase and CMCase specific activity (Fig.7.9) for transformed *G. thermoglucosidasius* DL33 after growth on 1% pretreated *M. x giganteus* was comparable to that of the mixed culture of TR1A subculture 6 for the supernatant from a 2 week old culture (section 5.5.1, Fig. 5. 13). However, the CMCase activity of the 2 week old culture supernatant from the TR1A subcultures was detectable on CMC native gels (section 5.5.2.1, Figures 5.14-

5.17), while that of transformed *G. thermoglucosidasius* DL33 supernatant from 3 week old cultures was not.

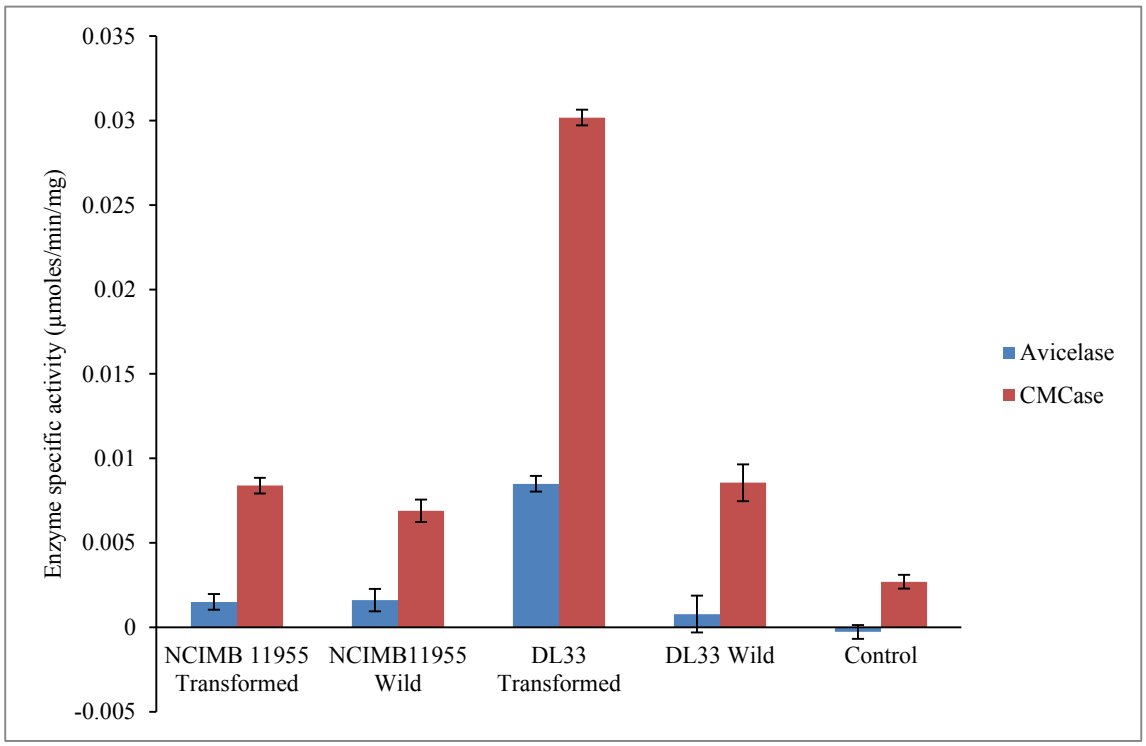


Figure 7.9 Assessment of avicelase and CMCase specific activity of transformed and wild-type *G. thermoglucosidasius* strains of NCIMB 11955 and DL33 and the control.
The supernatant was from 24 days old cultures, after incubation at 55°C with shaking at 250 rpm. The supernatant preparation and enzyme activity analysis was carried out as described in Fig. 7.5. The protein concentration of the supernatant was determined by the Biuret method. The error bars represent three analytical replicates.

7.7.2 Assessment of the xylanase specific activity of transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 and DL33 after growth on 1% pretreated *M. x giganteus* in RIM.

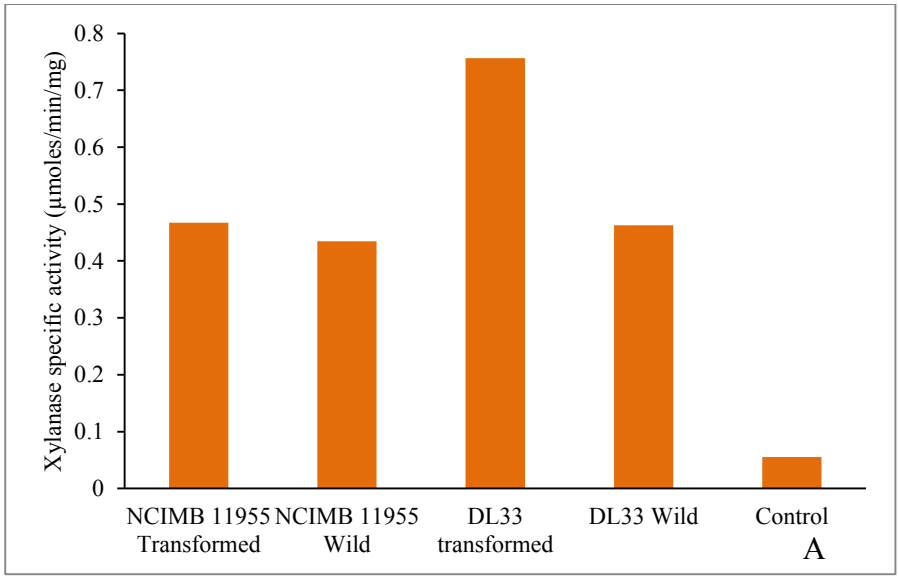
Synergistic effects between endoglucanase and xylanase were observed during a previous study in which hydrolysis of corn cobs and simple sugar yield were greater with the combined action of endoglucanase and xylanase enzymes than with separate enzymes (Dobrev and Zhekova, 2012). This suggests that expression of Csac_0137 may increase the degradation of the hemicellulose component of lignocellulosic biomass during growth on pretreated *M. x giganteus*. With this in mind, the xylanase specific activity of the same supernatants as those used in section 7.7.1 was compared.

The detected xylanase specific activity (Fig. 7.10A) was consistent with growth, as well as the avicelase and CMCase specific activity results (Figures 7.8, and 7.9, respectively). The results indicated that the xylanase specific activity of transformed *G. thermoglucosidasius* DL33 was higher than the rest of the strains (Fig. 7.10A).

The xylanase activity of the supernatant from these same samples was also assessed on 12% native PAGE gel containing 0.05% xylan from beechwood (Sigma). The supernatant from 6 week old TR1A subculture 8 from the study reported in section 5.5 of chapter 5 was also run on the same gel and used to compare with the xylanase activity of the transformed and wild-type strains (Fig. 7.10B and C).

Two clear bands (very faint) were evident (see arrow) in the lane corresponding to transformed *G. thermoglucosidasius* DL33 (Fig. 5.10C, lane TDL33), but were not present in the lane loaded with supernatant from wild-type strain of *G. thermoglucosidasius* DL33, (Fig. 7.10C, lane WDL33). Efforts to identify the enzymes giving the 2 extra bands arising from transformed *G. thermoglucosidasius* DL33 by mass spectrometry were not successful, probably due to low concentration of enzyme recovered.

The transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 showed much smaller clear zones than those of transformed or wild-type *G. thermoglucosidasius* DL33 (Fig. 7.10C), consistent with the activities detected by the DNS method (Fig. 7.10A), showing that the xylanase activity of transformed *G. thermoglucosidasius* DL33 was higher than its wild-type strain or the transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955.



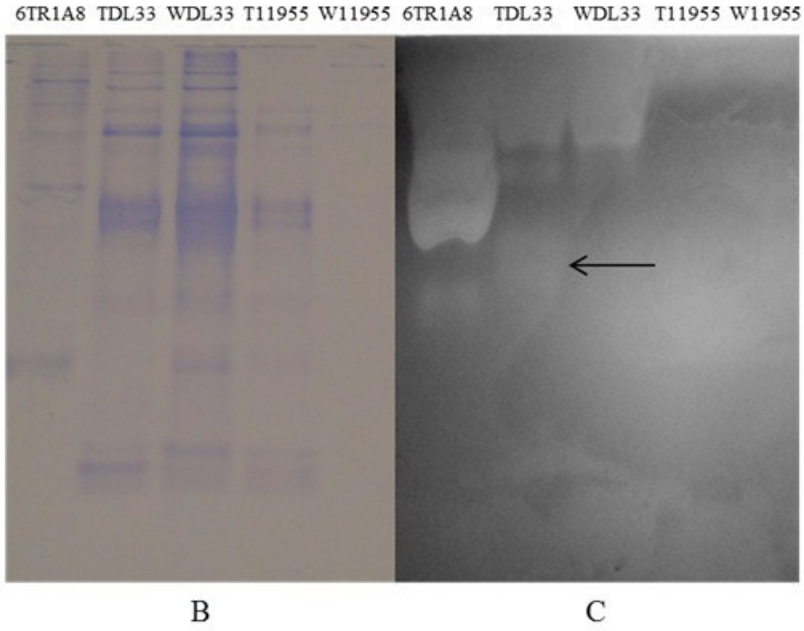


Figure 7.10 Xylanase activity of transformed and wild-type *G. thermoglucosidasius* strains of NCIMB 11955 and DL33.
Assessment of xylanase specific activity of the same supernatant samples from 24 day old cultures of transformed and wild-type *G. thermoglucosidasius* strains of NCIMB 11955 and DL33, and the control. The cultures were incubated at 55ºC with shaking at 250 rpm. Preparation of the supernatant and enzyme activity analysis was carried out as described in Fig. 7.5. The protein concentration of the supernatant was determined by the Biuret method. The DNS (3,5-dinitrosalicylic acid) method (Miller et al., 1960) was used to measure the enzyme activity for the results recorded in A. The same samples were analysed on 12% native PAGE gels containing 0.05% xylan from beechwood (Sigma), and stained with Coomassie brilliant blue (B) and Congo red (C). The samples loaded in the lanes in both B and C were supernatants from 6 week old TR1A subculture 8 (lane 6TR1A8), transformed *G. thermoglucosidasius* DL33 (lane TDL33), wild-type DL33 (lane WDL33), transformed *G. thermoglucosidasius* NCIMB 11955 (lane T11955) and wild-type *G. thermoglucosidasius* NCIMB 11955 (lane W11955), respectively.

7.8 Discussion.

Wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 were able to grow on xylan (Fig. 7.2), with *G. thermoglucosidasius* DL33 showing better growth than *G. thermoglucosidasius* NCIMB 11955, but neither of these strains grew on avicel or CMC.

Transformation of the two wild-type strains with a plasmid containing the Csac_0137 endoglucanase gene under the control of the cellobiose promoter was successful. However, expression of the Csac_0137 endoglucanase was not evident from enzyme activities in the supernatant from 24 h cultures on 0.2% CMC and 0.1% cellobiose in modified ASM. Indeed, the CMCase activity of the wild-type strains was actually slightly higher than the transformed strains for both *G. thermoglucosidasius* strains NCIMB 11955 and DL33 (Fig. 7.5). Although this endoglucanase might not be particularly active on CMC, it has subsequently been shown (Bartosiak-Jentys – unpublished) that the cellobiose promoter is not as strong as expected, so expression levels of heterologous genes using this promoter might generally be low.

However, the observed CMCase activity of transformed and wild strains of DL33 (~0.18 U/mg) was close to the CMCase activity (0.24 IU/mg) of the supernatant from a lignocellulose degrading consortia (Wongwilaiwalin et al., 2010). The avicelase, CMCase and xylanase activities (< 0.1, ~0.03, and ~0.8 U/mg, respectively) of transformed *G. thermoglucosidasius* strain DL33 after growth on 1% pretreated *M. x giganteus* were much lower than the TR1A subculture 8 activities recorded in Fig. 5.13C.

Both the transformed and wild strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 were able to grow on 2% cellobiose, showing that the strategy of using the cellobiose promoter was rational. *G. kaustophilus* and *G. thermoleovorans* also have genes that encode for β-glucosidase (Goh et al., 2014), which supports the argument that *Geobacillus* spp. are advantageous for engineering of better lignocellulose degrading strains, because they naturally produce β-glucosidase.

Interestingly, the specific β-glucosidase activity of the transformed strains after growth on 2% cellobiose was better than that of the wild-type strains in both examples studied. Although this could be associated with increased cell lysis in the cultures, there is a possibility that the heterologous, secreted Csac-0137 endoglucanase could contribute to the increased degradation of the cellobiose due to the ability of endoglucanases to degrade β-1,4-glycosidic bonds in cellobiose (Bauer et al., 1999). DNA or RNA levels in the supernatants from the cultures of

the transformed and wild-type strains could be analysed to provide evidence for, or rule out cell lysis.

When tested on 1% pretreated *M. x giganteus* transformed *G. thermoglucosidasius* DL33 grew better than all of the strains by day 24. Analysis of the supernatants from these cultures showed that the transformed *G. thermoglucosidasius* DL33 had the highest avicelase, CMCase and xylanase specific activities. These results suggest that Csac_0137 endoglucanase improved *G. thermoglucosidasius* DL33 xylanase activity during growth on pretreated *M. x giganteus*. This may be an indication that Csac_0137 endoglucanase and xylanase have a synergistic relationship in their activity.

Synergistic activity of lignocellulose degrading enzymes, such as endoglucanases, β-glucosidases, xylanases and β-xylosidases is required for complete deconstruction of complex lignocellulosic biomass (de Vries and Visser, 2001). It has been observed during previous studies that cellulases and xylanases act synergistically (Kumar and Wyman, 2009; Robison, 1984; Sanchez, 2009; Selig et al., 2008). Degradation of hemicellulose may involve synergistic actions among hemicellulases themselves or between hemicellulases and cellulases (Banerjee et al., 2010; Couturier et al., 2011; Fortes Gottschalk et al., 2010; Gao et al., 2011; Kumar et al., 2008).

The activity on CMC native PAGE gel was negative, while the xylanase native gel showed extra enzyme activity for the supernatant from the transformed *G. thermoglucosidasius* DL33 strain, visible as two faint clear zones. However, it was not possible to identify this protein by mass spectrometry due to the low protein recovery. Use of other ligno/cellulosic materials could improve the cellulase activity of the inserted Csac_0137 endoglucanase.

Despite the superior growth and enzyme activities, there was no visible degradation of the pretreated miscanthus by the transformed *G. thermoglucosidasius* DL33 strain at the end of 24 d incubation. Reducing the amount of miscanthus may produce more obvious reductions in biomass. However, it is probable that enzyme activities beyond the natural xylanase and recombinant Csac_0137 are needed to breakdown miscanthus, and the transformed strain of *G. thermoglucosidasius* DL33 may degrade lignocellulose more effectively in cocultures with other microorganisms.

8. Cocultures of transformed and wild-type strains of *Geobacillus thermoglucosidasius* NCIMB 11955 and DL33, and strains isolated from the TR1A mixed culture, on pretreated *M. x giganteus*.

8.1 Introduction.

The main focus of current research in cellulosic bioethanol is to overcome the recalcitrance of cellulosic feedstock to bioconversion. Cocultures are a promising solution to biomass hydrolysis and complete conversion of sugars for development of an economically feasible CBP (Svetlitchnyi et al., 2013). Cellulose hydrolysing microbial consortia have been reported to be stable (Harish et al., 2010) and resulted into improved cellulose utilisation (Argyros et al., 2011; Baskaran et al., 1995; Ljungdahl et al., 1981; Wiegel and Ljungdahl, 1981). However, these cocultures have not been developed to the industrial level.

Cocultivation of the transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 and strains isolated from compost during this study (chapter 6) was carried out to assess the ability of the resulting cocultures to secrete lignocellulolytic degrading enzymes and grow on pretreated *M. x giganteus.*

8.2 Co-cultivation of *G. thermoglucosidasius* NCIMB 11955 and DL33 strains with *Brevibacillus thermoruber* sp. strain TR1A_13, on 1% pretreated *M. x giganteus*.

Chapter 7 showed that the individual cultures of transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 did not grow very well on pretreated *M. x giganteus* as they probably did not secrete all of the necessary glycosyl hydrolases. Cocultures of these strains with bacteria isolated from the TR1A subcultures were established to look for improved ability to degrade pretreated *M. x giganteus*.

Cocultures of transformed *G. thermoglucosidasius* NCIMB 11955 and DL33 did not grow on 1% pretreated *M. x giganteus* in RIM even after 2 weeks of incubation. Various cocultures were set up involving the bacterial strains isolated from the TR1A subcultutures with the transformed and wild-type strains of *G. thermoglucosidasius* DL33 and NCIMB 11955 and grown on 1% pretreated *M. x giganteus* in RIM. The coculture involving transformed strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 with the isolate *B. themoruber* strain

TR1A_13 grew better than those with other strains. This coculture was subcultured into fresh medium, and was still able to grow well, with visible degaration of the pretreated miscanthus biomass particles. This coculture was therefore selected and used to compare growth, enzyme activity and degradation of the pretreated *M. x giganteus* by cocultures involving the transformed or wild-type strains of *G. thermoglucosidasius* DL33 and 11955.

B. thermoruber strains have been reported to produce thermostable cellulose hydrolysing enzymes (Liang et al., 2009; Rastogi et al., 2009). It was isolated from both the early and late subcultures of TR1A discussed in chapters 5 and 6. This suggests that it was a stable and fundamental member of the mixed microbial community that was responsible for the breaking down of the pretreated miscanthus in the TR1A subcultures. The isolate *B. themoruber* strain TR1A_13 also showed good growth on avicel, CMC and xylan (Fig. 6.5), but did not grow on pretreated *M. x giganteus* as an individual culture at 55^{o}C.

After an initial reduction in suspended protein concentration at the start of the incubation period (Fig. 8.1), the coculture of the transformed strains and the *B. thermoruber* strain TR1A_13 started growing by day 6 and initially gave a higher suspended protein concentration than cocultures of the wild-type strains and *B. thermoruber* strain TR1A_13. However, growth of the later increased between days 9 and 15 getting to similar protein concentrations as the recombinant coculture by day 18. The protein concentration in the recombinant and wild-type cocultures declined by day 21.

The individual culture of *B. thermoruber* sp. strain TR1A_13 started growing after day 9, but gave a lower suspended protein concentration (less than 2.5 mg/ml) than the transformed and wild-type cocultures throughout the incubation period (Fig. 8.1). Thus, cocultures of *G thermoglucosidasius* and TR1A_13 appeared to outperform TR1A_13 on its own and cocultures with the recombinant strains appeared to be superior to the wild-types.

In the TR1A subcultures degradation of pretreated *M. x giganteus* was always visible when cultures were grown for more than 2 weeks (chapter 5). The *G. thermoglucosidasius* cocultures were therefore left to grow for 6 weeks before assessing the degradation of miscanthus. Although the remaining pellet harvested at day 42 from the transformed and wild-type strains cocultures appeared partially degraded and finer than that of the individual culture of *B. thermoruber* sp. TR1A_13 or the control (results not shown), this was less than that previously observed in the TR1A subcultures.

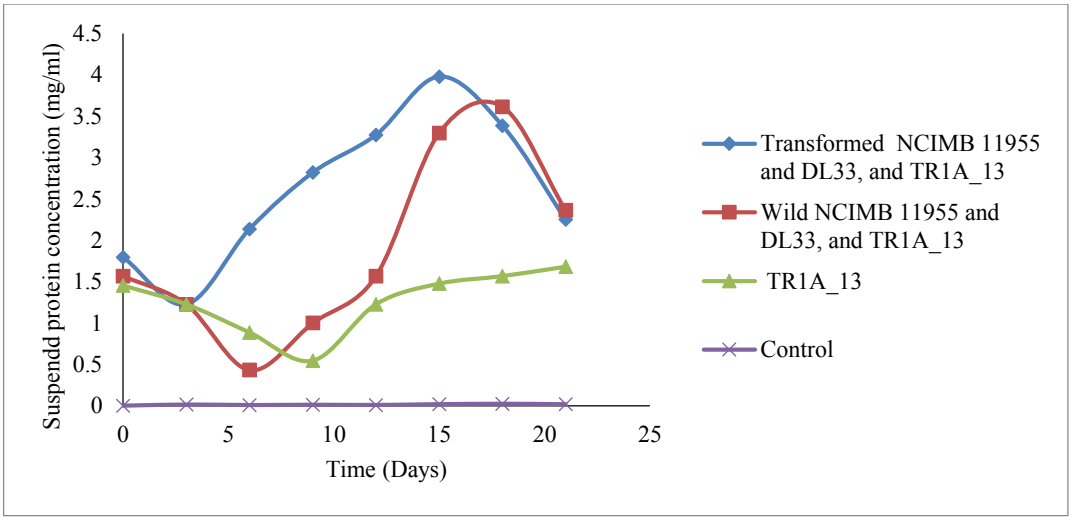


Figure 8.1 Growth of transformed or wild-type cocultures on pretreated *M. x giganteus*.
Cocultures of transformed or wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and *G. thermoglucosidasius* DL33 and *B. thermoruber* Strain TR1A_13, and the individual culture of *B. thermoruber* strain TR1A_13 on 1% pretreated *M. x giganteus* in RIM. The control was not inoculated. The cultures were incubated at 55°C with shaking at 250 rpm for 21 days. Growth was monitored by measuring the suspended cell protein concentration according to the protocol described by Ishida et al., (1997).

8.2.1 Assessment of the avicelase, β-glucosidase, CMCase and xylanase activity of cocultures of the transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 and DL33 with *B. thermoruber* TR1A_13, and the individual culture of *B. thermoruber* TR1A_13, after growth on 1% pretreated *M. x giganteus* in RIM.

The supernatants from the cultures used in section 8.2 were analysed for avicelase, β-glucosidase, CMCase and xylanase activity after 2 weeks of incubation. This revealed that there was more β-glucosidase and CMCase activity in the supernatant from the cocultures of the transformed strains than from the cocultures involving the wild-type strains for the 2 week old cultures (Fig. 8.2). Avicelase activity was the same for both cocultures, while the xylanase activity was higher in the wild-type coculture than the transformed coculture (Fig. 8.2). The supernatant from the individual culture of *B. thermoruber* TR1A_13 had higher β-glucosidase specific activity than its cocultures with the transformed and wild strains of *G. thermoglucosidasius* (Fig.8.2).

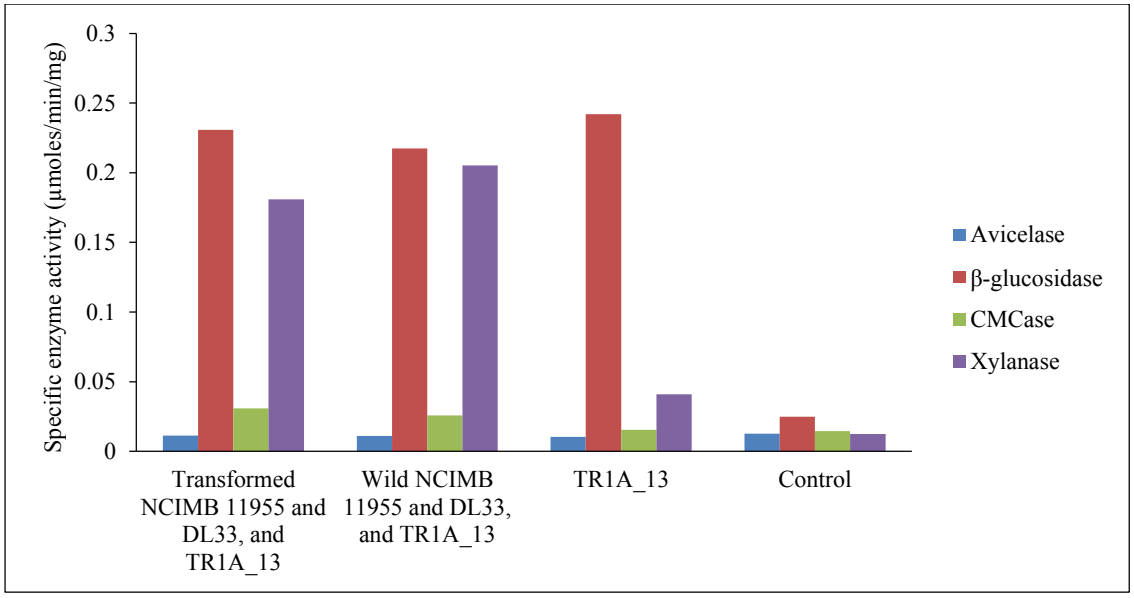


Figure 8.2 Assessment of the avicelase, β-glucosidase, CMCase and xylanase activity of the cocultures. The analysed supernatant was from the cocultures of the transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 with *B. thermoruber* TR1A_13, and of the individual culture of *B. thermoruber* TR1A_13 after growth on 1% pretreated *M. x giganteus* in RIM. The supernatants used for analysis were collected from 2 week old cultures. The DNS (3,5-dinitrosalicylic acid) method according to the protocol described by Miller et al., (1960) was used to assess the enzyme specific activity, after preparing and concentrating the supernatant as described in Fig.7.5 of section 7.5.1. The protein concentration was determined by the Biuret method.

8.2.1.1 Analysis of the CMCase and xylanase activity of the supernatant from the cocultures of transformed and wild *G. thermoglucosidasius* NCIMB 11955 and DL33, with *B. thermoruber* TR1A_13 on native PAGE gel.

The supernatants from the cultures used in section 8.2.1 were analysed for CMCase and xylanase activity on 12% native PAGE gel containing 0.05% CMC and xylan, respectively. The supernatant from a 2 week old culture of TR1A subculture 8 was also run on the same gels for comparison (Fig. 8.3A, B and C lane 2TR1A8). The supernatant from the individual culture of *B. thermoruber* TR1A_13 was also analysed on the gels (Fig. 8.3A, B and C lane TR1A13).

The cocultures containing both the transformed and wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 and *B. thermoruber* TR1A_13 showed clear zones on the xylan native gel, with the former showing extra bands indicated by the arrows in Fig. 8.3B, lane Trans Co. The fastest migrating protein which gave a clear zone indicated by the arrow (Fig. 8.3B, lane Trans Co) could be the Csac_0137 gene product, which has a predicted molecular weight of 39 KDa, and has also been reported to be better expressed on xylan (VanFossen et al., 2011). There were no clear zones produced by the supernatant from

the individual *B. thermoruber* TR1A_13 or the control (Fig. 8.3B, lanes TR1A13 and CTL, respectively).

Only the supernatant from the TR1A subculture 8 showed a clear zone on the CMC native PAGE gel (Fig. 8.3C, lane 2TR1A8). However, there were 4 faint clear bands in the upper regions of the CMC native PAGE gel, in about the same location as the upper clear zones observed on the xylan native PAGE gel in B, lanes Trans Co and Wild Co, for the supernatant from the transformed and wild-type cocultures (results not shown because the bands were too fant and might not show on picture).

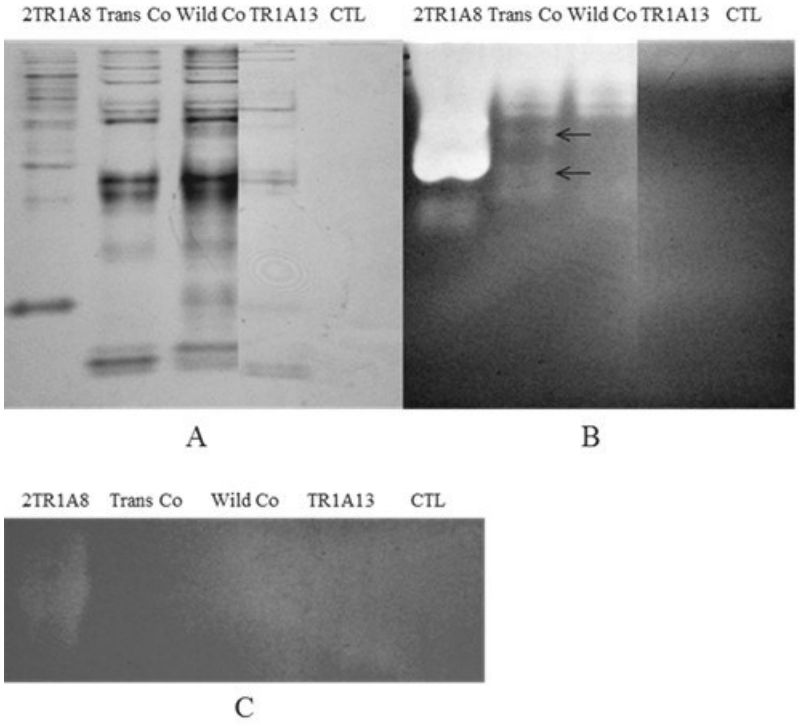


Figure 8.3 CMCase and xylanase native PAGE gels of the cocultures.
Coomassie brilliant blue stained 12% CMCase and xylanase native PAGE gels of the cocultures (A) and Congo red stained 12% native PAGE zymograms (B) for supernatants from cultures of TR1A subculture 8 (lane 2TR1A8), cocultures of the transformed (lane Trans Co) and wild (lane Wild Co) strains of *G. thermoglucosidasius* NCIMB 11955 and DL33, and *B. thermoruber* TR1A_13. Supernatant from an individual culture of *B. thermoruber* TR1A_13 (lane TR1A13) and a control (lane CTL) were also analysed. Supernatants from the same samples as those loaded in gel A were loaded in the corresponding lanes of gels B and C. The arrows in (Fig. 8.3B, lane Trans Co) show zones of clearing produced only by the supernatant from the coculture of the transformed strains of *G. thermoglucosidasius* NCIMB 11955 and DL33, and *B. thermoruber* TR1A_13, but not present in the lane containing the supernatant from the cocultures of wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33, and *B. thermoruber* TR1A_13 (Fig. 8.3B lane Wild Co). Gels A and B contained 0.05% xylan from beechwood (Sigma). The gel in C is a 12% native PAGE gel containing 0.05% CMC (Sigma). All the supernatants were prepared and concentrated as described in Fig. 7.5.

8.3. Assessment of growth and enzyme activity of the cocultures of transformed and wild-type *G. thermoglucosidasius* NCIMB 11955 or DL33 with *B. lichenformis* TR1A_17.

The results of section 6.3.2 show that *B. lichenformis* TR1A_17 could grow on pretreated *M. x giganteus* (Fig. 6.7B and C), producing high β-glucosidase and xylanase activities (Fig. 6.7D and F, lane TR1A_17). This isolate was therefore tested in another set of coculture combinations with the transformed and wild-type strains of *G. thermoglucosidasius*, to see whether expression of even a low level of recombinant Csac_0137 endoglucanase would improve growth on pretreated *M. x giganteus*. Separate cocultures of the transformed and wild strains of *G. thermoglucosidasius* NCIMB 11955 or DL33 and *B. lichenformis* TR1A_17 were set up, and growth, CMCase and xylanase activity compared after growth on 1% pretreated *M. x giganteus* in RIM.

All the cocultures showed good growth and all had a reddish pigmentation, except the coculture of transformed *G. thermoglucosidasius* NCIMB 11955 and *B. lichenformis* TR1A_17 which did not show much of this pigmentation. The reddish pigmentation of the transformed *G. thermoglucosidasius* DL33 and *B. lichenformis* TR1A_17 coculture was greater than that of its wild-type coculture, which in turn showed more pigmentation than that of the wild-type NCIMB 11955 and *B. lichenformis* TR1A_17 coculture (results not shown).

None of the four cocultures showed any CMCase activity on CMC zymograms (results not shown). All 4 cocultures showed xylanase activity on the xylan native PAGE gel (Fig. 8.4, gel B). The coculture of transformed *G. thermoglucosidasius* DL33 and *B. lichenformis* TR1A_17 produced the largest low molecular weight clearing zone (Fig. 8.4, gel B, lane T33co), followed closely by that of the coculture of wild-type *G. thermoglucosidasius* DL33 and *B. lichenformis* TR1A_17 (Fig. 8.4, gel B, lane W33co).

The extra xylanase activity in the coculture of transformed strain of *G. thermoglucosidasius* DL33 and *B. lichenformis* TR1A_17 was suspected to be due to the secreted Csac_0137 endoglucanase into the culture because of the small size of the protein, which is about 39 KDa (van de Werken et al., 2008; VanFossen et al., 2011), due to the the extra band in Fig. 8.4A, lane T33co (see arrow). The supernatant from the coculture of the wild-type strain of *G. thermoglucosidasius* NCIMB 11955 and *B. lichenformis* TR1A_17 showed 2 clear zones on the zymogram, 1 upper and 1 lower clear zone (Fig. 8.4, gel B, lane W11955co). This could

be an indication that the insertion of the Csac_0137 gene into *G. thermoglucosidasius* NCIMB 11955 actually affected its xylanase production, which could also be the reason for the low pigmentation observed in its coculture than that of its wild-type strain coculture with *B. lichenformis* TR1A_17.

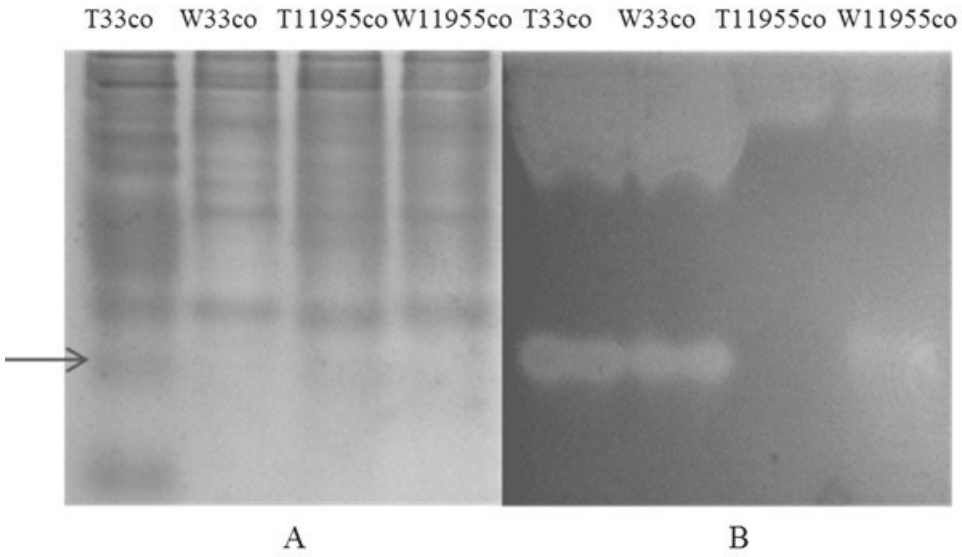


Figure 8.4 Xylanase activity of the cocultures.
Xylanase analysis of the supernatant from 3 day old cocultures of transformed *G. thermoglucosidasius* DL33 and *B. lichenformis* TR1A_17 (lane T33co), wild *G. thermoglucosidasius* DL33 and *B. lichenformis* TR1A_17 (lane W33co), transformed *G. thermoglucosidasius* NCIMB 11955 and *B. lichenformis* TR1A_17 (lane T11955co), and wild *G. thermoglucosidasius* NCIMB 11955 and *B. lichenformis* TR1A_17 (lane W11955co), analysed for xylanase activity on 12% native PAGE gels containing 0.05% xylan from beechwood (Sigma), stained with Coomassie brilliant blue stain (A), and Congo red stain (B). The cultures were grown on 1% pretreated *M. x giganteus* in RIM. The supernatant was prepared and analysed as described in Fig. 7.5.

8.4 Discussion.

The coculture of the recombinant *G. thermoglucosidasius* NCIMB 11955 and DL33 with *B. thermoruber* TR1A_13 grew better than the cocultures of their wild-type strains on pretreated *M. x giganteus* (Fig. 8.1) and both of these cocultures grew better than the individual cultures of any of the organisms (Fig. 7.8 of section 7.7 in chapter 7), and (Fig. 8.1).

More extensive degradation of pretreated miscanthus was observed with both the recombinant and the wild-type cocultures *G. thermoglucosidasius* NCIMB 11955 and DL33 with *B. thermoruber* TR1A_13, than with the individual culture of *B. thermoruber* TR1A_13. However, this was less than that observed in the TR1A subcultures (chapter 5). Involvement of more organisms which were able to support each other's growth symbiotically during

growth on pretreated *M. x giganteus* could have improved enzyme production by the participating microoganisms, allowing improved synergistic action of the enzymes during degradation of the pretreated miscanthus.

The cocultures involving the recombinant strains showed more β-glucosidase and CMCase specific activity than the wild-type cocultures (Fig. 8.2). However, the β-glucosidase activity of the individual culture of *B. thermoruber* sp. strain TR1A_13 was higher than that of its cocultures (Fig. 8.2). The xylanase activity of the individual culture of *B. thermoruber* sp. strain TR1A_13 was similarly low for both liquid (Fig.8.2) and native PAGE gel analysis (Fig. 8.3B, lane TR1A13). The CMCase was low for both the liquid analysis (Fig.8.2) and the native PAGE gel analysis (Fig. 8.3C). This shows that the endoglucanase Csac_0137 improved the β-glucosidase and xylanase activity of *G. thermoglucosidasius* NCIMB 11955 and DL33. The natural β-glucosidase expression of *B. thermoruber* TR1A_13 was affected during coculturing with the transformed or wild-type strains of *G. thermoglucosidasius* NCIMB 11955 and DL33 on pretreated miscanthus.

The coculture of transformed *G. thermoglucosidasius* DL33 and *B. lichenformis* sp. strain TR1A_17 grew best on pretreated *M. x giganteus* and produced better xylanase activity on the xylan zymograms than its wild coculture and both the recombinant and wild cocultures of *G. thermoglucosidasius* NCIMB 11955 (Fig. 8.4B). It was suspected that the extra xylanase activity in Fig. 8.4B, lane T33co was due to the secretion of the Csac_0137 endoglucanase which could have been part of the extra band pointed by an arrow in Fig. 8.4A, lane T33co, which unfortunately was not successfully identified during this study. However, insertion of Csac_0137 in *G. thermoglucosidasius* NCIMB 11955 seems to have affected the expression of the native xylanase by this bacterium.

9. General discussion and future work.

Pretreatment of *M. x giganteus.*

Given that the aim of this study was to evaluate thermophilic bacteria, with a particular focus on *Geobacillus* spp., for their ability to carry out consolidated bioprocessing it was important to use a biomass pretreatment method which left most of the polymeric carbohydrate intact. A suitable substrate and correct pretreatment methods are required for efficient saccharification (Chundawat et al., 2011), as different enzymes are said to degrade cellulosic materials to different extents (Coughlan, 1985). A suitable pretreatment method should result in opening up of the *M. x giganteus* structure of the lignocellulose, to make it more accessible to enzymes (Taherzadeh and Karimi, 2007; Yang and Wyman, 2008).

Acid pretreatment involves the use of high or low acid concentrations (Venkatesh and Pradeep, 2013). High acid concentration pretreatment results into complete degradation of cellulose and hemicellulose, but not lignin (Sun and Cheng, 2002). However, to be economical, this requires recycling of the acid. Low acid concentrations do not require recycling to be economic, but the process requires temperatures above 160^{o}C, leading to high energy costs (Gonzalez et al., 1986). Acidic pretreatment requires specialized vessels to withstand corrosion, especially at high temperatures (Venkatesh and Pradeep, 2013). The hemicelluloses get hydrolysed during weak acid pretreatment, increasing accessibility for enzymes to digest the cellulose (Kim et al., 2005), but lignin is not degraded (Venkatesh and Pradeep, 2013). Pretreatment of lignocellulosic biomass using low acid concentration also causes hydrolysis of pentose and hexose sugars leading to formation of products such as furfural and hydroxymethyl furfural, which are undesirable because microbial fermentation can be inhibited in their presence (Liu and Song, 2009).

The miscanthus biomass used for this study was pretreated by soaking in aqueous ammonia (SAA). Grasses such as miscanthus are susceptible to alkaline preatment methods, which is advantageous for integration with a CBP because the treatment is gentle (Taherzadeh and Karimi, 2007) and does not require any hi-tech machinery (Yang and Wyman, 2008). Alkaline treatment methods are carried out at normal temperatures (Venkatesh and Pradeep, 2013), and allow removal of lignin (Chang and Holtzapple, 2000; Kim and Lee, 2005), as well as acetyl groups and uronic acid from the hemicellulose (Chang and Holtzapple, 2000).

The SAA method results into breakage of the ester linkages between hemicellulose groups, increasing accessibility of the enzymes to the cellulose and the hemicellulose components and is able to retain high amounts of the xylan and glucan components (Dobrev and Zhekova, 2012; Kim and Lee, 2005; Murashima et al., 2003; Nunes et al., 2011). The compositional analysis and the saccharification results from this study showed that the pretreatment method (SAA) used for the miscanthus biomass was suitable for evaluating CBP as the polymeric sugars remained intact after the pretreatment process and were more accessible than the untreated material to enzyme degradation.

Preliminary studies of the WL isolates.

Preliminary studies were carried out to evaluate four thermophilic cellulolytic bacterial strains (WL strains) isolated on modified ASM + 0.5% CMC from wood compost previously obtained from a composting company in West London. During preliminary studies on the WL strains a procedure for reviving thermophilic bacteria from stock cultures stored at -80^{o}C was developed. Initially these thermophilic WL strains could not be revived by streaking the stock cultures directly on agar plates, but could be revived by aerobic pre-incubation in 2TY medium for at least 1 h at 55^{o}C. For WL14, 15 to 30 min incubation at 55^{o}C with shaking at 250 rpm was sufficient time for the cells to revive.

Research on *Mycobacterium tuberculosis* [the bacterium that causes tuberculosis (TB)] showed that this bacterium produced a set of proteins called resuscitation promoting factors (RPF) encoded in the genome (Cohen-Gonsaud et al., 2005), which enabled co-ordinated revival of populations of cells from dormancy, similar to those in *M. luteus* (Young et al., 2010). However, an experiment carried out to check for presence of growth/revival factors in the filtrate from the revived culture media of the WL strains did not provide evidence for such factors, suggesting that these cells were reviving independently. Further experiments could be carried out to test if growth was on the secreted material by filling holes in the 2TY agar plates with the filtrate from the cultures and inoculating the stock culture from the -80^{o}C around the hole. The growth of the culture could then be compared with that of the control containing fresh 2TY medium filled in the holes in place of the filtrate from the cultures.

Members of the *Geobacillus* spp. form endospores (Zeigler, 2014). The 16S rRNA gene analysis results showed that all four WL strains were *Geobacillus* spp. and could therefore have existed as spores in the stock cultures, needing time, sufficient nutrients and optimum

conditions to fully germinate into vegetative cells. *Bacillus subtilis* and related bacteria undergo sporulation in response to nutrient deprivation (Errington, 2003; Piggot and Hilbert, 2004; Stragier and Losick, 1996). The formed spores are durable and help the bacteria to survive harsh conditions (Hirano et al., 1991). During germination the spore requires sufficient hydration for successful breakdown of the cortex and removal of the spore coats (Segev et al., 2013). The pre-incubation of the WL cells in liquid medium during this study could have provided the cells with better hydration than on the agar plates.

Spore germination is said to be influenced by cell wall muropeptides, acids and sugars (Segev et al., 2013) which act as signaling molecules that bind to the spore membrane and signal to the spore to activate revival into a vegetative cell (Shah et al., 2008; Squeglia et al., 2011). The spore age and the incubation temperature, are said to influence the efficiency of germination (Segev et al., 2013). Further investigation of the underlying molecular processes involved in the successful revival of the WL strains might explain why incubation in the liquid medium favoured resuscitation, where as direct streaking of the stock cultures on the agar plates did not. This procedure was successfully used for reviving the -80^{o}C stock cultures of a second set of thermophilic bacterial strains isolated from domestic compost during this study.

The WL strains showed good growth on rich medium (2TY), with WL14 having the highest rate, while WL3 had the lowest rate. All four strains showed enzyme activity on avicel, cellobiose and CMC plates. The CMC plates showed largest clear zones around the bacterial colonies for the plates incubated for 24 h; after 8 day incubation the cellobiose plate showed the largest clear zones. However, the size of the clear zone on the plates not only reflects the amount of enzyme diffusing through the medium, but also the ease of substrate diffusion through the medium to the cells. This may be the case with cellobiose as β-glucosidase is commonly an intracellular enzyme and cellobiose is an easily diffusible small molecule. However, β-glucosidase has also been found to be secreted by some bacteria (Kajikawa and Masaki, 1999; Lo et al., 2009).

When grown on avicel, CMC and xylan as primary carbon sources in modified ASM liquid cultures, the WL strains did not show much growth on avicel or CMC over an 8 day incubation period; however, good growth was observed on xylan for all four strains. WL3 had the loweststt CMCase activity while WL14 had the highest. *Bacillus* spp. have been reported to produce high amounts of xylanases (Akhavan Sepahy et al., 2011; Schneider et al., 2000; Sushil et al., 2012), including thermophilic xylanases by *Geobacillus thermoleoevorans* (Sunna et al.,

1997). Another study reported production of thermophilic xylanase and arabinofuranosidase by *Geobacillus* spp. isolated from hot springs (Canakci et al., 2007).

A number of studies have also reported production of endoglucanases by *Geobacillus* spp. (Ng et al., 2009; Rastogi et al., 2010; Stathopoulou et al., 2012). However, reports of *Geobacillus* spp. producing good exoglucanase activity are very rare. Therefore, the WL strains were grown on avicel to assess their ability to degrade this substrate. All the four strains had avicelase activities. They were all shown to have some constitutive cellulase activity as low cellulase activities were detected in the control medium without carbon sources by the activity on the CMC native PAGE gels. The cells in the control were probably growing on the yeast extract in the medium.

Mass spectrometric analysis of samples taken from the clear bands on the Congo red native CMC PAGE gel revealed only one glycoside hydrolase present in the supernatant from both the control and the experiment culture, namely xylan-1,4-β-xylosidase from *Geobacillus* sp. (closest strain being Y412MC52). The other enzymes identified associated with the clear bands were NAD-dependent aldehyde dehydrogenase (MW 55.87 KDa) from *Geobacillus* sp. (closest strain being *G.kaustophilus* HTA426), Δ-1-pyrroline-5-carboxylate dehydrogenase (MW 56.55, 56.63 and 56.57 KDa) from *Geobacillus* spp. (closest strains being *G.kaustophilus* HTA426, Y412MC52 and WCH70). All these enzyme fragments were identified in samples taken from the zones of clearing for both the control and experiment supernatants, and showed a total ion confidence interval (C.I.) of 100%. (A confidence interval level closer to 100% is an indication that the identification of the protein is more likely to be correct).

These results indicated that no classical cellulases were detectable in the bands from the clear zones on the native CMC PAGE gel. Therefore the enzyme activities observed on the native CMC PAGE gels could be due to the activity of the xylosidase as the other enzymes were not typical glycoside hydrolases. However, it is also possible that the CMC active enzyme was not detected in the mass spectrometric assay due to poor recovery, proteolysis or fragmentation. Δ-1-Pyrroline-5-carboxylate dehydrogenase together with proline dehydrogenase PutB have been reported to catabolize L-proline to L-glutamate, allowing *Bacillus subtilis* to use L-proline as a sole source of carbon (Moses et al., 2012). Interestingly, Δ-1-pyrroline-5-carboxylate dehydrogenase and NAD-dependent aldehyde dehydrogenase from *Geobacillus* spp., glyceraldehyde-3-phosphate dehydrogenase from *Meiothermus* spp. and aldehyde dehydrogenases from *Micromonospora* sp., *Rhodomycrobium* sp. and *Streptomyces* sp. were

also detected in the supernatant from the TR1A subcultures. While this might reflect some involvement in glycoside hydrolysis this may simply reflect a degree of cell lysis and detection of intracellular proteins of similar gel mobility.

Slow growth was recorded for all the WL strains both on untreated and pretreated *M. x giganteus*, with no extensive degradation of miscanthus particles observed in any of the cultures. Growth on untreated miscanthus biomass could possibly be associated to the presence of the materials that could have leached out during the preatment process, while the growth on the pretreated miscanthus biomass could have been due to the presence of xylan oligomers resulting from the preatment process. Growth of WL14 on pretreated *M. x giganteus* was better than that of the other three WL strains, consistent with its higher xylanase activity. All four WL strains had high 16S rDNA sequence identities to *Geobacillus* spp. The WL 14 strain showed interesting results for growth and enzyme activity on polymeric carbon substrates and it may be informative to sequence its genome to study the glycoside hydrolases encoded. WL14 could also be further investigated in coculture experiments on the degradation of pretreated lignocellulose biomass.

New microbial isolations from domestic compost.

Consortia of thermophilic bacteria capable of degrading pretreated lignocellulosic biomass (*M. x giganteus*) were successfully enriched from domestic compost from the same composting company in West London where the WL strains were isolated. The strains were isolated on pretreated *M. x giganteus* in RIM and were called TR1A strains. The TR1A mixed microbial culture showed better growth on pretreated *M. x giaganteus* in buffered RIM than in unbuffered medium, and were able to degrade about 50% of the pretreated miscanthus biomass solid particles in 6 weeks, but could not degrade the untreated *M. x giganteus*. Compositional analysis results showed that the microbes in the mixed culture were able to use all the sugar components and were also able to degrade part of the lignin.

A stable mixed microbial community capable of degrading pretreated *M. x giganteus* was developed from the TR1A culture. Growth in the culture slightly dropped from about 0.6 mg/ml total cell protein in the first TR1A subculture to about 0.3 to 0.4 mg/ml total cell protein in the sixth TR1A subculture. The drop in growth could be due to dying off of microbial groups that could not use pretreated miscanthus biomass for growth. The stability of the mixed microbial community was shown by its ability to degrade pretreated *M. x giganteus* particles

in the subcultures, as the subcultures 6, 7 and 8 degraded about 50% of the pretreated miscanthus biomass. Stability of the degradation ability of the microbial community was also observed in a previous study (Haruta et al., 2002). Interestingly, the degradation ability was also tolerant to freezing at -80^{o}C, an observation also made in other studies (Haruta et al., 2002; Lv et al., 2008; Wongwilaiwalin et al., 2010).

The avicelase, CMCase and xylanase activity detected in the supernatant of the TR1A subcultures increased with subculturing (Fig. 5.13). Increase in enzyme activity was also confirmed by the greater CMCase activity detected on the CMC native PAGE gels for the supernatant from TR1A subculture 8 than that detected from the earlier subcultures (Figures 5.14 -5.17). These observations indicated that increasing the incubation period of the cultures on pretreated *M. x giganteus* allowed degradation of the pretreated miscanthus biomass particles. Longer incubation periods could also have created conditions that promoted secretion of enzymes involved in ligninolytic hydrolysis, an observation that was also reported during an earlier study (Chen et al., 2012a).

HPLC results showed that fermentation products (formate and lactate) were present in the supernatant from the TR1A mixed subculture 8. The unidentified products detected in the TR1A culture supernatant might give important information about the processes in this culture. Increasing the number of standards for HPLC analysis of the supernatant from the TR1A subcultures needs to be considered for future investigation to identify these unkown products.

Lack of detection of sugars in the TR1A mixed culture supernatant by HPLC shows that the degrading microorganisms were utilizing all the produced sugars for their own growth. A previous study reported a low concentration of glucose, less than 3 mg/ml, present in the mixed microbial culture (Haruta et al., 2002). Future work on the TR1A mixed culture should include studies on balancing the amount of pretreated miscanthus biomass to be used to allow detectable levels of sugars to remain in the culture that could be available for fermentation or production of other important products.

High ethanol concentration have been detected in supernatants from other mixed microbial cultures (Haruta et al., 2002), in contrast to the observations of this study, where no ethanol was detected. Use of other alcohol detection methods such as analysis of the gaseous phase by headspace GC analysis (Wei et al., 2013) or NMR (Kim et al., 2013) should be considered to analyse the amount of ethanol produced by the TR1A mixed culture grown on pretreated *M. x*

giganteus. Alcohol detection could also be improved by analysing samples taken at various stages of the subculture from as early as 5 day old cultures as reported by Haruta et al. (2002).

It was clear that many enzymes were involved in the degradation of the pretreated *M. x giganteus* biomass in the TR1A subcultures. Avicelase, CMCase and xylanase activities were detected in the supernatant from the mixed TR1A subcultures both by DNS method as well as on native PAGE gels containing CMC or xylan carbon substrates. A similar pattern of clear bands was observed on CMC and xylan containing native PAGE gels, therefore same enzyme groups could have been degrading cellulose as well as xylan in the TR1A subcultures.

Previous studies have reported degradation of hemicellulose by endoglucanases (Karboune et al., 2009). Cellulases are known to work synergistically with hemicellulases to achieve degradation of lignocellulosic biomass to sugars (Damude et al., 1996; Gao et al., 2011; Sizova et al., 2011). This could suggest that both enzyme groups could be present in the same clear band, as observed during this study, because more than one enzyme was detected in some of the bands. Other researchers have also reported that these lignocellulose degrading enzymes work better in groups than as individual enzymes (Himmel et al., 2007; Rojas et al., 2005; Tuncer and Ball, 2003a; Tuncer and Ball, 2003b).

The enzyme activities detected in the medium and on the gels were confirmed by the many different enzymes that were detected by mass spectrometry from the supernatant from the mixed microbial TR1A subcultures after growth on pretreated *M. x giganteus*. The results also showed that enzymes were produced at different times in the culture as different and sometimes same patterns of cleared zones were obtained from analyzing samples collected at different ages of the culture on native CMC PAGE gels. The xylan degrading enzymes were the largest group of the enzymes detected in the TR1A mixed microbial subcultures' supernatant by mass spectrometry, followed by the xylose isomerase, which is mostly an intracellular enzyme, but has also been reported to be secreted by a thermophilic *Bacillus* sp. (Chauthaiwale and Rao, 1994). Future work could also consider testing the TR1A culture supernatant for the presence of DNA or RNA to check if cells had lysed to release intracellular enzymes into the culture.

The enzymes identified by mass spectrometry showed a xylanase dominated group in the TR1A subculture 6 shifting to more xylose isomerase, as well as appearance of α-N-arabinofuranosidase and transaldolase in the TR1A subcultures 7 and 8, and endoglucanase A

in TR1A subculture 8. This could mean that the mixed microbial group in the late TR1A subcultures most likely consisted some of the cellulolytic bacteria which could purely depend on cellulose as a carbon source.

There is also a possibility that some of the cellulases could have been present as cellulosomes attached to the microbial cell walls and hence were not detected in the supernatant. Future studies on the TR1A mixed culture should look at the isolation of the cellulosomes by using methods for analyzing cellulosomes attached to the bacterial cell surfaces (Anderson et al., 2011), and also isolating the cellulosomes secreted in the culture by analyzing the suspended and attached fractions of the culture supernatant (Bayer et al., 1998). This will give more information on the glycoside hydrolases involved in the degradation of the pretreated *M. x giganteus* in the TR1A mixed microbial culture.

Future investigations could also consider testing the ability of the supernatant from the TR1A subcultures to saccharify different types of pretreated biomass and at elevated temperatures of up to 80^{o}C to check if the secreted enzymes could be used for the development of enzyme mixtures suitable for biorefinery processes. The supernatant from the TR1A subcultures could also be tested for ligninase activity to check for the ability of the secreted enzymes to degrade lignin.

Identification of bacteria isolated from the TR1A mixed microbial culture by 16S rRNA gene analysis.

The bacteria detected in the TR1A mixed culture isolated from the culture pellet DNA belonged to *Firmicutes, Bacteroidetes, Actinomycetes* and the *Thermus,* while those isolated as individual colonies from the liquid fraction belonged to *Actinomycetes* and *Firmicutes.* The microbial groups identified to be present in the TR1A mixed culture have been reported to produce glycoside hydrolases, as discussed in chapter 6. The individually isolated *Firmicutes* were mostly *Geobacillus* spp., consistent with the observation that this bacterial group was identified as the major producer of the enzymes detected in the TR1A mixed culture supernatant (Table 5.1).

The bacteria identified in the culture pellet DNA and those from the liquid fraction of the culture were different. Unless there is complete understanding of all the requirements of the mixed microbial community, it is not easy to isolated all the microbes present in the mixed

microbial culture, as earlier suggested by other researchers (Lee et al., 1996; Ward et al., 1998). The isolation and identification of the microbes in the TR1A mixed microbial culture needs to continue to give a complete composition of the microbial population. There is also a possibility that more groups could be detected in the pellet DNA if specific primers for other microbial groups such as fungi, actinomycetes and archaebacteria, could be used, as well as changing the conditions of PCR for amplification of the specific conserved sequences for each microbial group.

The *B. lichenformis* sp. strain TR1A_17 was the only isolate from the TR1A subcultures that was able to grow on pretreated *M. x giganteus* at 55^{o}C, and showed detectable β-glucosidase, xylanase activities, as well as low levels of avicelase and CMCase activities in the supernatant and agar plates with CMC, avicel, and xylan carbon sources. The best CMCase and xylanase activity was observed on the *B. lichenformis* strain TR1A_17 CMC and xylan plates incubated at 37^{o}C. This strain only grew at 55^{o}C on avicel and 2TY agar plates, but showed better avicelase activity on the avicel plate incubated at 55^{o}C than that incubated at 37^{o}C. However, xylanase activity was also detectable in the supernatant from the pretreated *M. x giganteus* culture at 55^{o}C on the native PAGE gel containing xylan, but no CMCase activity was detected on the CMC native PAGE gels. All these cultures were grown at pH7.

In a previous study another strain, *B. lichenformis* JK7*,* was found to record high activities of endoglucanase, followed by β-glucosidase, with xylanase recording the lowest activity, at optimum temperatures of 70^{o}C for the endoglucanase and 50^{o}C for both β-glucosidase and xylanase after growth on CMC and xylan (Seo et al., 2013). It has been reported to produce cellulases at temperature ranges of 50 to 55^{o}C and pH6.5 to 7 after growth on CMC and filter paper (Acharya and Chaudhary, 2012). An endoglucanase produced at an optimum temperature of 55^{o}C at pH6.1 was isolated from its culture in another study (Dhillon et al., 1985). These observations show that *B. lichenformis* spp. are capable of producing thermostable endoglucanases, as well as xylanases, depending on the carbon substrate.

B. lichenformis encodes a number of glycoside hydrolases in its genome (Table 6.1) and would be expected to degrade the pretreated *M. x giganteus* to greater extents, but there was no much physical degradation of the pretreated *M. x giganteus* particles observed in the culture. This observation seems to highlight the importance of cocultures, to help balance the conditions in the culture for better growth by the participating microorganisms through their symbiotic relations; this could result into secretion of sufficient levels of enzymes to promote synergistic

activities to achieve degradation of the lignocellulosic biomass to make the fermentable sugars available.

Expression of Csac_0137, an endoglucanase enzyme encoding gene from *Caldicellulosiruptor saccharolyticus* in *Geobacillus thermoglucosidasius* strains, NCIMB 11955 and DL33.

The wild-type strain of *G. thermoglucosidasius* DL33 grew better than the wild-type strain of *G. thermoglucosidasius* NCIM 11955 on xylan, but both wild-type strains were not able to grow on avicel or CMC. These two wild-type strains were successfully transformed with a Csac_0137 endoglucanase gene from *C. saccharolyticus* contained in a plasmid under the control of a cellobiose promoter. The inserted endoglucanase gene did not seem to improve the CMCase activities of the transformed strains after growth on 0.2% CMC + 0.1% cellobiose. However, both transformed strains were able to grow on 2% cellobiose. The transformed strains of *G. thermoglucosidasius* DL33 and NCIMB 11955 recorded more β-glucosidase activity than the wild-type strains, an indication that the secreted Csac_0137 could have contributed to the cellobiose degradation activities in the cultures of the transformed strains.

The transformed *G. thermoglucosidasius* DL33 strain recorded the highest growth and enzyme specific activities (avicelase, CMCase and xylanase) after growth on pretreated *M. x giganteus.* Enzyme activity analysis on native CMC PAGE gels showed no activity for all the strains, but the xylanase was positive on the xylan native PAGE gels, with transformed *G. thermoglucosidasius* DL33 showing more activity. This could be due to synergistic interactions between the endoglucanase and xylanase as earlier observed by Dobrev and Zhekova (2012). There was no visible degradation of the pretreated *M. x giganteus* particles in the cultures for all the strains. However, the improved growth and enzyme activities recorded by transformed *G. thermoglucosidasius* DL33 looked interesting, and could make this strain useful in cocultures with other microorganisms for better degradation of pretreated miscanthus biomass for CBP development.

Co-cultivation of transformed and wild-type strains of *Geobacillus thermoglucosidasius* NCIMB 11955 and DL33, and strains isolated from the TR1A mixed culture, on pretreated *M. x giganteus*.

Various cocultures were set up, involving the bacterial strains isolated from the TR1A subcultures with the transformed or wild-type strains of *G. thermoglucosidasius* DL33 and NCIMB 11955 and grown on pretreated *M. x giganteus*. The cocultures involving the transformed *G. thermoglucosidasius* strains of DL33 and NCIMB 11955 and *B. thermoruber* strain TR1A_13 grew better on pretreated *M. x giganteus*, and showed improved β-glucosidase

and CMCase activity compared to the wild cocultures or the individual cultures for these microorganisms. However, there was more degradation of the pretreated *M. x giganteus* particles visible in both the wild-type strains and the transformed strains cocultures, but the extent of degradation was less than that observed in the TR1A subcultures.

The findings of this study suggest that it is not easy for one microorganism, either isolated or created through engineering to produce all the enzymes required to degrade lignocellulosic materials. Thermophilic microbes have also been reported to produce enzyme yields which are too low for use at industrial level compared to mesophilic microbes (Bhalla et al., 2013a). Development of microbial consortia of similar composition to natural mixed microbial communities seems to be necessary for the release of the correct enzymes in the right amounts to achieve degradation of ligocellulosic biomass to release the sugars. Mixed culture systems are advantageous in overcoming the feedback regulation and metabolite repression in pure culture fermentation (Delatorre and Campillo, 1984; Lu et al., 2005; Lv et al., 2008; Soundar and Chandra, 1987). A previous study has also shown that natural microbial communities are effective for cellulose degradation (Lv et al., 2008).

The results from this study indicated that most of the isolated colonies from the TR1A subcultures were *Gebacillus* spp., therefore, members of this mixed microbial community were able to grow in the same culture with the *Geobacillus* spp. The enzymes identified in the TR1A culture (Table 5.1) at each sampling time showed that the *Geobacillus* spp. produced a variety of enzymes in the TR1A subculture. It might be possible to develop a coculture of various *Geobacillus* strains isolated from this mixed culture that could successfully grow on pretreated *M. x giganteus* and capable of degrading miscanthus.

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Appendix.

Peptides detected by mass spectrometry and their most closely related protein sequences.

The samples were prepared by the in-gel trypsin digest method or by following the method described in the ProteaseMAX™ Surfactant, Trypsin Enhancer from Promega. Determination of the enzyme sequence information of the isolated protein bands was by LC-mass spectrometry (MALDI and LC-MS/MS) and the derived partial peptide sequences were searched against protein databases. The position of the detected peptides are highlighted in the most closely related protein sequence.

TR1A subculture 6 (From Fig. 5.15, Table 5.1).

Xylan 1,4-β-xylosidase [*Geobacillus* sp. G11MC16] (MW 80145.5) Gel B, band 1 TR1A subculture 6

Detected Peptides

VGALIVDVPAGE
EGICLFPDGGWK
SGGLDLELARPPR
AGDLTFTIYSPVK
AFFGFEGTDPYTSMR
SNELIEKEWLSDDQK
SYYGNTQLLEHEGKPIWVVNEGEYR

Location of peptides in the protein sequence

MIMPNNLFFNAHHSPVGAFSSFTLGFPGKSGGLDLELARPPRQNVFIGVESLHESGLYHVLPFWETSGE
DESKRYDIENPDPNPQKPNILIPFAKEEIQREFHVATDTWKAGDLTFTIYSPVKAVPDPETADEEELKLA
LVPAVIVEMKIDNTNGTRARRAFFGFEGTDPYTSMRRIDETCPQLRGVGQGRIVGIVSKDEDVRSALHF
SMEDILTAQLEENWTFGLGKVGALIVDVPAGEKKTYQFAVCFYRGGYVTAGMDTSYFYTRFFNNIEEV
GLYALEKAEVLKEQSFRSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMM
NTFDLTVDQLFFELKMNPWTVKNVLDLYVERYSYEDGVRFPGEETEYPGGISFTHDMGVANTFLRPHH
SSYELYGLSGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDKRLAILEQCLESMVRRDHPDPEKRN
GIMGLDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFHDVGKEELAALAGEQA
EKCAATIVSYVTDDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALNKDGRFGEYIQALNAHLRYVL
REGICLFPDGGWKISSTSNNSWLSKIYLCQFIARHLLGWEWDEQGKRADAAHVAWLTHPTLSIWSWSD
QIIAGEIKASKYYPRGVTSILWLEEGE

Xylan 1,4-β-xylosidase [*Geobacillus* sp. Y412MC52] (MW 79815.5) Gel B, band 1 TR1A subculture 6

Detected Peptides

IDDTCPQLR
NVLDFYVER
VGALIVDVPAGEK
ILGWEWDEQGK
SGGLDLELARPPR
AFFGFEGTDPYTSMR

Location of peptides in the protein sequence

MPTNVFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESPHEPGLYHILPFAETAGEDES
KRYDIENPDPNPQKPNILIPFAKERIEREFRVATDTWKAGDLTLTIYSPVKAVPDPETASEEELKLALVPA
VIVEMTIDNTNGTRTRRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRILSIVSKDEGVRSALHFSMEDI
LTATLEENWTFGLGKVGALIVDVPAGEKKTYQFAVCFYRGGYVTAGMDASYFYTRFFHNIEEVGLYA
LEQAEVLKEQAFCSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNTFD
LTVDQLFFELKMNPWTVKNVLDFYVERYSYEDRVRFPGDGTEYPGGISFTHDMGVANTFSRPHYSSYE
LYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDRRFTILEQCLESMVRRDHPDPEKRNGVMG
LDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAVLAGKQAEKCA
ATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALKEDGRFGDYIRALRQHLQYVLREGIC
LFPDGGWKISSTSNNSWLSKIYLCQFIARRILGWEWDEQGKRADAAHVAWLTHPTLSIWSWSDQIIAGE
ISGSKYYPRGVTSILWLEEGE

β-xylosidase [*Geobacillus stearothermophilus* (MW 79848.5) Gel B, band 2 TR1A subculture 6

Detected Peptides

QHLQYVLR
IDDTCPQLR
NVLDFYVER
RIDDTCPQLR
ILGWEWDEQGK
EGICLFPDGGWK
SGGLDLELARPPR
ILGWEWDEQGKR
AFFGFEGTDPYTSMR
SNELIEKEWLSDDQK
SYYGNTQLLEHEGKPIWVVNEGEYR

Location of peptides in the protein sequence

MATNLFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESPHEPGLYHILPFAETAGEDES
KRYDIENPDPNPQKPNILIPFAKERIEREFRVATDTWKAGDLTLTIYSPVKAVPDPETASEEELKLALVPA
VIVEMTIDNTNGTRTRRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRILGIASKDEGVRSALHFSMEDI
LTATLEENWTFGLGKVGALIADVPAGEKKTYQFAVCFYRGGCVTAGMDASYFYTRFFHNIEEVGLYA
LEQAEVLKEQAFRSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNTFD
LTVDQLFFELKMNPWTVKNVLDFYVERYSYEDRVRFPGDETEYPGGISFTHDMGVANTFSRPHYSSYE
LYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDRRLTILEQCLESMVRRDHPDPEKRNGVMG
LDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAALAREQAEKCA
ATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALREDGRFGDYIRALRQHLQYVLREGIC
LFPDGGWKISSTSNNSWLSKIYLCQFIARRILGWEWDEQGKRADAAHVAWLTHPTLSIWSWSDQIIAGE
ISGSKYYPRGVTSILWLEEGE

β-xylosidase OS=*Paenibacillus* sp. DG-22 GN=xylA
PE=4 SV= (MW 78660.8) Gel B, band 2 TR1A subculture 6

Detected Peptides

YSYTDTVR
NIEAVAEYAAER
FVICFYR 39 - Carboxymethyl (C)[4]
NGVMGLDSSR
ILGLPWDEK
QNVYIGLER
GVTSILWLEE
TWAAYVAMEK
IYLCQFIAR - Carboxymethyl (C)[4]

TWAAYVAMEK
RILGLPWDEK
ISSTSDNSWLSK
NIEAVAEYAAER
EGVCLFPDGGWK - Carboxymethyl (C)[4]
LDDTSSLAGVGQGR
LGTDAWTAGDLTFK
RLDDTSSLAGVGQGR
SALHFSMEDILTEK
YDIENPDPNPDKPR
GGIVTAGLDTSYYYTR
EALVPAVLAELIVDNTR
RYDIENPDPNPDKPR
IIPAIEGLIFPYVTGCR - Carboxymethyl(C)[16]
MMNTFDLTVDQLFFELK
TMGGAEITTYDSLDVSLGQAR
CADTIVSHVQEDGTIPAVIGEGNNSK - Carboxymethyl (C)[1] [2]
TADEEELREALVPAVLAELIVDNTR
SYYGSTQLLELDGEPFWVVNEGEYR

Location of peptides in the protein sequence

MGRNDFFNAHHSPIGAFASFTLGFPGPGGGFDLELGRSPKQNVYIGLEREDGSSYDTLPFYESQEEDESK
RYDIENPDPNPDKPRLLHAWPKSQVKRDFRLGTDAWTAGDLTFKIYTQVRSVPDPKTADEEELREALV
PAVLAELIVDNTRSSRKRRAFFGFRGADPYSAMRRLDDTSSLAGVGQGRSIAIATDDKRVRSALHFSME
DILTEKLTENWTFGLGTVGALIADAEPGEKAVYRFVICFYRGGIVTAGLDTSYYYTRYFRNIEAVAEYA
AERFDALKASAERANGLLENGRLSEDQKFMMAHAIRSYYGSTQLLELDGEPFWVVNEGEYRMMNTF
DLTVDQLFFELKFNPWTVRNELDMFVKRYSYTDTVRFPGDETEYPGGISFTHDMGVANVLSRPGYSAY
ERYGLDGCFSHMTHEQLVNWVLCAAAYVERTGDRAWLEANLTVLESCLESMLNRDHPDPAQRNGV
MGLDSSRTMGGAEITTYDSLDVSLGQARNNIYLTGKTWAAYVAMEKLFKETGCGELAEVAGLQAQR
CADTIVSHVQEDGTIPAVIGEGNNSKIIPAIEGLIFPYVTGCREALDADGRFGAYIRALDRHFREVLREGV
CLFPDGGWKISSTSDNSWLSKIYLCQFIARRILGLPWDEKGARADAAHVKWLTHEELSVWSWSDQIIA
GEITGSKYYPRGVTSILWLEE

Glucoamylase OS=*Aspergillus niger* (glucan 1,4-α-glucosidase glaA-)(MW 68266.6) TR1A subculture 6 Gel B, band 3.

Detected Peptides

QGSLEVTDVSLDFFK
DLTWSYAALLTANNR
IESDDSVEWESDPNR
SIYTLNDGLSDSEAVAVGR
ALYSDAATGTYSSSSSTYSSIVDAVK

Location of peptides in the protein sequence

MSFRSLLALSGLVCTGLANVISKRATLDSWLSNEATVARTAILNNIGADGAWVSGADSGIVVASPSTDN
PDYFYTWTRDSGLVLKTLVDLFRNGDTSLLSTIENYISAQAIVQGISNPSGDLSSGAGLGEPKFNVDETA
YTGSWGRPQRDGPALRATAMIGFRQWLLDNGYTSTATDIVWPLVRNDLSYVAQYWNQTGYDLWEE
VNGSSFFTIAVQHRALVEGSAFATAVGSSCSWCDSQAPEILCYLQSFWTGSFILANFDSSRSGKDANTLL
GSIHTFDPEAACDDSTFQPCSPRALANHKEVVDSFRSIYTLNDGLSDSEAVAVGRYPEDTYYNGNPWFL
CTLAAAEQLYDALYQWDKQGSLEVTDVSLDFFKALYSDAATGTYSSSSSTYSSIVDAVKTFADGFVSI
VETHAASNGSMSEQYDKSDGEQLSARDLTWSYAALLTANNRRNSVVPASWGETSASSVPGTCAATSA
IGTYSSVTVTSWPSIVATGGTTTTATPTGSGSVTSTSKTTATASKTSTSTSSTSCTTPTAVAVTFDLTATT
TYGENIYLVGSISQLGDWETSDGIALSADKYTSGDPLWYVTVTLPAGESFEYKFIRIESDDSVEWESDPN
REYTVPQACGTSTATVTDTWR

Acid α-amylase OS=*Aspergillus niger* PE=1 SV=1. (MW 52902.1). Gel A

Detected Peptides

SLSDALHAR
TQSIYFLLTDR

Location of peptides in the protein sequence

MRLSTSSLFLSVSLLGKLALGLSAAEWRTQSIYFLLTDRFGRTDNSTTATCDTGDQIYCGGSWQGIINHL
DYIQGMGFTAIWISPITEQLPQDTADGEAYHGYWQQKIYDVNSNFGTADDLKSLSDALHARGMYLMV
DVVPNHMGYAGNGNDVDYSVFDPFDSSSYFHPYCLITDWDNLTMVQDCWEGDTIVSLPDLNTTETAV
RTIWYDWVADLVSNYSVDGLRIDSVLEVEPDFFPGYQEAAGVYCVGEVDNGNPALDCPYQKVLDGVL
NYPIYWQLLYAFESSSGSISNLYNMIKSVASDCSDPTLLGNFIENHDNPRFASYTSDYSQAKNVLSYIFLS
DGIPIVYAGEEQHYSGGKVPYNREATWLSGYDTSAELYTWIATTNAIRKLAISADSAYITYANDAFYTD
SNTIAMRKGTSGSQVITVLSNKGSSGSSYTLTLSGSGYTSGTKLIEAYTCTSVTVDSSGDIPVPMASGLPR
VLLPASVVDSSSLCGGSGRLYVE

Xylulokinase OS=*Thermobacillus composti* KWC4
GN=ThecoDRAFT_0903 PE=3 SV=1, also by *Paenibacillus curdlanolyticus* YK9
GN=PaecuDRAFT_1616 PE=3 SV=1 (MW 53694)

Xylulokinase OS=*Paenibacillus curdlanolyticus* YK9
GN=PaecuDRAFT_1616 PE=3 SV=1 (MW 53703.2)

Xylulose kinase OS=*Bacillus subtilis* GN=xylB PE=3
SV=2 (MW 55382.2). Gel A

Detected Peptide

VHFFNHGK

Location of peptide in the protein sequence

MSCVIGIDLGTSAVKALLVDRDGKVRAEASRNYPLFHEHTGWSEQRPEDWVEGTIGALRELISTSGVRP
DEVEGISFSGQMHGLVLLDEANRPVRNAILWNDTRTTAECREIERVLGPDLLGIARNPALEGFTLPKIL
WVKRHEPDVFAKAKRFLLPKDYVRYRLTGEIHMDYSDAAGTLLLDVAGKKWSTEILAAFDLPASFCPP
LVESHDHVGGVLPEVADQTGLAAGTKVFAGGADNACGAIGAGILSEGLTMCSIGTSGVILTYEQNRDT
DYAGKVHFFNHGKADNFYAMGVTLAAGYSLSWFKQTFAPNESFADFLRGVGDIKPGSGGLLFTPYLV
GERTPHADAVIRASFIGADGSHTRDHFARAVMEGITFSLNESMAIFREAGKPAGRVISIGGGAQNPVWL
QMQADIFGATVVALENEQGPGLGAAMLAAYGCGWFDSLDACAAKFVKHAASYDPNPEAVETYRGLF
DIYREIYTQTRGLNQALAAYRG

Xylose isomerase OS=*Thermobispora bispora* (strain
ATCC 19993 / DSM 43833 / CBS 139.67 / JCM 10125 / (MW 42842.5). Gel A

Detected Peptides

DPFGDATR
APLDPVETVHR
FAIEPKPNEPR
ADPEVQEALAAAR

Location of peptides in the protein sequence

MYTPTPEDRFSFGLWTVGWQARDPFGDATRAPLDPVETVHRLAELGAYGVTFHDDDLLAVEPDRTKA
IERFKKALSETGLKVPMATTNLFTHPIFKDGAFTSNDRDVRRYALRKVMRNLDLAAELGAKTYVCWG

GREGAESDAAKDVRAALDRYKEALDILCQYVIDKGYDIRFAIEPKPNEPRGDILLPTIGHALAFINELEH
PERVGLNPEVGHEQMAGLNFVHGIAQALWHGKLFHIDLNGQHGPRYDQDLIFGHGDLISAFFLVDLLE
HGGYDGPRHFDYKPMRTEDKEDVWESAAANMRMYLILKEKSKAFRADPEVQEALAAARVPDLSQPT
LAEGETIDDLLNEEFDPDAAAERGYHYTRLNQLAMEHLLGVRG

superoxide dismutase [*Meiothermus silvanus* DSM 9946] (MW 22966.6). Gel A

Detected Peptides

INEVPEDIR
FGSGWAWLVK
RINEVPEDIR

Location of peptides in the protein sequence

MAYPFKVPERPYAADALEPHIDTKTMEVHYQGHHVAYVNNLNAALEKHPELHSWELEDLLRRINEVP
EDIRTAVRNNGGGHHNHTLFWDILTPGGAKEPTGKLAEAINATFGSFDEFKKQLTQAGVTRFGSGWA
WLVKDKAGKLKIYSTANQDSPLMEGDTPLLGIDVWEHAYYLKYQNRRPEYLAAIWNVINWDKVAER
F

β-xylosidase [*Paenibacillus* sp. DG-22 (MW 78660.8). Gel A

Detected Peptides

YSYTDTVR
NIEAVAEYAAER
YDIENPDPNPDKPR
RYDIENPDPNPDKPR

Location of peptides in the protein sequence

MGRNDFFNAHHSPIGAFASFTLGFPGPGGGFDLELGRSPKQNVYIGLEREDGSSYDTLPFYESQEEDESK
RYDIENPDPNPDKPRLLHAWPKSQVKRDFRLGTDAWTAGDLTFKIYTQVRSVPDPKTADEEELREALV
PAVLAELIVDNTRSSRKRRAFFGFRGADPYSAMRRLDDTSSLAGVGQGRSIAIATDDKRVRSALHFSME
DILTEKLTENWTFGLGTVGALIADAEPGEKAVYRFVICFYRGGIVTAGLDTSYYYTRYFRNIEAVAEYA
AERFDALKASAERANGLLENGRLSEDQKFMMAHAIRSYYGSTQLLELDGEPFWVVNEGEYRMMNTF
DLTVDQLFFELKFNPWTVRNELDMFVKRYSYTDTVRFPGDETEYPGGISFTHDMGVANVLSRPGYSAY
ERYGLDGCFSHMTHEQLVNWVLCAAAYVERTGDRAWLEANLTVLESCLESMLNRDHPDPAQRNGV
MGLDSSRTMGGAEITTYDSLDVSLGQARNNIYLTGKTWAAYVAMEKLFKETGCGELAEVAGLQAQR
CADTIVSHVQEDGTIPAVIGEGNNSKIIPAIEGLIFPYVTGCREALDADGRFGAYIRALDRHFREVLREGV
CLFPDGGWKISSTSDNSWLSKIYLCQFIARRILGLPWDEKGARADAAHVKWLTHEELSVWSWSDQIIA
GEITGSKYYPRGVTSILWLEE

superoxide dismutase [*Paenibacillus sp.* oral taxon 786 str. D14 (MW 22486.3). Gel A

Detected Peptide

SVEELISNLDAVPESIR

Location of peptide in the protein sequence

MAHELPALPYPANALEPYIDEQTMIIHHDRHHNTYLTNLNAALESAPELQSKSVEELISNLDAVPESIRT
AVRNNGGGHANHSLFWKVIGPGGGGAPTGAVAAAIDSDLGGFDKFKEEFTKAATTRFGSGWAWLVV
GKDGKLAVTSTPNQDSPLFEGLTPILGLDVWEHAYYLKYQNKRPDYIAAFWNIINWDEVNRRYEAAK
K

Superoxide dismutase [Mn] *Geobacillus kaustophilus* HTA426] (MW 22944.4) or by [Geobacillus thermoleovorans] (MW 22915.4). Gel A

Detected Peptide

FGSFTAFKDEFSK

Location of peptide in the protein sequence

MPFELPALPYPYDALEPHIDKETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALPESIR
TAVRNNGGGHANHSLFWTILSPNGGGEPTGELAEAINKKFGSFTAFKDEFSKAAAGRFGSGWAWLVV
NNGELEITSTPNQDSPIMEGKTPILGLDVWEHAYYLKYQNRRPEYIAAFWNIVNWDEVAKRYSEAKAK

xylose isomerase [*Bacillus subtilis*] (MW 50220.8). Gel B

Detected Peptide

ELGAENYVFWGGR

Location of peptide in the protein sequence

MAQSHSSSINYFGSANKVVYEGKDSTNPLAFKYYNPQEVIGGKTLKEHLRFSIAYWHTFTADGTDVFG
AATMQRPWDHYKGMDLAKMRVEAAFEMFEKLDAPFFAFHDRDIAPEGSTLKETNQNLDMIMGMIKD
YMRNSGVKLLWNTANMFTNPRFVHGAATSCNADVFAYAAAQVKKGLETAKELGAENYVFWGGREG
YETLLNTDLKFELDNLARFMHMAVDYAKEIGYTGQFLIEPKPKEPTTHQYDTDAATTIAFLKQYGLDN
HFKLNLEANHATLAGHTFEHELRMARVHGLLGSVDANQGHPLLGWDTDEFPTDLYSTTLAMYEILQN
GGLGSGGLNFDAKVRRSSFEPDDLIYAHIAGMDAFARGLKVAHKLIEDRVFEDVIQHRYRSFTEGIGLEI
IEGRANFHTLEQYALNHKSIKNESGRQEKLKAILNQYILEV

xylose isomerase [*Geobacillus* sp. Y412MC52] (50474.2). Gel A

Detected Peptides

VFEQFIEER
AYFPNIGTIPYEGPESR

Location of peptides in the protein sequence

MAYFPNIGTIPYEGPESRNPLAFKFYNPEEKVGDKTMEEHLRFSVAYWHTFTGDGSDPFGVGNMIRPW
DKYSGMDLAKARVEAAFELFEKLNVPFFCFHDVDIAPEGETLSETYKNLDEIVDMIEEYMKTSKTKLL
WNTANLFSHPRFVHGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTDMKL
ELDNLARFLHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATALAFLQTYGLKDHFKFNIEANHA
TLAGHTFEHELRVARIHGMLGSVDANQGDTLLGWDTDEFPTDLYATTLAMYEILQNGGLGRGGLNFD
AKVRRGSFEPEDLFYAHIAGMDSFAIGLKVAHRLLEDRVFEQFIEERYKSYTEGIGREIVEGTADFKKLE
EYALQLGEIRNASGRLERLKTLLNQYLLEVSVPSVSRS

Xylan 1,4-β-xylosidase *Geobacillus* sp. (strainC56-T3). Gel A

Detected Peptides

VGALIVDVPAGEK
ILGWEWDEQGK
AGDLTFTIYSPVK

Location of peptides in the protein sequence

MPTNVFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESPHEPGLYHILPFAETAGEDES
KRYDIENPDPNPQKPNILIPFAKEEIKREFCVATDTWKAGDLTFTIYSPVKAVPDPETAAEEELKLALVP
AVIVEMTIDNTNGTRTRRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRILGIVSKDEGVRSALHFSME
DILTATLEENWTFGLGKVGALIVDVPAGEKKTYQFAVCFYRGGYVTAGMDASYFYTRFFHNIEEVGLY
ALEQAEVLKEQAFRSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNTF
DLTVDQLFFELKMNPWTVKNVLDFYVERYSYEDRVRFPGDETEYPGGISFTHDMGVANTFSRPHYSSY
ELYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDRRLTILEQCLESMVRRDHPDPEKRNGVM
GLDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAALAREQAEKC
AATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALREDGRFGDYIRALRQHLQYVLREGI
CLFPDGGWKISSTSNNSWLSKIYLCQFIARRILGWEWDEQGKRADAAHVAWLTHPTLSIWSWSDQIIA
GEISGSKYYPRGVTSILWLEEGE

TR1A subculture 7 (Fig. 5.16, Table 5.1).

RecName: Full=Endo-β-N-acetylglucosaminidase H;
AltName: Full=Mannosyl-glycoprotein endo-β-N from *Streptomyces plicatus* (Band 1 of TR1A subculture 7) (MW 33031.6). Gel B

Detected Peptides

ELYGSEAVRTP
ANMPDKIISLYNIGPAASR
AQLSPAAVEIGRTSR

Location of peptides in the protein sequence

MFTPVRRRVRTAALALSAAAALVLGSTAASGASATPSPAPAPAPAPVKQGPTSVAYVEVNNNSMLNV
GKYTLADGGGNAFDVAVIFAANINYDTGTKTAYLHFNENVQRVLDNAVTQIRPLQQQGIKVLLSVLG
NHQGAGFANFPSQQAASAFAKQLSDAVAKYGLDGVDFDDEYAEYGNNGTAQPNDSSFVHLVTALRA
NMPDKIISLYNIGPAASRLSYGGVDVSDKFDYAWNPYYGTWQVPGIALPKAQLSPAAVEIGRTSRSTVA
DLARRTVDEGYGVVYLTYNLDGGDRTADVSAFTRELYGSEAVRTP

Xylanase/ β-xylosidase/ 1,4-β-D-xylan xylohydrolase/ Xylan 1, (β-xylosidase gene) [*Geobacillus stearothermophilus*. Gel B band 1 of TR1A subculture 7 (MW 70551.9)

Detected Peptides

YSYEDR
VGALIVDVPAGEK
AGDLTFTIYSPVK

Location of peptides in the protein sequence

MPTNLFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVLIGVESLHESGLYHVLPFLETAEEDES
KRYDIENPDPNPQKPNILIPFAKEEIQREFHVATDTWKAGDLTFTIYSPVKAVPNPETADEEELKLALVP
AVIVEMTIDNTNGTRARRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRILSIVSKDEGVRSALHFSME
DILTAQLEENWTFGLGKVGALIVDVPAGEKKTYQFAVCFYRGGYVTAGMDASYFYTRFFQNIEEVGL
YALEQAEVLKEQSFRSNKLIEKEWLSDDQTFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNT
FDLTVDQLFFELKLNPWTVKNVLDLYVERYSYEDRVRFPGEETEYPSGISFTHDMGVANTFSRPHYSSY
ELYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDKRLAILEQCLESMVRRDHPDPEQRNGVM
GLDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAALAGEQAEKC
AATIVSHVTDDGYIPAIMGEGNDSKIIPAIEGLVFPYFTNCHEALDENGRFGAYIQALRNHLQYVLREGI
CLFPDGGWKISSTSNNSWLSKIYLCQFIARHILGWEWDEQGKRADAAHVAWLTHPTLSIWSWSDQIIA
GEITGSKYYPRGVTSILWLEEGE

β-xylosidase [*Geobacillus stearothermophilus* (MW 79848.5). Gel B band 1 TR1A subculture 7

Detected Peptides

QHLQYVLR
IDDTCPQLR
NVLDFYVER
IYLCQFIAR
ILGWEWDEQGK
EGICLFPDGGWK
SGGLDLELARPPR
ILGWEWDEQGKR
AFFGFEGTDPYTSMR

SNELIEKEWLSDDQK

Location of peptides in the protein sequence

MATNLFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESPHEPGLYHILPFAETAGEDES
KRYDIENPDPNPQKPNILIPFAKERIEREFRVATDTWKAGDLTLTIYSPVKAVPDPETASEEELKLALVPA
VIVEMTIDNTNGTRTRRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRILGIASKDEGVRSALHFSMEDI
LTATLEENWTFGLGKVGALIADVPAGEKKTYQFAVCFYRGGCVTAGMDASYFYTRFFHNIEEVGLYA
LEQAEVLKEQAFRSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNTFD
LTVDQLFFELKMNPWTVKNVLDFYVERYSYEDRVRFPGDETEYPGGISFTHDMGVANTFSRPHYSSYE
LYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDRRLTILEQCLESMVRRDHPDPEKRNGVMG
LDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAALAREQAEKCA
ATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALREDGRFGDYIRALRQHLQYVLREGIC
LFPDGGWKISSTSNNSWLSKIYLCQFIARRILGWEWDEQGKRADAAHVAWLTHPTLSIWSWSDQIIAGE
ISGSKYYPRGVTSILWLEEGE

Xylan 1,4-β-xylosidase [*Geobacillus* sp. Y412MC52] (MW 79815.5). Gel B band 1 of TR1A subculture 7.

Detected Peptides

QHLQYVLR
IDDTCPQLR
NVLDFYVER
IYLCQFIAR
VGALIVDVPAGEK
ILGWEWDEQGK
EGICLFPDGGWK
SGGLDLELARPPR
ILGWEWDEQGKR
AFFGFEGTDPYTSMR

Location of peptides in the protein sequence

MPTNVFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESPHEPGLYHILPFAETAGEDES
KRYDIENPDPNPQKPNILIPFAKERIEREFRVATDTWKAGDLTLTIYSPVKAVPDPETASEEELKLALVPA
VIVEMTIDNTNGTRTRRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRILSIVSKDEGVRSALHFSMEDI
LTATLEENWTFGLGKVGALIVDVPAGEKKTYQFAVCFYRGGYVTAGMDASYFYTRFFHNIEEVGLYA
LEQAEVLKEQAFCSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNTFD
LTVDQLFFELKMNPWTVKNVLDFYVERYSYEDRVRFPGDGTEYPGGISFTHDMGVANTFSRPHYSSYE
LYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDRRFTILEQCLESMVRRDHPDPEKRNGVMG
LDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAVLAGKQAEKCA
ATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALKEDGRFGDYIRALRQHLQYVLREGIC
LFPDGGWKISSTSNNSWLSKIYLCQFIARRILGWEWDEQGKRADAAHVAWLTHPTLSIWSWSDQIIAGE
ISGSKYYPRGVTSILWLEEGE

Enolase or 2-phosphoglycerate dehydrogenase or 2-phospho-D-glycerate hydro-lyase [*Meiothermus silvanus DSM 9946*]. Gel A

Detected Peptides

IQLVGDDLFVTNPAR
GYNTNVGDEGGFAPDLK

Location of peptides in the protein sequence

MTTIVELKAREVLDSRGNPTVEAEVTLEGGARGSAMVPSGASTGAHEALELRDGGPRYAGKGVLRAV
AAVNERIAPELIGYDALDQAGVDRAMLQLDGTPNKANLGANAILAVSLATARAAANALGLPLYRYLG

GVQGLTLPVPLMNVINGGKHADNNVDFQEFMLVPAGATSFREALRMGVETFHALKGVLKGRGYNTN
VGDEGGFAPDLKSNVEAVEVLLQAIEKAGYKPGQEISLALDPASTEFYKDGKYRLEAENKTLSSEEMV
AYWESWVGQYPIVSIEDGLAEDDWEAWKMLTERVGHKIQLVGDDLFVTNPARLAEGIEKGVGNAILV
KVNQIGTLSETLEAIALAQRSGYNAVISHRSGETEDTFIADLAVAVNAGQIKTGSASRSDRLAKYNQLL
RIEDELGVGARFLGYGVY

4-α-glucanotransferase from *Thermoproteus neutrophilus* V24Sta (MW 54947.8). Gel A

Detected Peptide

AAEDALPR

Location of peptide in the protein sequence

MLRGFGVLLHISSLPGGCLVGDLGPSAYRFADFLSEAEATYWQILPLSHTLPEYDDSPYSAASLLAGNP
ALVSLEKMAQLGLAKRAPPSCPPAERARFAEAWELKRRYLEEAFEGRLGWRDYEEFAARNSWWLEPY
GRYMALREAFGGPWTAWPAWARRPNADLPPRLERRADFYRYVQFHFWLQWEELKRYVNSLGVFIIG
DLPIYPALDSADVWEGQRYFKLAPDGAPLYVSGVPPDYYSPTGQLWGTPVYNWAELRRDRYVWWTR
RLTRLLSIFDYIRLDHFRGYAAYWEVPYGEPTAVRGRWAPGPGEELFRAAEDALPRLIAEDLGFITPDV
VELRYRLGIPGMRVLQFAWDGNPANEHKPHNYERNLVAYTGTHDNNTTLGWWREETTPRSRREALA
YMGGCRGGVSWCFIRLLFSTVADVAVVPMQDALGLGSEARMNKPGTARGNWKWRMAGDPPRAVA
ARLRRLARIYGR

1-pyrroline-5-carboxylate dehydrogenase [*Geobacillus thermodenitrificans* NG80-2] (MW 56574). Gel A

Detected Peptides

AIIVEDVYDQVLNR
EADADTAEAIDFMEYYGR

Location of peptides in the protein sequence

MVQPYKHEPFTDFTVDANRQAFLAALEKVEAELGREYPLIIGGERVMTEDKITSVNPANKAEVIGRVA
KANKELAERAMKTADEAFRTWSRTSPEARADILFRAAAIVRRRKHEFSAWLVKEAGKPWREADADTA
EAIDFMEYYGRQMLKLKDGIPVESRPGETNRFFYIPLGVGVVISPWNFPFAIMAGTTVAALVTGNTVLL
KPASATPVVAYKFAEVLEEAGLPAGVLNYIPGSGAEVGDYLVEHPRTRFISFTGSRDVGIRIYERAAKV
QPGQIWLKRVIAEMGGKDAIVVDKEADLELAAQSIVASAFGFSGQKCSACSRAIIVEDVYDQVLNRVV
ELTKQLNVGDPAEQATFMGPVIDQGAYNKIMEYIEIGKQEGRLMTGGEGDDSKGFFIQPTVFADVDPN
ARIMQEEIFGPVVAFAKARDFDHALEIANNTQYGLTGAVISRNRANLEKARHEFHVGNLYFNRGCTGA
IVGYQPFGGFNMSGTDSKAGGPDYLILHMQAKTVSEMF

xylose isomerase [Geobacillus thermodenitrificans NG80-2] (MW 50618.2) or *Bacillus* sp. NRRL B-14911 (MW 49648.5). Gel A

Detected Peptides

FYNPEEK
FFHMAVDYAK

Location of peptides in the protein sequence

MAYFPNIGKIAYEGPESRNPFAFKFYNPEEKVGGKTMEEHLRFSVAYWHTFTGDGSDPFGVGNMIRPW
DKYSGMDLAKARVEAAFELFEKLNVPFFCFHDVDIAPEGETLSETYKNLDEIVDMIEEYMKTSKTKLL
WNTANLFSHPRFVHGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTDMKL

ELDNLARFFHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATALAFLQTYGLKDYFKFNIEANHA
TLAGHTFEHELRVARIHGMLGSVDANQGDMLLGWDTDEFPTDLYATTLAMYEILQNGGLGRGGLNFD
AKVRRGSFEPEDLFYAHIAGMDSFAIGLKVAHRLLEDRVFEQFIEERYKSYTEGIGREIVEGTADFHKLE
QYALQLGEIRNTSGRLERLKTLLNQYLLEVSVPSKARL

Enolase (2-phosphoglycerate dehydratase) [Bacillus halodurans C-125]/ *Geobacillus* sp. Y4.1MC1 (MW 46318.4). Gel A

Detected Peptides

ALVPSGASTGEYEAVELR
SGETEDSTIADIAVATNAGQIK

Location of peptides in the protein sequence

MTIITDVYAREVLDSRGNPTVEVEVYLESGAMGRALVPSGASTGEYEAVELRDGGERFLGKGVLKAVE
NVNEVIAPELIGFDALDQIGIDQHMIELDGTENKGKLGANAILGVSMAVARAAANALDLPLYVYLGGF
NAKQLPVPMMNIINGGEHADNNVDIQEFMIMPVGAESFKEALRTGTEIFHSLKKVLKSKGYNTAVGDE
GGFAPNLSSNEEALQTIIEAIEQAGYTPGEQVKLAMDVASSELYNKEDGKYHLSGEGKVLSSEEMVAF
YEELVAKYPIISIEDGLDENDWEGHKMLTDRLGDKVQLVGDDLFVTNTKKLAQGIEQGVGNSILIKVN
QIGTLTETFDAIEMAKRAGYTAVISHRSGETEDSTIADIAVATNAGQIKTGAPSRTDRVAKYNQLLRIED
ELGNLAQYNGLQSFYNLKK

Δ-1-pyrroline-5-carboxylate dehydrogenase from *Geobacillus* sp. Y412MC52 (MW 56631.9). Gel A

Detected Peptides

DYPLVIGGER
HEPLTDFTVEANR
GFFIQPTVFADVDPNAR
EADADTAEAIDFMEYYGR

Location of peptides in the protein sequence

MVQPYRHEPLTDFTVEANREAFLAALKKVESELGRDYPLVIGGERVMTEDKIISINPANKTEVVGRVAK
ANKELAERAMKTADEAFRTWSRMSPEARADILFRAAAIVRRRKHEFSAWLVKEAGKPWREADADTA
EAIDFMEYYGRQMLKLKDGIPVESRPGETNRFFYIPLGVGVVISPWNFPFAIMAGTTVASLVTGNTVLL
KPASATPVVAYKFVEVLEEAGLPAGVLNYIPGSGAEVGDYLVDHPRTRFISFTGSRDVGIRIYERAAKV
HPGQIWLKRVIAEMGGKDAIVVDKEADLELAAQSIVASAFGFSGQKCSACSRAIVVQDVYDQVLNRV
VELTKQLNVGDPAEQATFMGPVIDQGAYNKIMEYIEIGKQEGRLMTGGEGDDSKGFFIQPTVFADVDP
NARIMQEEIFGPVVAFAKARDFDHALEIANNTEYGLTGAVISRNRANLEKARHEFHVGNLYFNRGCTG
AIVGYQPFGGFNMSGTDSKAGGPDYLILHMQAKTVSEMF

xylan 1,4-β-xylosidase (β xylosidase) from *Geobacillus thermodenitrificans* (MW 61879.8). Gel A

Detected Peptides

YNHAATIAR
ITEGPHLYK
IPLGEDIATLK
FWLIYTDVK
IDGYYYLLTAEGGTR
EIVVPDDVEYVYLR

Location of peptides in the protein sequence

MVKIKNPILTGFHPDPSICRAGDDYYIAVSTFEWFPGVRIYHSKDLKNWRLVARPLNRLSQLNMIGNPD
SGGIWAPHLSYSDGKFWLIYTDVKVVEGQWKDGHNYLVTCDTIDGEWSDPIYLNSSGFDPSLFHDEDG
RKYLVNMYWDHRVGHHPFYGIVLQEYSVEQKKLIGEPKIIFKGTDLRITEGPHLYKIDGYYYLLTAEGG
TRYNHAATIARSASLYGPYEVHPENPLITSWPYPRNPLQKAGHASIVHTHTDEWFLVHLTGRPLPREGQ
PLLDHRGYCPLGRETAIQRLEWKDGWPYVVGGNGPSLEIDGPNVEEVPWERDYDEKDDFDGDTLNHH
FQTLRIPLGEDIATLKARPGHLRLYGRESLTSRFTQAFVARRWQHFHFIAETKVAFRPTTIQQSAGLVNY
YNTQNWTTLQLTWHEEKGRILELMACDHLVVEQPLRGREIVVPDDVEYVYLRVNVQMTTYKYSYSF
DGVDWKEIPVTFESYKLSDDYIKSNAAFTGAFVGMHCRDGSGQNNYADFDYFLYKEL

Xylan 1,4-β-xylosidase [*Bacillus cellulosilyticus* DSM 2522] (MW 61516.2). Gel A

Detected Peptides

FWLIYTDVK
WQHFNFTAETK
IQNPILTGFNPDPSICR

Location of peptides in the protein sequence

MAKIQNPILTGFNPDPSICRAGEDYYIAVSTFEWFPGVGIYHSKDLKNWRLVSRPLNRLSQLNMMGNPD
SGGIWAPALSYSDGKFWLIYTDVKVTEGQWKDSHNYLVTCDTIDGEWSEPIYMNSSGFDPSLFHNDDG
KKYFVNMVWDHRVSHHNFYGIVLQEYSVEEKKLIGKKEVIFTGTDIKLTEAPHLYKVNGYYYLLTAEG
GTKYDHQATIARSKDLWGPYEVHPENPLITSFPYPRNPLQKAGHASIVETHTNEWFLVHLTGRPLPKEG
HALLDPRGYCPLGRETAIQRLEWKDDWPYVVGGNQPAAEIEGPAIDEVTWEKDVPEKDDFDGENLNL
HFQTLRIPLGEEIVSLKDKPGHLRIHGRESLTSKFTQAYVARRWQHFNFTAETKVAFQPETFQQAAGLV
NYYNTQNWTALQVTWHEEKGRILDLTTCDNFTFDQPLKGKEIVVPDHTEYVYMRVDVTTNTYRYSYS
FDGNEWVEIDIDFYSYKLSDDYIQGGGFFTGAFVGMQCQDTSGASLPADFDYFVYKEK

xylose isomerase [*Geobacillus kaustophilus* HTA426 (MW 50350.1). Gel A

Detected Peptides

VEAAFELFEK
FLHMAVDYAK
VFEQFIEER
EGYETLLNTDMK
LGAENYVFWGGR
KLEEYALQLGDIR
EIGFDGQFLIEPKPK
AYFPNIGTIPYEGPESR
FVHGAATSCNADVFAYAAAK
FNIEANHATLAGHTFEHELR

Location of peptides in the protein sequence

MAYFPNIGTIPYEGPESRNPLAFKFYNPDEKVGGKTMEEHLRFSVAYWHTFTGDGSDPFGVGNMIRPW
NTYSGMDLAKARVEAAFELFEKLNVPFFCFHDVDIAPEGETLSETYKNLDEIVDMIEEYMKTSKTKLL
WNTANLFSHPRFVHGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTDMKL
ELDNLARFLHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATALAFLQTYGLKDHFKFNIEANHA
TLAGHTFEHELRVARIHGMLGSVDANQGDTLLGWDTDEFPTDLYTTTLAMYEILQNGGLGRGGLNFD
AKVRRGSFEPEDLFYAHIAGMDSFAIGLKVAHRLLEDRVFEQFIEERYKSYTEGIGREIVEGTADFKKLE
EYALQLGDIRNTSGRLERLKTLLNQYLLEVSAPSGSRS

Enolase [*Paenibacillus* sp. JDR-2] (MW 45818.5). Gel A

Detected Peptide

SGESEDSTIADIAVATNAGQIK

Location of peptide in the protein sequence

MSIIVDVYAREVLDSRGNPTVEVEVSLESGGKGRAIVPSGASTGAYEAVELRDGDKGRYLGKGVEKAV
ENVNAIIAPEIIGLDALDQVAIDRKMIELDGTPNKAKLGANAILAVSMAVARAAADALNVSLYTYLGGF
NAKTLPVPMMNIINGGEHADNNIDVQEFMVLPVGAPTFKEALRIGAEIFHNLKSVLKDKGLNTAVGDE
GGFAPNLGSNEEAITTIIAAIELAGYKPGVDVFLGMDVASTEFYKDGKYHLEGEGKSFTSAEFVDLLAS
WVEKYPIITIEDGCSEDDWEGWKLLTDKLGGKVQLVGDDLFVTNTERLSDGIEKGVGNSILVKVNQIG
SLTETFDAIEMAKRAGYTAVISHRSGESEDSTIADIAVATNAGQIKTGAPSRTDRVAKYNQLLRIEDQLG
SVAQYAGKSAFYNLKNFK

Enolase [*Staphylococcus xylosus*] (MW 47086.9). Gel A

Detected Peptide

VQLVGDDLFVTNTVK

Location of peptide in the protein sequence

MPIITDVYAREVLDSRGNPTVEVEVLTESGAFGRALVPSGASTGEHEAVELRDGDKSRYLGKGVTKAV
DNVNEIIAPELIEGEFSVLEQVSIDKMMIQLDGTENKGKLGANAILGVSIAVARAAADLLGQPLYKYLG
GFNGKQLPVPMMNIVNGGSHSDAPIAFQEFMVLPVGAETFKESLRWGAEIFHNLKSILKNRGLETAVG
DEGGFAPKFEGTEDAVETILEAIKAVGLEPGKDVFLGFDCASSEFFEDGVYNYAKFEGENGAKRNAEE
QVDYLEELVNKYPIITIEDGMDENDWDGWKVLTDRIGDKVQLVGDDLFVTNTVKLSEGIEKGIGNSILI
KVNQIGTLTETFDAIEMAQKAGYTAVVSHRSGETEDTTISDIAVATNAGQIKTGSLSRTDRIAKYNQLL
RIEDELYETGKFDGLKSFYNLSK

Aldehyde dehydrogenase [*Rhodomicrobium vannielii* (strain ATCC 17100)]MW 55278.1. Gel A

Detected Peptides

YFASCIR
NLLVSYSPK
ETLAADIPLAIDHFR
IFQEEIFGPVVALTTFK

Location of peptides in the protein sequence

MIYAAPGTPGAIVTFKERYDNFIGGEWRSPLGGQYFESVTPITGKPFAQVARSQAEDIELALDAAHAAA
ERWGHTSVAERSLILNRIADRMEQNLEKLAYAESVDNGKPIRETLAADIPLAIDHFRYFASCIRAQEGTL
GQVDEDTVAYHFQEPLGVVGQIIPWNFPILMAAWKLAPALAAGNCVVLKPAEQTPIGILVWAEIIGDLL
PKGVLNIVNGFGLEAGKPLASSPRIAKIAFTGETSTGRLIMQYASQNLIPVTLELGGKSPNIFFDDVAAAD
DEFFDKAIEGFVMFALNQGEVCTCPSRALVQESLRDRFVDRALARVASIKQGNPLDTDTMIGAQASQE
QLHKILSYVNIGLEEGAKCLIGGERAYLGGELDTGFYVRPTVFEGNNSMRIFQEEIFGPVVALTTFKDEA
DALHLANDTVYGLGAGVWTRDGNRAYRFGRGIKAGRVWTNCYHLYPAHAAFGGYKQSGIGRETHH
MMLDHYQQTKNLLVSYSPKALGFF

Aldehyde Dehydrogenase [*Micromonospora aurantiaca*] (strain ATCC 27029) (MW 54546.3). Gel A.

Detected Peptides

NLLVSYSPK
VAFTGETTTGR
ETLAADLPLAIDHFR

Location of peptides in the protein sequence

MTRYDAPTHWQSRYDHYIGGEYVKPHGGKYFENPTPVTGQTFCEVARGTAEDVEKALDAAHGAADA
WGRTSVAERSLILNRIADRMQDNLESLAIAETWENGKPVRETLAADLPLAIDHFRYFAGAIRAQEGSLG
ELDDDTVAYHFHEPLGVVGQIIPWNFPILMAVWKLAPALAAGNAVVLKPAEQTPASIHYLLSLIGDLLP
PGVVNVVNGFGVEAGKPLASSPRVAKVAFTGETTTGRLIMQYASENIRPVTLELGGKSPNIFFDDVSAA
SDDFLDKALEGFTMFALNQGEVCTCPSRALIQQGHYADFLAAAVDRTRQITQGHPLDTDTMVGAQAS
NDQLEKILSYLDIGRQEGARVLTGGERADLGGELSGGYYVQPTIFEGDNSMRIFQEEIFGPVVSVTSFAD
LDDAVKIANDTLYGLGAGVWTRDLNTAYRAGRAIQAGRVWTNCYHAYPAHAAFGGYKQSGIGRENH
KMMLEHYQQTKNLLVSYSPKKLGFF

Aldehyde dehydrogenase [Streptomyces clavuligerus (strain ATCC 27064)] (MW 55304.8). Gel A.

Detected peptides

NLLVSYSPK
MMLDHYQQTK
ETLAADLPLAIDHFR

Location of peptides in the protein sequence

MTRFAAPGSEGAIVSYRSRYDHWIGGEYVAPARGEYFENPSPVNGLPFTEIARGTAEDVERALDAAHA
AAPAWGRTAPGERAGVLLRIADRMEAHLTELAVAESWENGKPVRETLAADLPLAIDHFRYFAGSLRA
QEGTLSELDDDTVAYHFHEPLGVVAQIIPWNFPILMASWKLAPALAAGNAVVLKPAEQTPASIHVWLD
LVADLLPDGVLNIVNGFGVEAGKPLASSPRVAKIAFTGETTTGRLIMQYASENIKPVTLELGGKSPNIFF
DDIWAADDELRDKALEGFTMFALNQGEVCTCPSRALIQSGHYREFLEAGVARTELIVPGHPLDTETMIG
AQASHDQLEKILSYLDIGRQEGAKVLTGGERITYDGEMAGGYYVQPTIFEGDNRMRVFQEEIFGPVVA
VTSFSDFDDAIKTANDTLYGLGAGVWTRDISTAYRAGRAIQAGRVWTNCYHAYPAHAAFGGYKQSGI
GREGHRMMLDHYQQTKNLLVSYSPKRLGFF

superoxide dismutase [*Meiothermus silvanus* DSM 9946] (MW 22966.6). Gel A

Detected Peptides

INEVPEDIR
FGSGWAWLVK
RINEVPEDIR

Location of peptides in the protein sequence

MAYPFKVPERPYAADALEPHIDTKTMEVHYQGHHVAYVNNLNAALEKHPELHSWELEDLLRRINEVP
EDIRTAVRNNGGGHHNHTLFWDILTPGGAKEPTGKLAEAINATFGSFDEFKKQLTQAGVTRFGSGWA
WLVKDKAGKLKIYSTANQDSPLMEGDTPLLGIDVWEHAYYLKYQNRRPEYLAAIWNVINWDKVAER
F

β-xylosidase/α-L-arabinfuranosidase, putative, gly43F [*Cellvibrio japonicus Ueda107* (MW 38086.4). Gel A

Detected Peptides

YYLYFPAR
YFEGPWMHK
YFEGPWMHK

Location of peptides in the protein sequence

MSTENEVVDYKALAARAISQPLVTHIYTADPSAHVFNGKVYIYPSHDIDAGIPFNDNGDHFGMEDYHV
LRMDSPEGKAEDCGVALHVKDVPWAERQMWAPDAITKDGKYYLYFPARARDGLFKIGVAIGDQPEG
PFVAEPEPIAGSYSIDPAVFGDDDGEFYLYFGGIWGGQLQKYRDNTYSEIHEEPTADQPALGARVARLS
ADMKSFVEASREVVILDEQGQPLLAGDNSRRYFEGPWMHKYQGKYYLSYSTGDTHFLCYATSDNPYG
PFTYQGQILTPVVGWTTHHSICEFEGKWYLFYHDSVLSEGVTHLRSVKVTELHYEADGKIKTIHPYRD

α-N-arabinofuranosidase [*Clostridium papyrosolvens* DSM 2782] (MW 36763.8). Gel A

Detected Peptides

QMWAPDAAFK
YFEGPWMHK
YFEGPWMHK

Location of peptides in the protein sequence

MEKIVKQKEPLVEHIYTADPSAHVFEGKIYIYPSHDLDEDIVSNDNGDQYMMEDYHILSLEDLNSPCVD
NGEALHMKDVPWVSKQMWAPDAAFKNNTYYLYFPARDKDGIFRIGVASSSSPAGPFTAQKEPIPGSFSI
DPAVLVDDDNRAYIYFGGLWGGQLEKWQTGSFSPDAEGPDVSAPAIGPRVAELSDDMLTFKEAPEEISI
VDEEGNPILAGDEDRRYFEGPWMHKYNGNYYLSYSTGTTHTIVYAVGNNPKGPFVFKGKILTPVVGW
TTHHSIVQYQDKWYLFYHDSSLSGGRDNKRCVKFTELKYNEDGTIQTIDPYK

Transaldolase [*Paenibacillus sp. oral taxon* 786 str. D14 (MW 24007.9). Gel A

Detected Peptides

AGATYVSPFLGR
VHNLDTQIIAASVR

Location of peptides in the protein sequence

MKFFVDTANLEDIKKAHKIGVLSGVTTNPSLVAKEGVKFEDRIEEILKAVPDVESVSAEVTPDAVTAEE
MIAQAEELIKINNHDPRITIKLPMTLAGLEATKYLAQKGVKTNVTLIFTVNQALLAARAGATYVSPFLG
RLDDISEDGVQLVSKVAELFRVHNLDTQIIAASVRHPDHVTRVALAGAHIATIPFAVIEQLAKHPLTDQG
LEKFAADWKKSVK

xylose isomerase [*Bacillus licheniformis* ATCC 14580] (MW 50220.8). Gel A

Detected Peptide

ELGAENYVFWGGR

Location of peptide in the protein sequence

MFFRNIGMIEYEGADSENPYAFKYYNPDEFVGGKTMKEHLRFAVAYWHTFDADGKDPFGDGTMFRA
WNRLTHPLDKAKARAEAAFEFFEKLGVPYFCFHDVDIVDEGATLRETFTYLDQMSSFLKEMMETSHV
QLLWNTANMFTHPRYVHGAATSCNADVYAYAAAKVKKGLDIAKELGAENYVFWGGREGYETLLNT
DMKLELENLSSFYRMAVEYAREIGFDGQFLIEPKPKEPTKHQYDFDAATTIAFLETYGLKDHFKLNLEA
NHATLAGHTFEHELRVAALHDMLGSIDANQGDLLLGWDTDEFPTDLYSAVLAMYEILKAGGFKTGGI
NFDAKVRRPSFADEDLFHAHIAGMDTYAVGLKVASRLLEDKALDQVIEERYESYTKGIGLEIKEGRTDL
KKLAAYALENDHIENQSGRQERLKATVNRYLLNALREAPAGKETH

Enolase [*Meiothermus silvanus* DSM 9946] (MW 44933.2). Gel A

Detected Peptides

IQLVGDDLFVTNPAR
GYNTNVGDEGGFAPDLK

Location of peptides in the protein sequence

MTTIVELKAREVLDSRGNPTVEAEVTLEGGARGSAMVPSGASTGAHEALELRDGGPRYAGKGVLRAV
AAVNERIAPELIGYDALDQAGVDRAMLQLDGTPNKANLGANAILAVSLATARAAANALGLPLYRYLG
GVQGLTLPVPLMNVINGGKHADNNVDFQEFMLVPAGATSFREALRMGVETFHALKGVLKGRGYNTN
VGDEGGFAPDLKSNVEAVEVLLQAIEKAGYKPGQEISLALDPASTEFYKDGKYRLEAENKTLSSEEMV
AYWESWVGQYPIVSIEDGLAEDDWEAWKMLTERVGHKIQLVGDDLFVTNPARLAEGIEKGVGNAILV
KVNQIGTLSETLEAIALAQRSGYNAVISHRSGETEDTFIADLAVAVNAGQIKTGSASRSDRLAKYNQLL
RIEDELGVGARFLGYGVY

TR1A subculture 8 (Fig. 5.17, Table 5.1).

Transaldolase [*Meiothermus silvanus* DSM 9946]. Gel B band 1 TR1A subculture 8 (MW 23373.3). Gel B

Detected Peptides

GLQQFLADWEK
VFQQLIQHPLTDK
MDLYLDTAEVSEIR

Location of peptides in the protein sequence

MDLYLDTAEVSEIREIASWGVLGGVTTNPSLVAKSGRGFEEVIREISAIVQGPVSAEVTAMEAPAMIAE
GRKLAAIDPNVVVKLPTIVEGLKACKALSAEGIRVNMTLIFSANQALLAAHAGAWCVSPFAGRLDDIS
WDGMDLVAEIAQIFDIHAIGTRVLAASIRHPMHVLQAAKAGAD
IATMPAKVFQQLIQHPLTDKGLQQFLADWEKSKEKAKQP

Mnanganese containing catalase from *Thermus aquaticus* Y51MC23]. Gel B band 2 TR1A subculture 8 (MW 33179.9)

Detected Peptides

FMDLGFHR
LQIELPMPK
EMIGYLLVR
YMDLGFHR

Location of peptides in the protein sequence

MFLRIDRLQIELPMPKEQDPNAAAAVQALLGGRFGEMSTLMNYMYQSFNFRGKKALKPYYDLIANIAT
EELGHIELVAATINSLLAKNPGKDLEEGVDPVTAPLGFAKDARNAAHFIAGGGNSLVMGAMGEHWHG
EYVFTSGNLILDLLHNFFLEVAARTHKLRVYEMTDNPVAREMIGYLLVRGGVHAAAYGKALETLTGV
EMNKMLPIPRIENSKIPEAKKFMDLGFHRNLYRFSQEDYKDLGLIWAGPSPEDGSEVVVVDGPPAGGPV
FDAGHDAAEFAPEFHPGELYEIAKKLYEKAK

xylose isomerase from *Geobacillus sp.* Y412MC52. Gel B band 2 TR1A subculture 8 (MW 50474.2)

Detected Peptides

VFEQFIEER
AYFPNIGTIPYEGPESR

Location of peptides in the protein sequence

MAYFPNIGTIPYEGPESRNPLAFKFYNPEEKVGDKTMEEHLRFSVAYWHTFTGDGSDPFGVGNMIRPW
DKYSGMDLAKARVEAAFELFEKLNVPFFCFHDVDIAPEGETLSETYKNLDEIVDMIEEYMKTSKTKLL
WNTANLFSHPRFVHGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTDMKL
ELDNLARFLHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATALAFLQTYGLKDHFKFNIEANHA
TLAGHTFEHELRVARIHGMLGSVDANQGDTLLGWDTDEFPTDLYATTLAMYEILQNGGLGRGGLNFD
AKVRRGSFEPEDLFYAHIAGMDSFAIGLKVAHRLLEDRVFEQFIEERYKSYTEGIGREIVEGTADFKKLE
EYALQLGEIRNASGRLERLKTLLNQYLLEVSVPSVSRS

Glyceraldehyde-3-phosphate dehydrogenase [*Meiothermus silvanus* DSM 9946]. Gel B band 2 TR1A subculture 8 (MW 35595.4)
Detected Peptides

VADLAQYIGK
GILAYTEEPLVSSDLK
VVSWYDNEWGYSCR
GILTTVHAYTASQSLVDAVK
DPAALPWGEIGADIVIESTGR
GDPHSSIFSALDTLVIGNMVK
VYEEKDPAALPWGEIGADIVIESTGR

Location of peptides in the protein sequence

MRVAINGFGRIGRQVFRILEERGVEIVGINDLSDNAILAHLFKYDSNYGRFPGTVSYDEQNLVVNGKTIR
VYEEKDPAALPWGEIGADIVIESTGRFTKLEAAEAHLKAGAKKVIISAPGKGDMLTVVMGVNEHMYDP
AKHHVISNASCTTNGLAPVAKVLNDKFGIEKGILTTVHAYTASQSLVDAVKDDPRDARAAAINIVPSET
GAAKAVGLVIPELKGKFTGMAFRVPTSTVSVVDFTAILHREASKEEINAAMKEAAEGPMKGILAYTEEP
LVSSDLKGDPHSSIFSALDTLVIGNMVKVVSWYDNEWGYSCRVADLAQYIGKRL

NAD-dependent aldehyde dehydrogenase [*Geobacillus kaustophilus* HTA426] (MW 55870.5). Gel A

Detected Peptides

DINTAYR
YFASCIR Carboxymethyl (C)[5]
YENFIGGK
NLLVSYSPK
ILSYIDIGK
RYENFIGGK
VAFTGETTTGR
QEGAELLIGGER
MMLDHYQQTK
AADIELALDAAHAAK
ETLAADIPLAIDHFR
ALIHESIYDAFMER
MIYAQPGQPGALVTFK
MIYAQPGQPGALVTFK
IFQEEIFGPVLAVTTFK
AQEGTISEIDHDTVAYHFK
ILSYIDIGKQEGAELLIGGER
QGNPLDTETMIGAQASSEQLEK
DHDEALSIANETLYGLGAGVWTR

Location of peptides in the protein sequence

MIYAQPGQPGALVTFKKRYENFIGGKWVPPVDGEYFENITPITGQPYCEVPRSKAADIELALDAAHAAK
DAWGRTSPAERARLLNKIADRMEENLEMLAVAETWENGKPIRETLAADIPLAIDHFRYFASCIRAQEGT
ISEIDHDTVAYHFKEPLGVVGQIIPWNFPILMAAWKLAPALAAGNCVVLKPAEQTPTSILVLIELIEDLLP
PGVVNIVNGFGLEAGKPLASNPRVAKVAFTGETTTGRLIMQYASQNIVPVTLELGGKSPNIFFADVMDK
DDEFLDKALEGFTMFALNQGEVCTCPSRALIHESIYDAFMERALERVKQIKQGNPLDTETMIGAQASSE
QLEKILSYIDIGKQEGAELLIGGERNMLEGELAGGYYVKPTIFKGHNKMRIFQEEIFGPVLAVTTFKDHD
EALSIANETLYGLGAGVWTRDINTAYRFGRGIQAGRVWTNCYHVYPAHAAFGGYKMSGIGRETHKM
MLDHYQQTKNLLVSYSPKKLGLF

Glyceraldehyde-3-phosphate dehydrogenase
[*Meiothermus ruber* DSM 1279. Gel A band 2 TR1A subculture 8 (MW 35353.4)

Detected Peptides

VADLAQYIGK
GILAYTEEPLVSSDLK
VVSWYDNEWGYSCR
GLLTTVHAYTASQSLVDAVK
GVEVVGINDLSDNAILAHLFK

Location of peptides in the protein sequence

MKIGINGFGRIGRQVFRILQERGVEVVGINDLSDNAILAHLFKYDSNYGRFPGTVSYDEKTITVNGKTIR
VYEEKDPANIPWGEIGADIVIESTGRFTKLEAAEAHLKAGAKKVIISAPGKGDMLTVVMGVNEHMYDP
AKHHVISNASCTTNGLAPVAKVLNDHFGIEKGLLTTVHAYTASQSLVDAVKDDPRDARAAALNIVPSE
TGAAKAVGLVIPELKGKFGGMAFRVPTSTVSVVDFTAILSKEASKEEINAAMKAAAEGPMKGILAYTE
EPLVSSDLKGDPHSSIFSALDTLVVGNLVKVVSWYDNEWGYSCRVADLAQYIGKKL

Xylan 1,4-β-xylosidase [*Geobacillus sp.* Y412MC52*]*. (MW 79866). Gel B band 3 TR1A subculture 8

Detected Peptides

QHLQYVLR
NVLDFYVER
VGALIVDVPAGEK
AFFGFEGTDPYTSMR
LALVPAVIVEMTIDNTNGTR
LALVPAVIVEMTIDNTNGTR
TMGGAEITTYDSLDVSLGQAR
IIPAIEGLVFPYFTNCHEALK

Location of peptides in the protein sequence

MPTNVFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESPHEPGLYHILPFAETAGEDES
KRYDIENPDPNPQKPNILIPFAKERIEREFRVATDTWKAGDLTLTIYSPVKAVPDPETASEEELKLALVPA
VIVEMTIDNTNGTRTRRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRILSIVSKDEGVRSALHFSMEDI
LTATLEENWTFGLGKVGALIVDVPAGEKKTYQFAVCFYRGGYVTAGMDASYFYTRFFHNIEEVGLYA
LEQAEVLKEQAFCSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNTFD
LTVDQLFFELKMNPWTVKNVLDFYVERYSYEDRVRFPGDGTEYPGGISFTHDMGVANTFSRPHYSSYE
LYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDRRFTILEQCLESMVRRDHPDPEKRNGVMG
LDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAVLAGKQAEKCA
ATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALKEDGRFGDYIRALRQHLQYVLREGIC
LFPDGGWKISSTSNNSWLSKIYLCQFIARRILGWEWDEQGKRADAAHVAWLTHPTLSIWSWSDQIIAGE
ISGSKYYPRGVTSILWLEEGE

β-xylosidase [*Geobacillus stearothermophilus*] (MW 79850). Gel B band 3 TR1A subculture 8

Detected Peptides

QHLQYVLR
NVLDFYVER
LTILEQCLESMVR
AFFGFEGTDPYTSMR
LALVPAVIVEMTIDNTNGTR
LALVPAVIVEMTIDNTNGTR

TMGGAEITTYDSLDVSLGQAR

Location of peptides in the protein sequence

MATNLFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPRQNVFIGVESPHEPGLYHILPFAETAGEDES
KRYDIENPDPNPQKPNILIPFAKERIEREFRVATDTWKAGDLTLTIYSPVKAVPDPETASEEELKLALVPA
VIVEMTIDNTNGTRTRRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRILGIASKDEGVRSALHFSMEDI
LTATLEENWTFGLGKVGALIADVPAGEKKTYQFAVCFYRGGCVTAGMDASYFYTRFFHNIEEVGLYA
LEQAEVLKEQAFRSNELIEKEWLSDDQKFMMAHAIRSYYGNTQLLEHEGKPIWVVNEGEYRMMNTFD
LTVDQLFFELKMNPWTVKNVLDFYVERYSYEDRVRFPGDETEYPGGISFTHDMGVANTFSRPHYSSYE
LYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDRRLTILEQCLESMVRRDHPDPEKRNGVMG
LDSTRTMGGAEITTYDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEELAALAREQAEKCA
ATIVSHVTEDGYIPAVMGEGNDSKIIPAIEGLVFPYFTNCHEALREDGRFGDYIRALRQHLQYVLREGIC
LFPDGGWKISSTSNNSWLSKIYLCQFIARRILGWEWDEQGKRADAAHVAWLTHPTLSIWSWSDQIIAGE
ISGSKYYPRGVTSILWLEEGE

RecName: Full=Endoglucanase A; AltName:
Full=Endo-1,4-β-glucanase A; AltName:
Full=Cellulase A; from *Thermobispora bispora* DSM 43833/ Uncultured *Bacteroidetes* bacterium DNA, fosmid clone: JFF029_C06 (MW 46980)
Gel B band 3 TR1A subculture 8

Detected Peptides

AWIDEIAAGLR
DCGGPSAGGAPNHTAYR Carboxymethyl (C)[2] [2]
NGNGPLGSEWCDPPGR Carboxymethyl(C)[11

Location of peptides in the protein sequence

MSRIRRFLATALAAATAGVGAIVTAIASAGPAHAYDSPFYVDPQSNAAKWVAANPNDPRTPVIRDRIA
AVPTGRWFANYNPSTVRAEVDAYVGAAAAAGKIPIMVVYAMPNRDCGGPSAGGAPNHTAYRAWIDE
IAAGLRNRPAVIILEPDALPIMTNCMSPSEQAEVQASAVGAGKKFKAASSQAKVYFDAGHDAWVPADE
MASRLRGADIANSADGIALNVSNYRYTSGLISYAKSVLSAIGASHLRAVIDTSRNGNGPLGSEWCDPPG
RATGTWSTTDTGDPAIDAFLWIKPPGEADGCIATPGVFVPDRAYELAMNAAPPTYSPSPTPSTPSPSPSQ
SDPGSPSPSPSQPPAGRACEATYALVNQWPGGFQAEVTVKNTGSSPINGWTVQWTLPSGQSITQLWNG
DLSTSGSNVTVRNVSWNGNVPAGGSTSFGFLGSGTGQLSSSITCSAS

superoxide dismutase [*Meiothermus silvanus* DSM 9946] (MW 22970). Gel B band 4 TR1A subculture 8

Detected Peptides

INEVPEDIR
FGSGWAWLVK
RINEVPEDIR
NNGGGHHNHTLFWDILTPGGAK
IYSTANQDSPLMEGDTPLLGIDVWEHAYYLK
IYSTANQDSPLMEGDTPLLGIDVWEHAYYLK

Location of peptides in the protein sequence

MAYPFKVPERPYAADALEPHIDTKTMEVHYQGHHVAYVNNLNAALEKHPELHSWELEDLLRRINEVP
EDIRTAVRNNGGGHHNHTLFWDILTPGGAKEPTGKLAEAINATFGSFDEFKKQLTQAGVTRFGSGWA

WLVKDKAGKLKIYSTANQDSPLMEGDTPLLGIDVWEHAYYLKYQNRRPEYLAAIWNVINWDKVAER
F

Catalase [*Geobacillus* sp. Y4.1MC1] (MW 55481.7). Gel A

Detected Peptides

FSTVAGELGSADTVR
VGVNHNLLPINRPR
IAGENPDYHTEDLYNAIEK
FDNNGGGSVNYEPNSFGGPTEVPEHK

Location of peptides in the protein sequence

MADTKKLTTSWGAPVGDNQNSITAGNPGPTLIQDVHLIEKLAHFNRERVPERVVHAKGAGAHGYFEV
TNDMSKYTKAKVFNGVGKRTPVFVRFSTVAGELGSADTVRDPRGFAVKFYTEEGNYDIVGNNTPIFFI
RDAIKFPDFIHTQKRDPRTHLKNPTAMWDFWSLSPESLHQVTYLFGDRGIPLTYRHMNGYGSHTFKWV
NEKGEAVWVKYHFKTNQGVKNMDPELAVKIAGENPDYHTEDLYNAIEKGDYPSWTLYVQIMPLEDA
KTYRFNPFDVTKVWSHKDYPLIEVGRMVLNRNPENYFAEVEQATFSPGNLVPGVEPSPDKMLQARLFA
YADAHRYRVGVNHNLLPINRPRVEVNNYQRDGFMRFDNNGGGSVNYEPNSFGGPTEVPEHKTTPFPV
SGVAESVPYDDDDHYTQAGDLYRLMSEEEKARLVKNIVESLKQVTKEEIKLRQIRHFYKADPDYGRRV
AEGLGLQIPDDVTTNA

Xylose isomerase from *Bacillus subtilis* (MW 13358.4). Gel A

Detected Peptide

VEAAFEMFEK

Location of peptide in the protein sequence

MAQSHSSSVNYFGSVNKVVFEGKASTNPLAFKYYNPQEVIGGKTMKEHLRFSIAYWHTFTADGTDVF
GAATMQRPWDHYKGMDLARARVEAAFEMFEKLDAPFFAFHDRDIAPEGSTLKETNQNLDIIVGMIKD
YMRDSNVKLLWNTANMFTNPRFVHGAATSCNADVFAYAAAQVKKGLETAKELGAENYVFWGGREG
YETLLNTDLKFELDNLARFMHMAVDYAKEIEYTGQFLIEPKPKEPTTHQYDTDAATTIAFLKQYGLDN
HFKLNLEANHATLAGHTFEHELRMARVHGLLGSVDANQGHPLLGWDTDEFPTDLYSTTLAMYEILQN
GGLGSGGLNFDAKVRRSSFEPDDLVYAHIAGMDAFARGLKVAHKLIEDRVFEDVIQHRYRSFTEGIGLE
ITEGRANFHTLEQYALNNKTIKNESGRQERLKPILNQ

xylose isomerase [*Geobacillus kaustophilus* HTA426] (MW 50350.1). Gel A

Detected Peptides

VEAAFELFEK
FLHMAVDYAK
VFEQFIEER
FLHMAVDYAK
LGAENYVFWGGR
EGYETLLNTDMK
LEEYALQLGDIR
KLEEYALQLGDIR
LLWNTANLFSHPR
EIGFDGQFLIEPKPK
NLDEIVDMIEEYMK
AYFPNIGTIPYEGPESR

FVHGAATSCNADVFAYAAAK
FNIEANHATLAGHTFEHELR

Location of peptides in the protein sequence

MAYFPNIGTIPYEGPESRNPLAFKFYNPDEKVGGKTMEEHLRFSVAYWHTFTGDGSDPFGVGNMIRPW
NTYSGMDLAKARVEAAFELFEKLNVPFFCFHDVDIAPEGETLSETYKNLDEIVDMIEEYMKTSKTKLL
WNTANLFSHPRFVHGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTDMKL
ELDNLARFLHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATALAFLQTYGLKDHFKFNIEANHA
TLAGHTFEHELRVARIHGMLGSVDANQGDTLLGWDTDEFPTDLYTTTLAMYEILQNGGLGRGGLNFD
AKVRRGSFEPEDLFYAHIAGMDSFAIGLKVAHRLLEDRVFEQFIEERYKSYTEGIGREIVEGTADFKKLE
EYALQLGDIRNTSGRLERLKTLLNQYLLEVSAPSGSRS

xylose isomerase [*Geobacillus* sp. Y412MC52] (MW 50474.2). Gel A

Detected Peptides

FYNPEEK
VEAAFELFE
FLHMAVDYAK
VFEQFIEER
FLHMAVDYAK
LGAENYVFWGGR
EGYETLLNTDMK
LLWNTANLFSHPR
EIGFDGQFLIEPKPK
NLDEIVDMIEEYMK
AYFPNIGTIPYEGPESR
FVHGAATSCNADVFAYAAAK
FNIEANHATLAGHTFEHELR

Location of peptides in the protein sequence

MAYFPNIGTIPYEGPESRNPLAFKFYNPEEKVGDKTMEEHLRFSVAYWHTFTGDGSDPFGVGNMIRPW
DKYSGMDLAKARVEAAFELFEKLNVPFFCFHDVDIAPEGETLSETYKNLDEIVDMIEEYMKTSKTKLL
WNTANLFSHPRFVHGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTDMKL
ELDNLARFLHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATALAFLQTYGLKDHFKFNIEANHA
TLAGHTFEHELRVARIHGMLGSVDANQGDTLLGWDTDEFPTDLYATTLAMYEILQNGGLGRGGLNFD
AKVRRGSFEPEDLFYAHIAGMDSFAIGLKVAHRLLEDRVFEQFIEERYKSYTEGIGREIVEGTADFKKLE
EYALQLGEIRNASGRLERLKTLLNQYLLEVSVPSVSRS

Transaldolase [*Paenibacillus* sp. JDR-2] (MW 23281.3). Gel A

Detected peptides

AGATYISPFVGR
LGLVDGVTTNPSLIAK

Location of peptides in the protein sequence

MKFFLDTANVEEIRRIARLGLVDGVTTNPSLIAKEGRDFKEVIQEIAGFIHGPISAEVIGTTSEEMLMEAF
DIADWAPNIVIKLPMTEDGLYATRALSEKGIKTNVTLIFTAAQGLIAAKAGATYISPFVGRLDDIGTDGI
GLIRDLRTILHTYGMHAEIIAASIRHIGHVEQAALAGAHIATIPGALLPSLWKHPLTDAGIAKFLSDWDS
RKPNS

Xylose isomerase [*Thermoanaerobacterium thermosulfurigenes/ Thermoanaerobacterium thermosaccharolyticum* (strain ATCC DSM 571](MW 50.312). Gel A

Detected Peptides

FLHMAVDYAK
EIGFEGQFLIEPKPK

Location of peptides in the protein sequence

MNKYFENVSKIKYEGPKSNNPYSFKFYNPEEVIDGKTMEEHLRFSIAYWHTFTADGTDQFGKATMQRP
WNHYTDPMDIAKARVEAAFEFFDKINAPYFCFHDRDIAPEGDTLRETNKNLDTIVAMIKDYLKTSKTK
VLWGTANLFSNPRFVHGASTSCNADVFAYSAAQVKKALEITKELGGENYVFWGGREGYETLLNTDME
FELDNFARFLHMAVDYAKEIGFEGQFLIEPKPKEPTKHQYDFDVANVLAFLRKYDLDKYFKVNIEANH
ATLAFHDFQHELRYARINGVLGSIDANTGDMLLGWDTDQFPTDIRMTTLAMYEVIKMGGFDKGGLNF
DAKVRRASFEPEDLFLGHIAGMDAFAKGFKVAYKLVKDRVFDKFIEERYASYKDGIGADIVSGKADFR
SLEKYALERSQIVNKSGRQELLESILNQYLFAE

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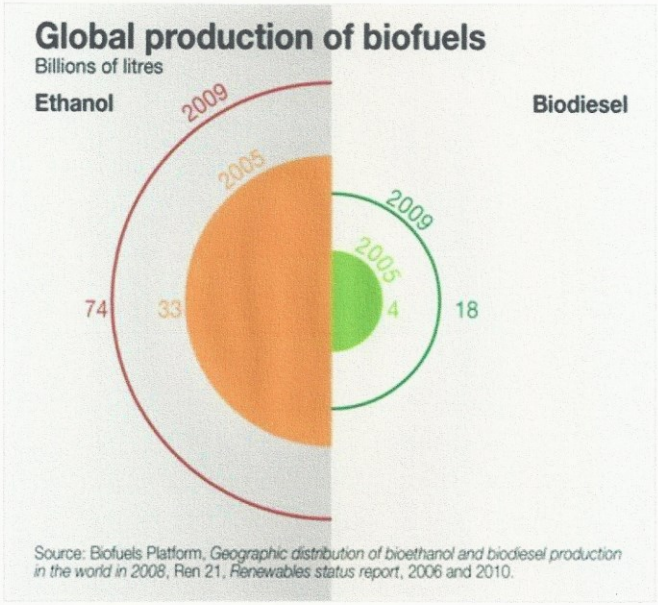
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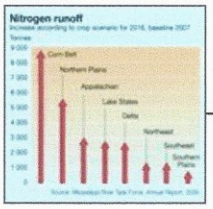
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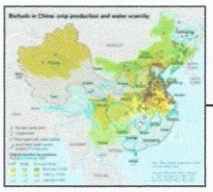
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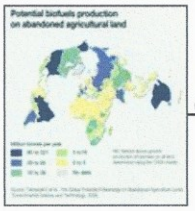
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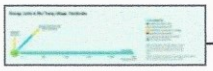
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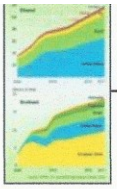
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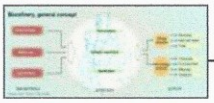
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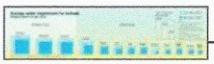
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Dear Agripina, I am happy for you to use the figure in your PhD thesis. Best regards, Paul

Prof. Paul Knox,
Centre for Plant Sciences
Faculty of Biological Sciences
University of Leeds
Leeds LS2 9JT
United Kingdom

Tel: +44-113-3433169
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Sent: 08 January 2014 11:20
To: Paul Knox
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Dear Paul,
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Kind regards
Agripina Banda

From: Banda, Agripina
Sent: 01 June 2011 11:39
To: 'Paul Knox'
Subject: RE: Thank you

Hi,
Thank you very much.
Best regards
Agripina Banda
From: Paul Knox [mailto:J.P.Knox@leeds.ac.uk]
Sent: 01 June 2011 09:35
To: Banda, Agripina
Subject: RE: permission to use Figure 1 (Schematic Outline of the Occurrence of the Major Cell....) from Cell Wall Biology: Perspectives from Cell Wall Imaging (2010) journal.
Dear Agripina, I am happy for you to use this figure. Best regards, Paul

Prof. Paul Knox,
Centre for Plant Sciences
Faculty of Biological Sciences
University of Leeds
Leeds LS2 9JT
United Kingdom

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From: Banda, Agripina [mailto:a.banda09@imperial.ac.uk]
Sent: 30 May 2011 10:41

From: Emilie David [edavid@aaas.org]
Sent: 10 January 2014 16:06
To: Banda, Agripina
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Emilie David

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Sent: Wednesday, January 08, 2014 10:12 AM
To: Emilie David
Subject: RE: Permission to use Figure 1a from "Somerville, C., Youngs, H., Taylor, C., Davis, S.C. &Long, S.P. (2010). Feedstocks for lignocellulosic biofuels. Science 329, 790-792"

Hi,

My name is Agripina Banda (Mrs). I am a PhD student at Imperial College London, South Kensington, Division of Biology, Post Code-SW7 2AZ. My cell number is 079543358491.

I have read your article [Somerville, C., Youngs, H., Taylor, C., Davis, S.C. & Long, S.P. (2010). Feedstocks for lignocellulosic biofuels. Science 329, 790-792] and I found it very helpful. I am writing a thesis on Evaluation of consolidated bioprocessing. I am asking for permission to use figure 1a of your article in my PhD thesis. I first requested for permission to use this figure during the first week of june 2011 (see message from your office below this mail). I have just realised I did not get a response. I am still asking for permission to use figure 1 from the above mentioned article in my thesis.

Thank you.

Agripina Banda

From: Mailer-Daemon@aaas.org [Mailer-Daemon@aaas.org] on behalf of Emilie David [edavid@aaas.org]
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Subject: RE: Permission to use Figure 1a from "Somerville, C., Youngs, H., Taylor, C., Davis, S.C. &Long, S.P. (2010). Feedstocks for lignocellulosic biofuels. Science 329, 790-792"

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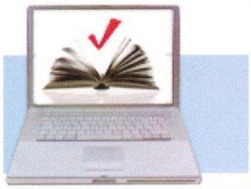
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