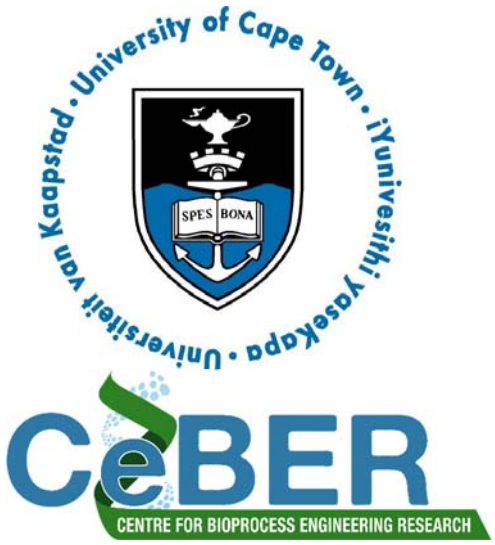

Enhanced bioethanol fermentation from mixed xylose and glucose using free and immobilized cultures: mathematical model and experimental observation

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Abstract

Bioethanol plays a significant role in the world of liquid biofuel. However, majority of bioethanol is produced from edible food crops such as corn and sugarcane that causes an increase in demand for vacant lands for food production and, subsequently, increase in the cost of food manufacturing. Therefore, alternative raw materials for bioethanol production are sought after, such as sugarcane bagasse which is a waste material from the sugar industry. South Africa, a net sugar exporter, has a large potential to produce bioethanol from sugarcane bagasse. This research focuses on the study of the production of bioethanol from glucose and xylose which are the two most abundant sugars in hydrolysed sugarcane bagasse. To date, no suitable wild type organisms can concomitantly ferment both glucose and xylose to ethanol efficiently.

Options to address the co-fermentation of glucose and xylose include genetic modification of the selected microorganism to include both pathways - limitation in the understanding of the metabolic pathways regulations - or utilization of two microorganisms in co-culture or sequential culture e.g. *Zymomonas mobilis* and *Pichia stipitis* for efficient fermentation of glucose and xylose respectively. In this study, the dual micro-organism route is explored. There are numerous problems associated with co-culturing. Xylose, a non-preferred carbon source is only converted if the glucose concentration is adequately low due to catabolite repression. In order to increase xylose conversion, a low glucose concentration is required. Therefore, two stage sequential fermentation either in one or two reactors was tested. A high inoculum of suspended or immobilized *Z. mobilis* was inoculated in the first stage to convert the glucose rapidly. Varying reactor configuration, including the continuous fluidized bed, continuous stirred tank reactor (CSTR) and stirred batch reactor were considered. The products and residual substrate from this fermentation was then directed to a second stage, using either a CSTR or stirred batch configuration, with a high inoculum of *P. stipitis* in suspension culture for conversion of xylose. When immobilized, *Z. mobilis* was entrapped in calcium alginate beads. On the issue of ethanol tolerance, *P. stipitis* is generally more easily inhibited by ethanol (threshold ethanol concentration of 35 g L^{-1}) compared to other ethanol producing strains such as *Z. mobilis* (threshold ethanol concentration of 127 g L^{-1}) and *Saccharomyces cerevisiae* (threshold ethanol concentration of 118.2 g L^{-1}). In order to overcome this, a continuous bioprocess was investigated to keep ethanol concentrations in Stage II below 35 g L^{-1} to prevent inhibition of metabolic reactions in *P. stipitis*. Further, ethanol fermentation by *Z. mobilis* requires obligate anaerobic

conditions while xylose conversion by *P. stipitis* is optimum under microaerobic conditions. Therefore, oxygen was sparged into the second *P. stipitis* stage only.

The following components were carried out in this project to improve the kinetic model and to find accurate kinetic data in the selected process of the two stage sequential fermentation. Firstly, where kinetic parameters were not available in literature, the kinetic parameter relationships of glucose and xylose utilization between different constructs of the same species were examined, for example, a wild type and engineered strain. This approach was used for glucose conversion using wild type *Z. mobilis*, owing to the ill-fit of available kinetic parameters with experimental results. In this study, the correction factors on estimated kinetic parameters from linear and non-linear regression when a xylose fermentation route was inserted recombinantly (*S. cerevisiae* RWB 217) into the native culture (*S. cerevisiae* CEN.PK 113-7D) were determined. From kinetic parameters of an engineered strain with the xylose-fermenting pathway (*Z. mobilis* ZM4 (pZB5)) and the correction factors, kinetic parameters of the wild-type *Z. mobilis* ZM4 were determined. Predicted rates of *Z. mobilis* ZM4 were then validated with experimental data generated in this study.

Then, the optimum initial biomass concentration required to provide a faster volumetric rate of sugar utilisation and ethanol production, as well as the optimum oxygenation level for xylose conversion using *P. stipitis* achieved through appropriate aeration were investigated through experimental observation and using a MATLAB mathematical model developed through combination of the Andrews and Levenspiel's models, with oxygen, substrate, cell and product terms. Experiments were carried out to validate the kinetic model and data under anaerobic and microaerobic growth conditions in a batch process. The results showed that both increasing the initial biomass concentration (3 g L^{-1}) and operating under optimum oxygenation levels (0.1 vvm) benefitted the ethanol production and yield by *P. stipitis* from xylose. It was also concluded that the addition of the oxygen effective factors in the developed model allowed for optimization of aeration in the fermentation system.

Next, the custom kinetic model for fermentation process of bioethanol production was developed in Aspen Custom Modeller (ACM) and embedded in Aspen Plus. The model includes equations of vapour-liquid equilibrium (VLE), mass balance, and energy balance (e.g. molecular weight, thermodynamic phase equilibria, kinetic equation). The obtained results showed better agreement between industrial data and kinetic model (1% differences) than a stoichiometric model (9% differences). The simulation showed that ACM integrated into Aspen Plus allowed for complex biological processes to be accurately predicted for biomass growth, ethanol production and sugar consumption. Finding suitable microorganisms and process conditions for efficient glucose and xylose conversion is still currently a challenge and requires optimization. Therefore, this research focusses on improving the conversion of glucose and xylose to bioethanol, with specific emphasis on the fermentation systems used to maximize biomass efficiency, and

ethanol yields and productivities. Manipulation of process conditions ranging from operation conditions (e.g. batch, fed-batch, continuous), process parameters (aeration, temperature, pH), immobilization technique and type of microorganism initially using kinetic models and thereafter validating with experimental data, therefore, offers a quick and strong foundation in improving bioethanol yields and productivities.

February 2019

Declaration

I declare that this thesis is my own unaided work, both in concept and execution and that, apart from the normal guidance from my supervisors, I have received no other assistance. Neither the substance nor any part of this thesis has been submitted in the past, or is being, or is to be submitted for a degree at this University or at any other university.

I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following publications in my PhD thesis, and, where co-authorships are involved, my co-authors have agreed that I may include the publications.

The list of papers presented in this thesis and published / wil or prepared to be published in international peer-reviewed journals are:

Nosrati-ghods, N., Harrison, S.T.L., Isafiade, A.J., Tai, S.L., 2018. Ethanol from Biomass Hydrolysates by Efficient Fermentation of Glucose and Xylose – A Review 1–19. doi:10.1002/cben.201800009.

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Table of content

Contents

Abstract i
Declaration iv
Acknowledgement v
Table of content vi
List of figures x
List of tables xiv
Nomenclature xvi
1. Introduction 1
2. Literature review 5
Paper title: 7
Ethanol from biomass hydrolysates by efficient fermentation of glucose and xylose – a review 7
2A.1. Introduction 7
2A.1.1. Background 7
2A.1.2. Objective 8
2A.1.3. Challenges 8
2A.1.4. Approaches and limitations 9
2A.1.5. Way forward - optimization of process condition 10
2A.1.5.1. Initial total sugar concentration and a ratio of glucose to xylose 10
2A.1.5.2. Aeration condition 11
2A.1.5.3. Reactor configuration 11
2A.1.5.4. Immobilization technique 14
2A.2. Metabolic engineering of preferred ethanol producers for utilization of C5 and C6 sugars 15
2A.2.1. Recombinant *S. cerevisiae* 16
2A.2.2. Recombinant *Z. mobilis* 21
2A.2.3. Recombinant *E. coli* 22
2A.2.4. Recombinant *Klebsiella oxytoca* 24
2A.2.5. Fermentation performance of recombinant cells 25
2A.3. Co-culturing 26
2A.3.1. Co-culture of *P. stipitis* & *S. cerevisiae* 28
2A.3.2. Co-culture of *P. stipitis* & *S. diastaticus* 29
2A.3.3. Co-culture of *P. stipitis* & *K. marxianus* 29
2A.3.4. Co-culture of *P. stipitis* & *Z. mobilis* 29
2A.3.5. Co-culture of *K. marxianus* & *S. cerevisiae* 30
2A.3.6. Fermentation performance of co-culture 30
2A.4. Sequential batch culture 31
2A.4.1. Sequential batch culture: single bioreactor 33
2A.4.2. Sequential batch culture: two chambered bioreactor 34
2A.4.3. Fermentation performance of sequential batch culture 34
2A.5. Conclusions 34
2A.6. Acknowledgments 36
2A.7. Declaration of interest 36
2A.8. Supporting Information 36
2A.9. References 37

Paper title: 46
Mathematical modelling of bioethanol fermentation processes from glucose or/and xylose – a review ... 46
2B.1. Introduction 48
2B.2. Unsegregated Kinetic Model 49
2B.3. Effective aspects 60
2B.3.1. Limitary effects 60
2B.3.2. Inhibitory effects 61
2B.3.3. Advanced inhibitory effects 66
2B.4. Modelling from literature experimental values 68
2B.5. Conclusion 72
2B.6. Acknowledgments 73
2B.7. Declaration of interest 73
2B.8. Nomenclature: 73
2B.9. References 76
3. Research approach 81
3.1. Objective 82
3.2. Hypothesis 82
3.3. Key Questions 83
3.4. Approach 83
3.4.1. Preliminary set-up 83
3.4.2. Modelling set-up 84
3.4.3. Experimental set-up 84
3.5. Limitations of the study 84
3.6. Novel & substantial contribution 85
3.7. References: 86
4. Kinetic model and kinetic parameters of *Z. mobilis* for bioethanol fermentation using wild and engineered strains from glucose and xylose 87
Paper title: 89
Kinetic data analysis and mathematical modelling of intra (wild type vs engineered) and inter species (*Saccharomyces cerevisiae* vs *Zymomonas mobilis*) dependency for bioethanol production from glucose or/and xylose 89
4.1. Introduction 90
4.2. Materials and Methods 92
4.2.1. Kinetic models 92
4.2.1.1. Biomass production rate 93
4.2.1.2. Substrate uptake rate 94
4.2.1.3. Ethanol production rate 94
4.2.2. Mass balance 94
4.2.3. Parameter estimation 95
4.2.4. Experimental work for validation 95
4.2.4.1. Strains 95
4.2.4.2. Culture storage 95
4.2.4.3. Synthetic media 96
4.2.4.4. Inoculum preparation and fermentation studies 96
4.2.4.5. Analytical procedures 96

4.3. Results and Discussion 97
4.3.1. Modelling from literature experimental values using *S. cerevisiae* 97
4.3.2. Modelling from literature experimental values using *Z. mobilis* 99
4.3.3. Validating modelling with experiments of this study using wild type of *Z. mobilis* 102
4.3.4. Sensitivity analysis 103
4.4. Conclusion 105
4.5. Declaration of interest 106
4.6. Acknowledgments 106
4.7. Nomenclature 107
4.8. References 109
5. Kinetic model and kinetic parameters of *P. stipitis* for bioethanol fermentation from xylose 112
Paper title: 114
Analysis of ethanol production from xylose using *Pichia stipitis* in microaerobic condition through experimental observations and kinetic modelling 114
5.1. Introduction 116
5.2. Material and Methods 118
5.2.1. Strains 118
5.2.2. Culture storage 118
5.2.3. Synthetic media 118
5.2.4. Inoculum preparation and fermentation studies 118
5.2.5. Analytical procedures 118
5.2.6. Kinetic model 119
5.2.6.1. Biomass production rate 119
5.2.6.2. Substrate and oxygen uptake rate 119
5.2.6.3. Ethanol production rate 120
5.2.7. Mass balance 120
5.2.8. Parameter estimation 120
5.2.9. Statistical analysis 121
5.3. Results and Discussion 121
5.3.1. Kinetic constants of *Pichia stipitis* 121
5.3.2. Validating modelling with experimental data of this study 122
5.3.2.1. Initial biomass concentration of 0.3 g L^{-1} 123
5.3.2.2. Initial biomass concentration of 1.5 g L^{-1} 125
5.3.2.3. The initial biomass concentration of 3 g L^{-1} 127
5.3.3. Comparison of performance 128
5.3.4. Statistical analysis 128
5.4. Conclusion 129
5.5. Declaration of interest 129
5.6. Acknowledgment 130
5.7. Nomenclature: 130
5.8. References: 132
6. Kinetic model of bioethanol fermentation using Aspen Custom Modeller (ACM) 134
Paper title: 135
Embedding the equation-oriented kinetic model for bioethanol fermentation in a sequential modular flowsheet simulator 135

6.1. Introduction 136
6.2. Materials and Methods 137
6.2.1. Experimental work for validation 137
6.2.1.1. Strains 137
6.2.1.2. Culture storage 137
6.2.1.3. Synthetic media 137
6.2.1.4. Inoculum preparation and fermentation studies 138
6.2.1.5. Analytical procedures 138
6.2.2. Computational methods 138
6.2.2.1. Kinetic models 139
6.2.2.1.1. Biomass production rate 139
6.2.2.2. Kinetic constants 140
6.2.2.3. Stoichiometry 140
6.2.2.4. Mass balance 141
6.2.2.5. Aspen Tech interfaces (ACM – Aspen Plus) 141
6.2.2.6. Process of interfacing 141
6.3. Results and discussion 143
6.3.1. Comparing kinetic and stoichiometry model with literature experimental data and experiment of this study 143
6.3.2. Comparing kinetic and stoichiometry model with industrial data 144
6.4. Conclusion 145
6.5. Nomenclature: 146
6.6. References: 148
7. Co-fermentation for bioethanol production from mixed glucose and xylose using *Zymomonas mobilis* and *Pichia stipitis* 149
7.1. Introduction 149
7.2. Material and Methods 153
7.2.1. Microorganisms and culture storage 153
7.2.2. Synthetic media 153
7.2.3. Inoculum preparation and fermentation studies 154
7.2.4. Immobilization 154
7.2.5. Reactor configuration 155
7.2.6. Analytical procedures 155
7.3. Results and Discussion 155
7.3.1. Sequential batch culture of the high inoculum sizes of *Z. mobilis* and *P. stipitis* in one batch stirred tank reactor 155
7.3.2. Two stage fermentation in two bioreactors in a continuous process 158
7.4. Conclusion 161
7.5. References 163
8. Conclusions and Recommendations 165
References 171
Appendix A: Supplementary information for Chapter 2 186
Appendix B: Aspen custom Modeller code of Chapter 6 214

List of figures

Figure 2.1.Metabolism in recombinant *S. cerevisiae*, *Z. mobilis*, and *E. coli* (Altintas et al., 2006; Karhumaa et al., 2005; Nikolaev, 2010). Dashed lines denote *Z. mobilis*, dotted lines denote *S. cerevisiae*, dashed-dotted lines denote *E. coli*, and solid lines denote all three microorganisms. 16

Figure 2.2.Ethanol productivity (g L^{-1} h^{-1}) and yield (g g^{-1}) of engineered (a) *S. cerevisiae* in batch anaerobic culture, (b) *Z. mobilis*, (c) *E. coli*, (d) *K. oxytoca* (Table I in appendix A)..... 20

Figure 2.3.Ethanol productivity (g L^{-1} h^{-1}) of different co-culture vs yield (g g^{-1}) (Table II in appendix A)..... 27

Figure 2.4.Ethanol yield (g g^{-1}) and productivity (g L^{-1} h^{-1}) of two-stage fermentation in one and two bioreactors (Table III in appendix A)..... 32

Figure 2.5.Glucose concentration vs time for the initial glucose concentration of 80 g L^{-1} using *Saccharomyces yeast* 1400. The R2 represents correlation coefficient denoted in Equation D. The open square symbol denotes the experimental data (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995). Labels with grey symbols and lines denote the respective models used in the comparison. 70

Figure 2.6.Ethanol production vs time for the initial glucose concentration of 80 g L^{-1} using *Saccharomyces yeast* 1400. The R2 represents correlation coefficient denoted in Equation D. The open square symbol denotes the experimental data (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995). Labels with grey symbols and lines denote the respective models used in the comparison. 71

Figure 2.7.Biomass concentration vs time for the initial glucose concentration of 80 g L^{-1} using *Saccharomyces yeast* 1400. The R2 represents correlation coefficient denoted in Equation D. The open square symbol denotes the experimental data (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995). Labels with grey symbols and lines denote the respective models used in the comparison. 71

Figure 4.1.Metabolism in selected recombinant and wild type *S. cerevisiae,* and *Z. mobilis* (Bothast et al., 1999; Dien et al., 2003; Kuyper et al., 2005a, 2005b; Matsushika et al., 2009; Senthilkumar and Gunasekaran, 2005; Zhang et al., 1995). Dashed lines denote *Z. mobilis*, dotted lines denote *S. cerevisiae* and solid lines denote both microorganisms..... 92

Figure 4.2.The concentration of ethanol and glucose over time (*S. cerevisiae* CEN.PK 113-7D). The solid line (—) denotes substrate concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote glucose concentration and open circles (○) denote ethanol concentration in the experiment (Kuyper et al., 2005a). The Correlation coefficient of glucose (0.9) and ethnaol (0.95) 99

Figure 4.3.The concentration of ethanol, xylose and glucose over time (*S. cerevisiae* RWB217). The solid lines (—) denote substrate concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote glucose concentration, open diamonds (◊) denote xylose concentration and open circles (○) denote ethanol

concentration in the experiments (Kuyper et al., 2005a, 2005b). The correlation of coefficient of glucose (0.94), xylose (0.94) and ethanol (0.97)..... 99

Figure 4.4. a) The concentration of glucose and xylose over time (*Z. mobilis* ZM4 (pZB5)). The solid lines (—) denote substrate concentration in modelling. Half-filled squares (◧) and half-filled diamonds (⬖) denote 59.3 g L^{-1} glucose and 63.2 g L^{-1} xylose in the experiment (Leksawasdi et al., 2001). The average correlation coefficient for the substrate for 59.3/63.2 g L^{-1} glucose/xylose (0.98 ± 0.01). b) The concentration of ethanol and biomass over time (*Z. mobilis* ZM4 (pZB5)). The dotted line (…) denotes biomass concentration and dashed lines (--) denote ethanol concentration in modelling. Half-filled triangles (◭) using 59.3 g L^{-1} glucose and 63.2 g L^{-1} xylose denote biomass concentration in experiment. Half-filled circles (◐) using 65 g L^{-1} glucose and 65 g L^{-1} xylose denote ethanol concentration in the experiment (Leksawasdi et al., 2001). The correlation coefficient for ethanol at 59.3/63.2 g L^{-1} glucose/xylose (0.98). The correlation coefficient for biomass at 59.3/63.2 g L^{-1} glucose/xylose (0.75). 101

Figure 4.5.The concentration of ethanol, biomass and glucose over time (*Z. mobilis* ZM4). The solid lines (—) denote substrate concentration, dotted lines (…) denote biomass concentration and dashed lines (--) denote ethanol concentration in modelling. Open squares (□) denote 100 g L^{-1} glucose, full-filled squares (■) denote 150 g L^{-1} glucose and half-filled squares (◧) denote 200 g L^{-1} glucose in experiment. Open triangles (Δ) using 100 g L^{-1} glucose, full-filled triangles (▲) using 150 g L^{-1} glucose and half-filled triangles (◭) using 200 g L^{-1} glucose denote biomass concentration in experiment. Open circles (○) using 100 g L^{-1} glucose, full-filled circles (●) using 150 g L^{-1} glucose and half-filled circles (◐) using 200 g L^{-1} glucose denote ethanol concentration in the experiment (Lee and Rogers, 1983). The average correlation coefficient of glucose (0.97±0.048), biomass (0.82±0.34) and ethanol (0.977±0.011)..... 102

Figure 4.6.The concentration of glucose, biomass and ethanol over time (*Z. mobilis* ATCC 31821). The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote glucose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration in the experiment of this study. Correlation coefficient of glucose (0.98), biomass (0.96) and ethanol (0.99). 103

Figure 5.1.Xylose, ethanol and biomass concentration versus time under the anaerobic condition, the initial biomass concentration of 0.3 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study. 123

Figure 5.2.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.1 vvm), the initial biomass concentration of 0.3 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study. 124

Figure 5.3.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.2 vvm), the initial biomass concentration of 0.3 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study. 124

Figure 5.4.Xylose, ethanol and biomass concentration versus time under the anaerobic condition, the initial biomass concentration of 1.5 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study. 125

Figure 5.5.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.1 vvm), the initial biomass concentration of 1.5 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study. 126

Figure 5.6.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.2 vvm), the initial biomass concentration of 1.5 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study. 126

Figure 5.7.Xylose, ethanol and biomass concentration versus time under the anaerobic condition, the initial biomass of 3 g L^{-1}, free cell. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study. 127

Figure 5.8.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.1 vvm), the initial biomass of 3 g L^{-1}, free cell. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study. 128

Figure 6.1.The custom model block of a process unit operation in a sequential modular flowsheet simulator for bioethanol production process from sugarcane (Dias et al., 2010) 137

Figure 6.2.Comparison of the kinetic and stoichiometry model of fermentation data on ethanol, microorganisms and residual sugars with experiment of this study for bioethanol production from glucose 143

Figure 6.3.Ethanol production from 170 g L^{-1} glucose using *Z. mobilis* (Lee et al., 1980). a) square (■) is for glucose concentration, dashed line (---) is for glucose concentration using

stoichiometry model, solid line (—) is for glucose concentration using kinetic model; b) circle (●) is for ethanol concentration, dashed line (---) is for ethanol concentration using stoichiometry model, solid line (—) is for ethanol concentration using kinetic model; c) triangle (▲) is for biomass concentration in experimental result, dashed line (---) is for biomass concentration using stoichiometry model, solid line (—) is for biomass concentration using kinetic model. 144

Figure 6.4.Comparison of the kinetic and stoichiometry model of fermentation data on glycerol, acids, residual sugars and microorganisms over ethanol production with industrial data for bioethanol production from glucose (Bonomi et al., 2011) 145

Figure 7. 1. The process of immobilization in this study (adapted from Becerra et al., 2001; Fu et al., 2009)..... 154

Figure 7. 2. Fermentation profile of the sequential batch suspended cultures (xylose, glucose, ethanol and biomass concentration versus time). Circle (●) is for glucose concentration, square (■) is for xylose concentration, triangle (▲) is for ethanol concentration, line (—) is for biomass concentration. 156

Figure 7. 3. Fermentation profile of the sequential batch immobilized cultures (xylose, glucose and ethanol concentration versus time). Circle (●) is for glucose concentration, square (■) is for xylose concentration, triangle (▲) is for ethanol concentration. 157

Figure 7. 4. Schematic diagram of two CSTR in series 159

Figure 7. 5. Schematic diagram of CSTR and fluidized bed reactor in series 159

List of tables

Table 2.1.Effects of initial total sugar concentration on the fermentative performance of pure cultures (Laplace et al., 1991) 10

Table 2.2.Fermentation of xylose by *C. shehatae* (Abbi et al., 1996) 12

Table 2.3.Performance of *P. stipitis* and *Sp. passalidarum* fermentation (Nakanishi et al., 2017) 13

Table 2.4.Summary of some modified Monod models for biomass and ethanol production rate 51

Table 2.5.Kinetic constants and yield in terms of inhibitory effect of exogenous and endogenous ethanol (Hoppe and Hansford, 1982) 64

Table 2.6.Kinetic constants of *Saccharomyces yeast* 1400 (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1999, 1995) 68

Table 2.7.Correlation coefficient results (modelling and experiment) 69

Table 4.1.Initial concentrations of *S. cerevisiae* and *Z. mobilis* strains from literature experimental results which was used for modelling of this study 93

Table 4.2.Kinetic constants of *S. cerevisiae* (CEN.PK 113-7D and RWB 217) and the respective correction factors denoted in boxes. [Estimated] denotes estimated values from this study using a non-linear regression fit to literature experimental data (Kuyper et al., 2005a, 2005b, 2004, 2003) 98

Table 4.3.Kinetic constants of *Zymomonas mobilis* (ZM4 and ZM4 (pZB5)), and the respective estimated correction factors denoted in box. [Estimated] denotes estimated values in this study from a non-linear regression fit to the literature experimental data (Leksawasdi et al., 2001) 100

Table 4.4.Sensitivity analysis of fermentation time using *S. cerevisiae* by varying each model parameters by 10% 104

Table 4.5.Sensitivity analysis of fermentation time using *Zymomonas mobilis* by varying each model parameters by 10% 104

Table 5.1.Kinetic constants obtained from literature and experiment of this study 121

Table 5.2.The initial concentrations, ethanol yield and productivity, a correlation coefficient 122

Table 5. 3. Summary of Anova result. 129

Table 6.1.Initial concentrations of *Z. mobilis* strains which was used for modelling and experiment of this study 138

Table 6.2.Kinetics of *Z. mobilis* and *S. cerevisiae* 140

Table 7.1 a. Physicochem conditions and immobilization technique for bioethanol fermentation by *Zymomonas mobilis* for glucose conversion and by *Pichia stipitis* for xylose conversion 150

Table 7.1 b. Aeration conditions of bioethanol fermentation by *Zymomonas mobilis* for glucose conversion and by *Pichia stipitis* for xylose conversion 151

Table 7.1 c. Reactor configuration of bioethanol fermentation by *Zymomonas mobilis* for glucose conversion and by *Pichia stipitis* for xylose conversion 151

Table 7. 1 d. Substrate and product conditions of bioethanol fermentation by *Zymomonas mobilis* for glucose conversion and by *Pichia stipitis* for xylose conversion 152

Table 7. 2. Ethanol yield (Ysp) and ethanol productivity (Qp) on glucose across three inoculum sizes of Saccharomyces cerevisiae UFPEDA 1238 (Wanderley et al., 2014)..... 156

Table 7. 3. Comparison of different fermentation parameters for bioethanol production at different conditions in batch process using pure sugars..... 158

Table 7. 4. Comparison of different fermentation parameters for bioethanol production at different conditions during continuous culture using pure sugars 160

Nomenclature

C_x: Cell concentration in medium (mass/unit volume)

C_{xyl}: Xylose concentration (mass/unit volume)

C_{glu}: Glucose concentration (mass/unit volume)

C_{x0}: Initial cell concentration in medium (mass/unit volume)

C_{xyl0}: Initial xylose concentration (mass/unit volume)

C_{glu0}: Initial glucose concentration (mass/unit volume)

C_o: Dissolved oxygen concentration in medium during the fermentation (mass/unit volume)

C_o^*: Dissolved oxygen concentration at saturation (mass/unit volume) (C_o^* at 0.21 % v/v O_2 and 30 ºC: 7.5 mg L^{-1})

$C_{x,max}$: Maximum cell concentration in medium (mass/unit volume)

$C_{AC,max}$: Maximum Acetic Acid concentration in substrate above which cells do not grow (mass/unit volume) (mass/unit volume)

C_{Ac}: Acetic Acid concentration (mass/unit volume)

D: Dilution rate (1/unit time)

dp: Predicted values of experimental data

$\overline{dp}$: Average of experimental values

F: Feed molar flow (mole/unit time)

H_{in}: Feed enthalpy (energy unit/mole/unit time)

H_L: Liquid enthalpy (energy unit/mole/unit time)

H_v: Vapour enthalpy (energy unit/mole/unit time)

J: Order of inhibition by substrate (dimensionless)

$K_{s,glu}$: Glucose limitation constant for substrate uptake rate (mass/unit volume)

$K_{s,xyl:}$ Xylose limitation constant for substrate uptake rate (mass/unit volume)

$K_{x,glu:}$ Glucose limitation constant for biomass production rate (mass/unit volume)

$K_{x,xyl:}$ Xylose limitation constant for biomass production rate (mass/unit volume)

$K_{is,glu:}$ Glucose inhibition constant for substrate uptake rate (mass/unit volume)

$K_{is,xyl}$: Xylose inhibition constant for substrate uptake rate (mass/unit volume)

$K_{ix,glu:}$ Glucose inhibition constant for biomass production rate (mass/unit volume)

$K_{ix,xyl}$: Xylose inhibition constant for biomass production rate (mass/unit volume)

$K_{x:}$ Substrate limitation constant for biomass production rate (mass/unit volume)

$K_{xb:}$ Substrate limitation constant for biomass production rate based on Blackman model (mass/unit volume)

$K_{x,glu:}$ Glucose limitation constant for biomass production rate (mass/unit volume)

$K_{x,xyl:}$ Xylose limitation constant for biomass production rate (mass/unit volume)

K_p: Substrate limitation constant for product formation rate (mass/unit volume)

$K_{p,glu}$: Glucose limitation constant for product formation rate (mass/unit volume)

$K_{p,xyl:}$ Xylose limitation constant for product formation rate (mass/unit volume)

$K_{o:}$ Oxygen limitation constant of substrate for biomass production rate (mass/unit volume)

$K_{o,glu:}$ Oxygen limitation constant of glucose for biomass production rate (mass/unit volume)

$K_{o,xyl}$: Oxygen limitation constant of xylose for biomass production rate (mass/unit volume)

$K'_{isx,}$ K_{isx}, K_{isx1}, $K_{isx2:}$ Substrate inhibition constant for biomass production rate (mass/unit volume)

$K'_{isp,}$ K_{isp}: Substrate inhibition constant for product formation rate (mass/unit volume)

$K'_{iss,}$ K_{iss}: Substrate inhibition constant for substrate uptake rate (mass/unit volume)

$K_{ipx:}$ Product inhibition constant of substrate for biomass production rate (mass/unit volume)

$K_{ipx,glu:}$ Product inhibition constant of glucose for biomass production rate (mass/unit volume)

$K_{ipx,xyl}$: Product inhibition constant of xylose for biomass production rate (mass/unit volume)

K_{ipp}: Product inhibition constant of substrate for product formation rate (mass/unit volume)

$K_{ipp,glu}$: Product inhibition constant of glucose for product formation rate (mass/unit volume)

$K_{ipp,xyl}$: Product inhibition constant of xylose for product formation rate (mass/unit volume)

k_{ipp1}, k_{ipx1}, k_{ipp2}, $k_{ipx2:}$ Empirical constants

$K_{isg:}$ Inhibition constant of xylose by glucose for substrate uptake rate (mass/unit volume)

K_{ixg}: Inhibition constant of xylose by glucose for biomass production rate (mass/unit volume)

K_{ipg}: Inhibition constant of xylose by glucose for product formation rate (mass/unit volume)

K_D: Empirical constants (cell wall permeability, substrate diffusion, and cell size) (mass/unit volume)

K_{P1}, K_{P2}: Production rate constants (mass/unit volume)

$K_L a$: Volumetric oxygen transfer coefficient (1/unit time)

$L_{mole\text{-}fraction:}$ Liquid mole fraction (dimensionless)

$L_{molar\text{-}flow}$: liquid molar flow (mole/unit time)

m_p: Product maintenance coefficient (1/unit time)

mx: Acetic Acid inhibition constant in substrate for growth of biomass (dimensionless)

m_x: Maintenance energy coefficient of biomass (g-substrate/ g-biomass/ unit time)

m_o: Maintenance energy coefficient of oxygen (g-substrate/ g-oxygen/ unit time)

MW_e: Molecular weight of ethanol (mass/mole)

MW_{glu}: Molecular weight of glucose (mass/mole)

np: Number of experimental points

n: Kinetic constant in Moser model (dimensionless)

P: Product concentration (mass/unit volume)

$\bar{P}$: Average of product concentration (mass/unit volume)

P_{ip}: Inhibition of product to growth for product formation from substrate (mass/unit volume)

P_{ix}: Inhibition of product to growth of biomass from substrate (mass/unit volume)

P_0: Initial product concentration (mass/unit volume)

P_f: Output product concentration (mass/unit volume)

P_{tot}: Total vapour pressur (unit pressure)

$P_{x,max}$: Maximum product concentration in substrate above which cells do not grow (mass/unit volume)

$P_{p,max}$: Maximum product concentration in substrate above which cells do not produce product (mass/unit volume)

$P_{m,x,\ glu}$: Maximum ethanol concentration above which cells do not grow in glucose (mass/unit volume)

$P_{m,x,xyl}$: Maximum ethanol concentration above which cells do not grow in xylose (mass/unit volume)

$P_{m,s,\ glu}$: Maximum ethanol concentration above which glucose do not uptake (mass/unit volume)

$P_{m,s,xyl}$: Maximum ethanol concentration above which xylose do not uptake (mass/unit volume)

$P_{ix,\ glu}$: Minimum ethanol concentration above which cells production is affected negatively when grown in glucose (mass/unit volume)

$P_{ix,xyl}$: Minimum ethanol concentration above which cells production is affected negatively when grown in xylose (mass/unit volume)

$P_{is,\ glu}$: Minimum ethanol concentration above which glucose consumption is affected negatively (mass/unit volume)

$P_{is,xyl}$: Minimum ethanol concentration above which xylose consumption is affected negatively (mass/unit volume)

$q_{m,glu:}$ Maximum specific glucose utilization rate (1/unit time)

$q_{m,xyl}$: Maximum specific xylose utilization rate (1/unit time)

$q_{glu:}$ Specific glucose utilization rate (1/unit time)

q_{xyl}: Specific xylose utilization rate (1/unit time)

Q: Heat (unit energy/unit time)

r_s: Substrate consumption rate (mass/unit volume/unit time)

r_x: Biomass production rate (mass/unit volume/unit time)

r_e: Ethanol production rate (mass/unit volume/unit time)

$r_{e,m}$: Maximum ethanol production rate (mass/unit volume/unit time)

r_o: Oxygen consumption rate (mass/unit volume/unit time)

r_{glu}: Glucose consumption rate (mass/unit volume/unit time)

r_{rate}: Reaction rate (mass /unit volume/unit time)

R_{vol}: Reactor volume (unit volume)

S_{xyl}: Xylose concentration (mass/unit volume)

S_{glu}: Glucose concentration (mass/unit volume)

S_c: Threshold substrate concentration below that the organism grows apparently without inhibition (mass/unit volume)

S: Substrate concentration (mass/unit volume)

S_0: Input substrate concentration (mass/unit volume)

S_f: Output substrate concentration (mass/unit volume)

S_{ip}: Inhibition of substrate for product formation from substrate (mass/unit volume)

S_{ix}: Inhibition of substrate to growth of biomass from substrate (mass/unit volume)

$S_{x,maxl}$: Maximum substrate concentration in substrate above which cells do not grow (mass/unit volume)

t: Time

t_L: Lag time (time)

τ: Residence time (time)

VVM: volume of air/volume of medium/time (unit volume/ unit volume/ unit time)

V: Vapour molar flow (mole/unit time)

X: Cell concentration in medium (mass/unit volume)

$X_{x,max}$: Maximum biomass concentration in substrate above which cells do not grow (mass/unit volume)

X_P: Predicted values of model

y: Vapor mole fraction (dimensionless)

Y_{xp}: Ethanol yield constant from biomass (g-product/ g-biomass)

Y_{xs}: Substrate yield constant from biomass (g-substrate/g-biomass)

Y_{SP},$Y_{P/S}$: Product yield constant from substrate (g-product/g-substrate)

$Y_{SX:}$ Cell yield constant from substrate (g-cells/g-substrate)

Y_{SX}^{max}: Maximum cell yield constant from xylose (g-biomass/g-substrate)

$Y_{SP,\,glu}$: Product yield constant from glucose (g-product/g-glucose)

$Y_{SP,\,xyl}$: Product yield constant from xylose (g-product/g-xylose)

Y_{ox}^{max}: Maximum cell yield constant from oxygen in xylose (g-bimass/g-oxygen)

Z: Inlet mole fraction (dimensionless)

μ_{max}: Maximum specific growth rate in substrate (1/unit time)

$\mu_{max,xyl}$, $\mu_{m,xyl}$: Maximum specific growth rate in xylose (1/unit time)

$\mu_{max,glu}$, $\mu_{m,glu}$: Maximum specific growth rate in glucose (1/unit time)

μ: Specific growth rate in substrate (1/unit time),

$\mu_{xyl:}$ Specific growth rate in xylose (1/unit time),

μ_{glu}: Specific growth rate in glucose (1/unit time)

NOMENCLAUTURE

$\nu_{max:}$ Maximum specific rate of product formation in substrate (1/unit time)

$\nu_{max,xyl:}$ Maximum specific rate of product formation in xylose (1/unit time)

$\nu_{max,glu}$: Maximum specific rate of product formation in glucose (1/unit time)

ν: Specific rate of product formation in substrate (1/unit time)

ν_{xyl}: Specific rate of product formation in xylose (1/unit time)

ν_{glu}: Specific rate of product formation in glucose (1/unit time)

α: Correction factor (optional, dimensionless)

γx: Substrate inhibition constant for growth of biomass (dimensionless)

β'p, βp: Product inhibition constant in substrate for product formation (dimensionless)

βs: Product inhibition constant in glucose for glucose consumption (dimensionless)

βs': Product inhibition constant in xylose for xylose consumption (dimensionless)

βx: Product inhibition constant in glucose for growth of biomass (dimensionless)

βx': Product inhibition constant in xylose for growth of biomass (dimensionless)

αx: Biomass inhibition constant in substrate for growth of biomass (dimensionless)

$\alpha_{ix,oxy}$: Oxygen inhibition constant in xylose for growth of biomass (mass/unit volume)

$\alpha_{x,oxy}$: Product limitation constant in xylose for growth of biomass (mass/unit volume)

γp: Substrate inhibition constant for substrate uptake (dimensionless)

ω: Degree of substrate inhibition for growth (dimensionless)

ω': Degree of substrate inhibition for product formation (dimensionless)

ϕ: Coefficient of consumption activity (dimensionless)

γ: Activity coefficient (dimensionless)

1. Introduction

Currently, rising fuel costs, dependency on fossil supplies, dwindling crude oil reserves and increasing eco-friendly consciousness has pushed the exploration for sustainable, renewable, useful, and profitable energy sources with reduced emissions of greenhouse gases and reduced environmental burden (Nigam and Singh, 2011). Climate change and global warming are identified as extreme environmental warnings (Raupach and Canadell, 2010). Biomass can assist as a notable substitute raw material to counter the current and upcoming fuel claims (Srirangan et al., 2012). All fuel made from biomass is labelled biofuel (Srirangan et al., 2012). The two best known liquid biofuels are biodiesel and bioethanol; these are intended to substitute (Srirangan et al., 2012) or supplement fossil fuels until improved transportation technology is in place. Bioethanol is used as a fuel additive, gasoline enhancer, platform chemical and also for pharmaceutical and beverage uses (Jong et al., 2012). With the current interest, economical production of ethanol from cellulosic resources by direct bioconversion has been launched in countries like Brazil, Canada and USA (Manochio et al., 2017).

Ethanol can be produced from the following three categories of renewable biomass (Balat et al., 2008):

- Sucrose-based feedstocks, for instance, sugar cane, sugar beet, sweet sorghum and fruits;
- Starchy materials such as corn, wheat, rice, potatoes, cassava, and sweet potatoes;
- Lignocellulosic biomass such as wood, straw, bagasse, and grasses.

First generation bioethanol is made from sugar feedstock or from starch-rich materials, competing with food and feeds. Second generation bioethanol is produced from lignocellulosic feedstocks (Mohr and Raman, 2013). The advantage of first generation crops is their long-term availability (Mohr and Raman, 2013). As these crops are also foodstuffs, diverting major amounts of energy from food products is expected to deplete the finite amount of land available for producing food for the burgeoning world population and increase food prices. To avoid this, second generation crops such as lignocellulosic biomass have been suggested for liquid biofuel production (Mohr and Raman, 2013). On converting the cellulose and hemicellulose fractions of lignocellulose to sugars by pretreatment and hydrolysis, hexose and pentose sugars are released. Glucose and xylose are the first and second most abundant sugars in nature respectively for the production of bioethanol (Lachke, 2002). A complete conversion of glucose and xylose from bagasse to ethanol is a precondition for increasing the effectiveness of an industrial process for bioethanol production (Krishnan et al., 2000).

This research focuses on the production of bioethanol from xylose and glucose hydrolysed from the hemicellulose and cellulose fraction of bagasse in the pretreatment and hydrolysis steps. For simplicity, the inhibitory compounds resulting from the pretreatment and hydrolysis of lignocellulosic material such as

acetic acid, furfural and p-hydroxybenzoic acid do not form part of the scope of this thesis but will be considered the in subsequent study. A high and efficient integrated conversion of glucose and xylose is necessary for the commercial viability of bioethanol production.

This Ph.D. thesis includes an introduction followed by Chapter 2A which presents a literature review of different approaches to bioethanol fermentation from glucose and xylose (e.g. engineered culture, co-cultures, sequential cultures). This chapter leads to the selection of a continuous two stage sequential fermentation process to overcome the issue of catabolite repression, low ethanol tolerance of the xylose-fermenting microorganisms and understanding of complex metabolic pathway regulations required during genetic engineering of microorganism. The suggested configuration also allows for optimum aeration levels while using different microorganisms. In Chapter 2B, the major available kinetic models for bioethanol fermentation from glucose, xylose, mixture of glucose and xylose were reviewed and assessed. The comparison between modelling and experimental data showed that effective factors of substrate limitation (e.g. sugars and oxygen), product inhibition, and substrate inhibition (e.g. sugars and oxygen) should be considered to improve the sensitivity and accuracy of the kinetic models in terms of the objective of the model, specific microorganism, and process conditions. Better agreement was found between modelling and experiment using simpler equations and considering three important effective factors of substrate limitation, substrate inhibition and product inhibition when grown in standard growth medium. Chapter 3 identifies the literature gaps in a consolidated way, refines the thesis scope, and justifies the research approach, hypotheses, key questions, limitations, common methods and novel contributions. In Chapter 4, the suitable kinetic model and kinetic parameters for bioethanol production with glucose using *Z. mobilis* were selected from intra (wild type vs engineered) and inter (*S. cerevisiae* vs *Z. mobilis*) species kinetic parameters dependency when the same xylose utilization pathway was engineered in these strainsand then validated with experimental data. In Chapter 5, the kinetic parameters for xylose conversion using *P. stipitis* were estimated for the best fit between experimental data and modelling. This study involved the development of a kinetic model that includes oxygen inhibition and oxygen limitation constants for bioethanol production from xylose using MATLAB. The initial biomass concentration was varied in Chapter 5 to show the role of initial biomass concentration in ethanol productivity. In Chapter 6, the kinetic models developed were transferred and modelled in Aspen Custom Modeller (ACM) for bioethanol fermentation. This model was then embedded in an Aspen Plus flowsheet instead of using a stoichiometric or the build-in kinetic model in Aspen Plus. Better agreement with industrial data was found with the developed ACM kinetic model than the stoichiometricAspen Plus model. In Chapter 7, the suggested two stage sequential fermentation process of Chapter 2A was tested. A high inoculum concentration of suspended or immobilized *Z. mobilis* in continuous fluidized bed or CSTR was inoculated in the first stage to convert the glucose; thereafter, a high inoculum concentration of *P. stipitis* was inoculated into the second

stage as a suspension culture for conversion of xylose using CSTR. A continuous process was investigated to overcome low ethanol tolerance of *P. stipitis*. A low glucose concentration was used to increase xylose conversion and to prevent catabolite repression. Also, a low oxygen flowrate was only sparged into the second *P. stipitis* stage to provide microaerobic conditions in the 2nd stage while maintaining anaerobic conditions in the first stage. Integrating discussion and conclusions are presented in Chapter 8 to close the thesis. Of the chapters based on a publication, a preamble is included to establish the context of the findings with respect to the integrated thesis as well as each author's contribution.

The thesis is presented as a series of papers addressing the literature review, mathematical model, kinetic parameters, methodology, research findings through experimental observations and mathematical modelling as were mentioned above.

Two papers were accepted and presented at the 10th World Congress of Chemical Engineering ("Kinetic data analysis and mathematical modelling of intra (wild type vs engineered) and inter species (*Saccharomyces cerevisiae* vs *Zymomonas mobilis*) dependency for bioethanol production from glucose or/and xylose"; "Embedding equation oriented kinetic model in a fermentation process for bioethanol production in a sequential modular flowsheet simulator"). One paper has been accepted and published in the peer-review journal of ChemBioEng Reviews (Ethanol from biomass hydrolysates by efficient fermentation of glucose and xylose – a review) and two draft manuscripts are in preparation for submission ("Mathematical modelling of bioethanol fermentation processes from glucose or/and xylose – a review"; "Analysis of ethanol production from xylose using *Pichia stipitis* in microaerobic condition through experimental observations and kinetic modelling"). Chapter 7 presents the novel processes for efficient bioethanol fermentation from the mixture of glucose and xylose using *Zymomonas mobilis* and *Pichia stipitis,* most importantly suggesting sequential continuous culture of two microorganisms in two reactors in series. Each paper, presented as part of a continuous narrative, addresses components of the objectives detailed in the research approach provided in Chapter 3.

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2. Literature review

This chapter is composed of two papers. In the first paper of this chapter, ethanol production from xylose and glucose leading to definition of approach (2A.1-2A.9) and in the second paper of this chapter, modelling of ethanol fermentation (2B.1-2B.9) were reviewed.

The first paper, entitled **Ethanol from biomass hydrolysates by efficient fermentation of glucose & xylose–a review**, presents a review of various approaches to bioethanol production from a mixture of glucose and xylose. Owing to the absence of a naturally occurring microbial species that ferments both glucose and xylose simultaneously to ethanol, the potential of genetic modification to achieve engineered strains capable of this, co-culture with wild type or recombinant strains and sequential culture are reviewed. Co-fermentation of bioethanol production via co-culture explores a range of paired microorganisms to achieve both glucose and xylose metabolism, including *P. tannophilus & Z. mobilis, P. stipitis & Z. mobilis, K. marxianus & S. cerevisiae, P. stipitis & K. marxianus, P. stipitis & S. cerevisiae, P. stipitis* & respiratory *deficient mutant of S. diastaticus, C. shehatae & S. cerevisiae, P. tonnophilis & S. cerevisiae, E. coli & S. cerevisiae are reviewed.* Engineered cultures, including recombinant *S. cerevisiae*, recombinant *Z. mobilis*, recombinant *E. coli*, recombinant *Klebsiella oxytoca* with metabolic pathways for both xylose and glucose are reviewed. Finally, sequential culture in either one or two reactors was reviewed. The pros and cons, fermentation modes, fermentation performance and fermentation condition of every approach were assessed in this paper. Finally, the issues of the different affinities for oxygen among the different microorganisms, the low ethanol tolerance of xylose-fermenting microorganisms, and the catabolite repression on the xylose assimilation from glucose were also discussed in the recommendations section. Two-stage fermentation in two reactors along with manipulation of process conditions was recommended in this paper. This leads to the novelty of my PhD project and defines the approach to the research, further specified in Chapter 3.

The second paper, entitled **Mathematical modelling of bioethanol fermentation processes from glucose or/and xylose – a review**, is a review of major available kinetic models for fermentation of bioethanol production from glucose and/or xylose. This paper presents a brief description of unstructured (Black box) and structured (metabolic pathway, white box) models. This paper focused on the unstructured and non-segregated model, as the structured model is demanding for information in experimental cell metabolism, difficult to use with process tools like software sensors or controllers, and problematic with model parameter verification and model evaluation. In order to improve the accuracy and sensitivity of the kinetic models, effective factors to describe substrate limitation, product inhibition and substrate inhibition were

considered. Comparison between the major available kinetic models in literature and experimental data for bioethanol fermentation from glucose or xylose or both, obtained from the literature, was carried out. The one which has the best agreement with experimental data was suggested. The suitable kinetic models, based on this review, were then used and improved in the project for both the modelling of the experimental data generated (Chapters 4 and 5) and the further scenario analysis to propose the integrated process, tested in Chapter 7. This is laid out in objectives (1) and (2), defined in Chapter 3.

Author contribution in both papers:

The manuscript was conceptualised by Nosaibeh Nosrati Ghods (NNG), Prof. Susan T.L. Harrison (STLH) and Dr. Siew L. Tai (SLT). It was written by NNG and edited by STLH, Associate Prof. Adeniyi J. Isafiade (AJI) and SLT. STLH and SLT helped in the interpretation of the literature reviewed and the integration of the knowledge into comprehensive form, shedding new understanding of the topic for this review article. STLH approved the final draft. The financial support of the National Research Foundation of South Africa Competitive Programme for Rated Researchers (CPRR:87744), kindly prepared by Prof. Duncan M. Fraser and Associate Prof. Adeniyi J. Isafiade; the financial support of the research development grant from the University Research Council (URC) of the University of Cape Town, the financial support of Centre for Bioprocess Engineering Research (CeBER) through the SARChI Chair in Bioprocess Engineering (GUN 64778) of Prof. Susan T.L. Harrison are gratefully acknowledged.

Paper title:

Ethanol from biomass hydrolysates by efficient fermentation of glucose and xylose – a review

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Abstract:

Hydrolysis of biomass yields xylose & glucose for fermentation to ethanol. Fermentation performance of these hydrolysates is limited by lack of industrially suitable cells to convert both glucose & xylose efficiently. To solve this issue, several methods have been suggested, e.g. co-cultivation of two or more species, engineering strains for enhanced substrate utilization, use of sequential culture. Challenges of co-culture include slower xylose fermentation due to varying affinities for oxygen, lower ethanol tolerance of xylose-fermenters and catabolite repression. Although successful engineering of microorganisms is demonstrated, there is limitation in understanding of the metabolic pathways regulations. Alternatively, sequential batch culture was suggested, but its productivity needs to be improved. Optimizing process conditions, e.g. process configuration, immobilization technique, cell type enables improved yield and productivity. This paper reviews approaches and conditions sought to improve glucose & xylose conversion from lignocellulosic hydrolysates to ethanol, with specific emphasis on microbial system used to maximize biomass resource efficiency, ethanol yield and productivity.

Keywords: co-culture, ethanol production, fermentation, glucose and xylose, resource efficiency

2A.1. Introduction

2A.1.1. Background

Renewable biomass has major potential as renewable feedstock for liquid fuel and bio-based platform chemicals (Srirangan et al., 2012), with potential for conversion to bioethanol, biodiesel, and biobutanol (Srirangan et al., 2012). Bioethanol is well recognized as a potential liquid biofuel and fuel additive in gasoline. It can be used in the pharmaceutical and beverage industries and as a platform chemical, providing the building block for polyethylene and bio-PET (Jong et al., 2012). Economic production of ethanol from cellulosic resources by direct bioconversion has been implemented in Brazil, EU and USA (Manochio et

al., 2017). First generation bioethanol is made from sugar or starch-rich feedstocks, while second generation bioethanol may be produced from lignocellulose (Mohr and Raman, 2013). The advantage of first generation crops is long-term availability and high yields, but they compete with food supply (Mohr and Raman, 2013). Second-generation lignocellulosic crops or crop residues provide alternative. For example, waste bagasse from the sugar industry comprise 43.8% cellulose, 28.6% hemicellulose, 23.5% lignin (Pereira et al., 2011). Cellulose is a straight chain glucose polymer with β1-4 linkages (Pulidindi et al., 2014). Hemicellulose contains mostly xylose, with traces of mannose, galactose, arabinose, and glucose (Menon and Rao, 2012). Pre-treatment removes lignin and hemicellulose, reduce the crystallinity of cellulose, increase the porosity of the lignocellulosic materials and plays a key role in simplifying the downstream processing (Kumar et al., 2009). The fraction of lignin, hemicellulose and cellulose in feedstocks have an effect on the selection of the pre-treatment methods used (Crimes, 2015). Various pre-treatment methods available include biological, physical, chemical, physicochemical and Pulsed-Electric-Field Pretreatment (Kumar et al., 2009). Delignification is required before hydrolysis to remove the lignin fraction from the solid biomass (Crimes, 2015). During hydrolysis, the glycosidic bonds that hold the sugar monomers together are broken using enzymes or chemicals (e.g. acids) or a combination of both (Crimes et al., 2015). It is noted that hydrolysis of hemicellulose to C-5 sugars is more complicated than cellulose to C-6 sugars (Crimes, 2015). After hydrolysis, detoxification (e.g. neutralization, overliming, adsorption, ion exchange, the use of enzymes and electrodialysis) is required before fermentation to remove inhibitors that were introduced during the pre-treatment method (Crimes, 2015). The final product is a fermentable C6 and C5-rich sugar solutions derived from the hydrolysis of cellulose and hemicellulose, respectively, available for bioethanol fermentation. Glucose and xylose are the most abundant fermentable sugars in nature for bioethanol production (Lachke, 2002).

2A.1.2. Objective

The present review aims to provide an overview of the current approaches to fermentation for bioethanol production from glucose and xylose bearing hydrolysates, considering process setups and conditions. Several parameters such as ethanol yield ($Y_{p/s}$, g-ethanol g-consumed-sugar^{-1}) and volumetric ethanol productivity (Q_p, g-ethanol L^{-1} h^{-1}) are used to evaluate fermentation processes.

2A.1.3. Challenges

Although *Saccharomyces cerevisiae* and *Zymomonas mobilis* are the benchmark microorganisms for bioethanol production (Banerjee, 2010) demonstrating high ethanol tolerance and yield, they cannot ferment xylose (Banerjee, 2010). Conversely, the yeast *Pichia stipitis* and *Kluyveromyces marxianus* ferment both xylose and glucose, albeit at lower ethanol yields and with additional challenges. They are

sensitive to low concentrations of inhibitors in hydrolysates (*i.e.*, acetate) and cannot grow without oxygen. Pentoses enter the pentose phosphate pathway prior to glycolysis. Ethanol production occurs only at an optimum oxygen level and low ethanol tolerance exists (Slininger et al., 1987). Since no suitable microorganisms are available to convert both xylose and glucose to ethanol efficiently, co-culturing and sequential culturing have been explored, in tandem with construction of genetically engineered microorganisms.

2A.1.4. Approaches and limitations

In co-culture experiments, the combined utilization of two microorganisms may show advantages due to synergistic action of their metabolic pathways (Bader et al., 2010). According to a US Department of Energy (DOE) study in 2005, co-culture bioconversion to ethanol is plausible with potential high-payoff. Combinations of microorganisms showing promise include co-culture of immobilized *Z. mobilis* and suspended *Pichia stipitis* (Fu et al., 2009), co-culture of ethanologenic *E. coli* K011 and *S. cerevisiae* (Okuda et al., 2008), co-culture of *Z. mobilis* and *Candida tropicalis* for ethanol production from hydrolysed agricultural wastes (Patlea and Lal, 2008), co-culture of *S. cerevisiae* and *Pachysolen tannophilis* (Qian et al., 2006), and co-culture of restricted catabolite repressed mutant *P. stipitis* and respiratory-deficient mutant *S. cerevisiae* (Kordowska-Wiater and Targoński, 2002). Co-culture has shown slow xylose fermentation compared to glucose because of catabolite repression, low ethanol tolerance of the xylose-fermenters and varying oxygen requirement between the combinations of microorganisms (Grootjen et al., 1991a; Hamidimotlagh et al., 2007; Kordowska-Wiater and Targoński, 2002; Jean M. Laplace et al., 1993; Lebeau et al., 1997).

Development of recombinant yeast and bacterial strains capable of high-level ethanol production from hemicellulosic sugars has been sought through two methods. First, metabolism of conventional ethanol-producing species (*S. cerevisiae* and *Z. mobilis*) is extended to metabolize xylose and arabinose (Dumsday et al., 1997). Second, genes for ethanol production were added into microorganisms like *E. coli* and *Klebsiella oxytoca* which produce little or no ethanol but have native ability to ferment pentose and hexose sugars (Dumsday et al., 1997). Recombinant *E. coli* and *Z. mobilis* are the most suitable strains for use with hemicellulosic hydrolysates (H.G. Lawford and Rousseau, 1991). Despite developments using engineered strains, limitations remain with limited research on the metabolic network model.

Alternatively, sequential fermentation, growing microorganisms in series rather than simultaneously, combines efficient conversion of pentoses and hexoses to ethanol in a simple and powerful approach (Grootjen et al., 1991a) by allowing hexose to be utilized first followed by pentose conversion. In sequential processes, xylose-fermenters like *P. stipitis* are cultivated at optimum specific oxygen uptake rate for xylose fermentation subsequent to anaerobic conditions for glucose-fermenters (M. Taniguchi et al., 1997).

However, sequential cultures are mostly batch processes with optimization constraints. Beck et al. (1990) evaluated two microbial combinations (*S. cerevisiae* and *P. tannophilus*, *S. cerevisiae* and *E. coli*), comparing co-culture and sequential culture under set conditions (30 °C, 100 rpm, working volume 100 ml) (Beck et al., 1990). Increased ethanol yields resulted in sequential fermentation of *S. cerevisiae* and *E. coli* (sequential: 0.51 g g^{-1}; co-culture: 0.43 g g^{-1} from 125 g L^{-1} substrate) (Beck et al., 1990).

2A.1.5. Way forward - optimization of process condition

Optimizing process conditions such as initial total sugar concentration, ratio of glucose to xylose, aeration conditions, and immobilization technique offers an alternative or extension to improving yields and productivity of bioethanol through enhancing microbial capacity in the sequential culture.

2A.1.5.1. Initial total sugar concentration and a ratio of glucose to xylose

For efficient consumption of glucose and xylose, both initial total sugar concentrations and ratio of glucose to xylose are important. Laplace et al. (Laplace et al., 1991) investigated the effects of initial total sugar concentration on the fermentative performance of pure cultures of *P. stipitis*, *C. shehatae*, *S. cerevisiae*, and *Z. mobilis*.

Table 2.1.Effects of initial total sugar concentration on the fermentative performance of pure cultures (Laplace et al., 1991)

P. stipitis	**Initial xylose concentration** (g L^{-1})	35	85	114			
	Ethanol yield (g g^{-1})	0.44	0.44	0.45			
	Ethanol productivity (g g^{-1} h^{-1})	0.18	0.19	0.14			
	Used xylose (%)	100	100	87			
C. shehatae	**Initial xylose concentration** (g L^{-1})	20	50	80	110	170	250
	Ethanol yield (g g^{-1})	0.40	0.40	0.35	0.33	0.32	0.25
	Ethanol productivity (g g^{-1} h^{-1})	0.19	0.20	0.17	0.13	0.18	0.10
	Used xylose (%)	100	100	88	72	58	49
S. cerevisiae	**Initial glucose concentration** (g L^{-1})	20	50	80	110	170	250
	Ethanol yield (g g^{-1})	0.43	0.46	0.47	0.41	0.42	0.43
	Ethanol productivity (g g^{-1} h^{-1})	0.40	0.65	0.47	0.34	0.22	0.16
	Used glucose (%)	100	100	100	84	82	33
Z. mobilis	**Initial glucose concentration** (g L^{-1})	20	50	80	110	170	250
	Ethanol yield (g g^{-1})	0.49	0.48	0.48	0.50	0.30	0.0
	Ethanol productivity (g g^{-1} h^{-1})	3.05	3.60	1.35	1.20	1.30	0.0
	Used glucose (%)	100	100	100	84	89	0

Table 2.1 shows there is an optimum value for ethanol yield and productivity because of substrate and product inhibition. Beck et al. (Beck et al., 1990) showed an increase in ethanol yield with increasing substrate concentration until substrate inhibition occurs. In sequential culture of *S. cerevisiae* and *E. coli*, initial sugar concentration of 50 g L^{-1} gave an ethanol yield of 0.41 g g^{-1}; while initial sugars of 125 g L^{-1} and 200 g L^{-1} gave ethanol yields of 0.51 and 0.41 g g^{-1} respectively (Beck et al., 1990). The most common ratio of glucose and xylose tested is 2:1 (glucose: xylose) with ethanol yield of 0.45-0.5 g g^{-1} and average productivity of 0.9 g $L^{-1}h^{-1}$ (Chaudhary and Ghosh, 2014; Hanly and Henson, 2013; Kordowska-Wiater and Targoński, 2002; Jean M. Laplace et al., 1993; Lawford and Rousseau, 2002; Lebeau et al., 1997; Masayuki Taniguchi et al., 1997; Taniguchi and Tanaka, 2004), approximating the composition in sugarcane bagasse or other biomass hydrolysates; ratios of 4:1 (De Bari et al., 2004) with ethanol yield of 0.396 g g^{-1} and productivity of 0.89 g $L^{-1}h^{-1}$, 3:1 (Watanabe et al., 2007) with ethanol yield of 0.43 g g^{-1}, and 1:1 (Jeppsson et al., 2003; Kuyper et al., 2005a, 2005b; Mohagheghi et al., 2002; Zaldivar et al., 2002; Zhang et al., 1995) with ethanol yield of 0.41-0.43 g g^{-1} and productivity of 0.2-0.61 g $L^{-1}h^{-1}$ are also reported.

2A.1.5.2. Aeration condition

Glucose and xylose can be fermented aerobically or anaerobically depending on the microbial strain. For example, *P. stipitis* cells grown aerobically assimilated xylose 27 times faster than under anaerobic conditions (Balat et al., 2008; Fu et al., 2009; Gírio et al., 2010; Skoog et al., 1990), indicating high energy requirements for conversion or transport of xylose (Skoog et al., 1990). *Z. mobilis* and *S. cerevisiae,* require anaerobic conditions for high ethanol yields (Balat et al., 2008; Fu et al., 2009; Gírio et al., 2010; Yang et al., 2009). Anaerobic fermentation of *Z. mobilis* consumed glucose more quickly, grew more rapidly and showed increased ethanol yield and productivity over aerobic cultures (Yang et al., 2009). Typically, the preference for bioethanol production is anaerobic for glucose conversion and microaerobic for xylose conversion. Skoog et al. (1990) showed maximum specific ethanol yield and productivity for xylose fermentation by *P. stipitis* when oxygen transfer rates (OTR) were below 1 mmol/lh (Skoog et al., 1990).

2A.1.5.3. Reactor configuration

Reactor configuration (e.g. batch, fed-batch and continuous) plays a key role in fermentation (Vogel and Todaro, 2009). A material balance across these reactors using Monod kinetics demonstrates that the productivity of continuous culture exceeds fed-batch that, in turn, exceeds batch culture. Extending duration of the ethanol production phase, e.g. through fed-batch culture, increases the yield. Abbi et al. (Abbi et al., 1996) demonstrated these findings for the fermentation of xylose and rice straw hydrolysate by *C. shehatae* in batch, fed-batch, and continuous process (Table 2.2). Jeppsson et al. (Jeppsson et al., 2003) compared

batch and continuous processes of engineered *S. cerevisiae*. The ethanol yield in a continuous process (0.39 g g^{-1}) was higher than batch (0.31 g g^{-1}) (Jeppsson et al., 2003). The volumetric productivity of ethanol in fed-batch and continuous systems is higher due to the elimination of long lag-time and downtime associated with batch systems, production at optimum biomass concentration over the majority of the process, and elimination of accumulation and inhibition of products. In fed-batch cultures, final ethanol concentration was determined by initial sugar concentration, rather than nature of biomass (free and immobilized cells) (Gaur, 2006). Catabolite repression favors consumption of glucose over other sugars in most yeasts of interest. For example, when residual glucose concentrations exceed 2.3 g L^{-1} using *P. stipitis*, xylose fermentation is suppressed (Grootjen et al., 1991b). This occurs in the early stages of batch culture. Further, an ethanol concentration above 35 g L^{-1} inhibits xylose fermentation with wild-type xylose-fermenting strains (Hamidimotlagh et al., 2007). To obtain a glucose concentration of less than 2.3 g L^{-1}, while avoiding accumulation of inhibiting metabolites and maintaining ethanol concentration below 35 g L^{-1}, a continuous process is suggested (Lebeau et al., 1997). In fed-batch, control of glucose below 2.3 g L^{-1} is also possible, but ethanol inhibition of xylose-fermenting species is still prevalent. Ethanol yield and productivity of *Spathaspora passalidarum* is comparable to *P. stipitis* as shown in Table 2.3 (Nakanishi et al., 2017), where both have similar glucose tolerance of 35% (w/v) (Rodrussamee et al., 2018) but *S. passalidrarum* with better ethanol tolerance (40 g L^{-1}) (Su et al., 2015) than *P. stipitis* (35 g L^{-1}) (Hamidimotlagh et al., 2007). Wash out and contamination is a critical issue for continuous production of ethanol, even at low dilution rates, since the extracellular product is favored over cell growth (Taherzadeh and Karimi, 2007). Comparison between fed-batch (2.0–3.9 × 10^7 % bacterial-rods mL^{-1}) and continuous (3.8–9.9 × 107 % bacterial-rods mL^{-1}) processes showed that fed-batch processes contained less contaminants (Lopes et al., 2016). In order to overcome this, recycling yeast cells with acid treatment (e.g. diluted sulfuric acid, pH 2.0–2.5 for 1–2 h) is the common procedure in Brazil to kill bacteria to prevent sugar losses due to bacterial contamination (Lopes et al., 2016).

Table 2.2.Fermentation of xylose by *C. shehatae* (Abbi et al., 1996)

	Yield (g ethanol produced/g used-xylose)		**Productivity (g L^{-1} h^{-1})**	
	Free cell	**Immobilized cell**	**Free cell**	**Immobilized cell**
Batch	0.42	0.50	0.15	0.14
Fed-batch	0.47	0.50	0.20	0.24
Continuous/Dilution rate (h^{-1})	-	0.33/0.75	-	0.33/0.75

Ultimately, yield is increased by extension of the ethanol production phase of the cell. Hence, cell retention (immobilization) and cell recycle (using centrifuges or microfiltration) are of interest (Chen, 2011).

Table 2.3.Performance of *P. stipitis* and *Sp. passalidarum* fermentation (Nakanishi et al., 2017)

Microorganism	Condition	Carbon source	Ethanol productivity ($g\ L^{-1}h^{-1}$)	Ethanol yield [g (g sugar used^{-1})]	Reference
P. stipitis Y-7124	Bioreactor- sequential batch cell recycle (10th recycle), residual glucose (0.0 g L^{-1}), residual xylose (16.4 ± 0.76 g L^{-1}), microaerobic (0.1 vvm)	Glucose (15.5 ± 0.07 g L^{-1}) and xylose (64.4 ± 3.46 g L^{-1})	1.50	0.47	(Santos et al., 2016)
P. stipitis Y-7124	Bioreactor- batch, xylose consumption (85 %), 0.7 vvm and 100 RPM	Detoxified sugarcane bagasse hydrolysate (50 g L^{-1} xylose, 5.0 g L^{-1} glucose, 2.7 g L^{-1} acetic acid)	0.10	0.16	(Dussán et al., 2016)
P. stipitis Y-7124	Flask, 2.5–4.7 $mmol_{O2}L^{-1}h^{-1}$, 0.34–0.4 $mmol_{O2}$ $g_{CDW}^{-1}h^{-1}$ (CDW: cell dry weight), residual xylose (50.3 g L^{-1})	Xylose (140 g L^{-1})	0.73	0.39	(Su et al., 2015)
P. stipitis Y-7124	Flask, 100% xylose conversion	Xylose (75.1 g L^{-1})	0.49	0.48	(Günan Yücel and Aksu, 2015)
P. stipitis Y-7124	Flask, 100% conversion of reducing sugars	Detoxified sugar beet pulp hydrolysate, Initial reducing sugar concentration: 48.2 g L^{-1}	0.25	0.12	(Günan Yücel and Aksu, 2015)

Microorganism	Condition	Carbon source	Ethanol productivity (g $L^{-1}h^{-1}$)	Ethanol yield [g (g sugar $used^{-1}$)]	Reference
P. stipitis Y-7124	Bioreactor-batch, 100% xylose conversion, oxygen-limited	Xylose (120 g L^{-1})	0.23	0.46	(Farias et al., 2014)
P. stipitis Y-7124	Bioreactor-continuous, dilution of 0.008 h^{-1}, 100% xylose conversion, oxygen-limited	Xylose (95 g L^{-1})	0.33	0.43	(Farias et al., 2014)
P. stipitis Y-7124	Bioreactor, 250 rpm, 0.25 vvm	Glucose (5 g L^{-1}), xylose (30 g L^{-1}) and arabinose (5 g L^{-1})	0.32	0.32	(Silva et al., 2011)
P. stipitis Y-7124	Bioreactor- sequential fed batch cell recycle (3rd fed-batch)	Sugarcane bagasse hydrolysate, pretreated with NaOH/AQ	0.36	0.32	(Nakanishi et al., 2017)
Sp. passalidarum 27907	Bioreactor, batch, 90% N2 and 10% air (2.1% dO2) from the start, and the dO2 controller was set to a range of 400 to 500 rpm	Maple hemicellulosic hydrolysate (65 g/l of xylose and 35 g/l of glucose)	0.64	0.34	(Long et al., 2012)

2A.1.5.4. Immobilization technique

Immobilization has several advantages over free cell cultivation, including relative ease of product separation, reuse of biocatalyst and high volumetric output (Ghorbani et al., 2011). Immobilization approaches include adsorption (to *diethylaminoethylcellulose* (DEAE)-cellulose and bone char), covalent cross-linking (with glutaraldehyde to an immobilization matrix), and entrapment (in calcium alginate, polyacrylamide, κ-carrageenan and agar) (Bickerstaff, 1997). Entrapment in calcium alginate is one of the most common methods for whole cell immobilization because of ease and non-toxicity (Rosevear, 2008). Immobilization reduces the risk of contamination in continuous processes as the dilution rate can exceed specific growth rate washing out opportunistic contaminants. It enhances xylose and glucose conversion by reducing catabolite repression owing to diffusion limitation restricting glucose concentration inside the beads. Similarly, it reduces ethanol inhibition and concentrates the cells into a small volume, thus increasing ethanol yield and productivity (Abbi et al., 1996), (Table 2.2).

2A.2. Metabolic engineering of preferred ethanol producers for utilization of C5 and C6 sugars

To maximize resource productivity of ethanol from lignocellulosic hydrolysates, metabolic engineering of the preferred industrial microbes *S. cerevisiae* and *E.* coli, preferred ethanol producer such as *Z. mobilis,* and preferred microbe for pulp and paper streams *K. oxytoca* has been considered to widen its substrate utilization range for ethanol production.

S. cerevisiae is versatile and well characterized as an industrial microorganism (Taylor et al., 2009). It gives high ethanol yield and high productivity from hexose sugars (up to 90% of theoretical yields) (Banerjee, 2010; Harun et al., 2010; K. Ohta et al., 1991; Kazuyoshi Ohta et al., 1991). It is also adapted to tolerate process stresses including inhibition from by-products and ethanol, osmotic and pH stress and extreme growth conditions (e.g. low temperature, anaerobiocity) (Banerjee, 2010). *Z. mobilis* uses the Entner-Doudoroff (ED) pathway, fermenting glucose more efficiently and rapidly than *S. cerevisiae* in which glucose is assimilated through the Embden-Meyerhof-Parnas (EMP) pathway (Figure 2.1) (Altintas et al., 2006; Karhumaa et al., 2005; Nikolaev, 2010). The ED pathway yields half the ATP per mole of glucose compared to the EMP pathway (Zhang et al., 1995). Hence, *Z. mobilis* produces less biomass than *S. cerevisiae* but maintains a high glucose flux through the ED pathway, channelling more carbon towards fermentation products (Zhang et al., 1995). A high ethanol yield (0.5 g g^{-1}) and productivity (3.6 g $g^{-1}h^{-1}$) were achieved by *Z. mobilis* from glucose owing to its unique physiology (Sadik and Halema, 2014). While *S. cerevisiae* remains preferred by industry because of its hardiness, industrial scale trials of 586 m^3 using *Z. mobilis* have been successful (Dien et al., 2003). However, neither *S. cerevisiae* nor *Z. mobilis* are well suited for conversion of lignocellulosic biomass, owing to the poor use of xylose (Dien et al., 2003). Hence, metabolic engineering has been proposed.

E. coli has several advantages as a biocatalyst for ethanol production, including the ability to ferment a wide spectrum of sugars, no requirements for complex growth factors and prior industrial application (e.g. recombinant protein production) (Hahn-Hägerdal et al., 2007). Major disadvantages of using *E. coli* cultures are its narrow neutral pH growth range (ca.pH 6.0–8.0) and that ethanol is not a central product in *E. coli* (Hahn-Hägerdal et al., 2007).

K. oxytoca is an enteric bacterium found growing in pulp and paper streams (Dien et al., 2003), and capable of growing at pH 5.0 and temperatures of 35°C. *K. oxytoca* grows on a wide variety of sugars including hexoses, pentoses, cellobiose and cellotriose (Dien et al., 2003), making it especially appealing for cellulose fermentation. Simultaneous Saccharification and Fermentation (SSF) cultures with cellobiose-fermenting organisms require less cellulase addition for cellulose hydrolysis (Freer and Detroy, 1983). Using cellobiose-fermenting strains reduces the growth of some contaminants by eliminating glucose from the fermentation broth (Dien et al., 2003). Performances of *S. cerevisiae, Z. mobilis, K. oxytoca* and *E.* coli are compared in Figures 2.2a, 2.2b, 2.2c, 2.2d and Table I in appendix A in which species exhibiting both high

yields and productivities are preferred (e.g. recombinant *Z. mobilis* and *E. coli*) (H.G. Lawford and Rousseau, 1991).

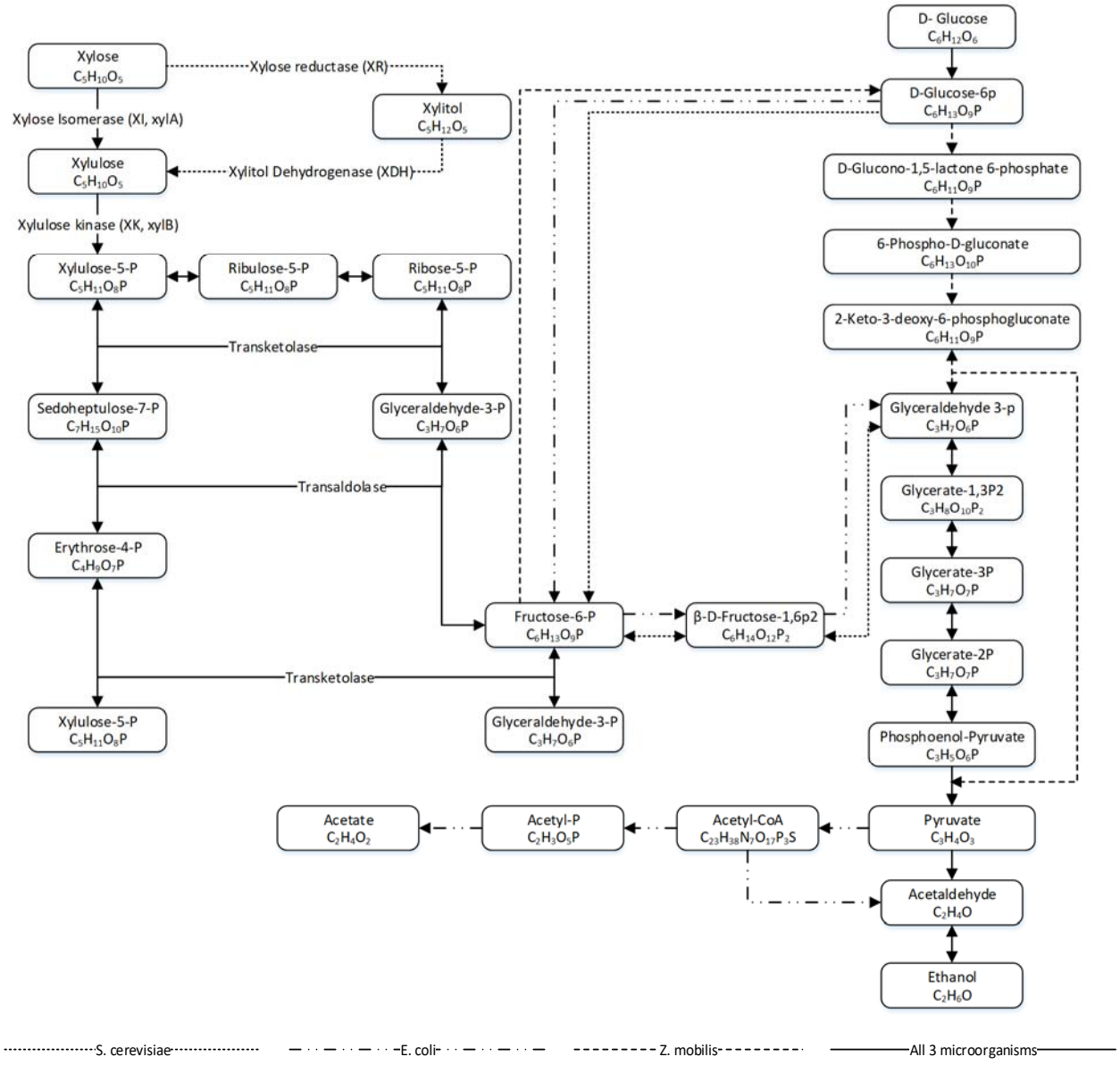


Figure 2.1.Metabolism in recombinant *S. cerevisiae*, *Z. mobilis*, and *E. coli* (Altintas et al., 2006; Karhumaa et al., 2005; Nikolaev, 2010). Dashed lines denote *Z. mobilis*, dotted lines denote *S. cerevisiae*, dashed-dotted lines denote *E. coli*, and solid lines denote all three microorganisms.

2A.2.1. Recombinant *S. cerevisiae*

The first strategy explored for construction of a xylose-fermenting *S. cerevisiae* was to insert the initial steps of the xylose catabolism pathway from *P. stipitis* in *S. cerevisiae*. By incorporating the xylose-fermenting enzymes xylose reductase (XR) encoded by *XYL1* and xylitol dehydrogenase (XDH) encoded by *XYL2* in the initial metabolism, xylose is converted to xylulose (B. Hahn-Hägerdal et al., 1994); xylulose

is then readily catabolized in *S. cerevisiae* (Figure 2.1). The XR of *P. stipitis* has the strongest NADH/NADPH dependent activity of all known xylose reductases (Amore et al., 1991; Takuma et al., 1991). Chen and Ho (Chen and Ho, 1993) and Ho et al. (Ho et al., 1998; Ho and Tsao, 1998) constructed recombinant *Saccharomyces* strains containing plasmid-borne xylose reductase (*XYL1* with *XYL1.1* (NADPH-dependent)) and xylitol dehydrogenase genes (*XYL2*) from *P. stipitis* and extra copies of the *S. cerevisiae* xylulokinase gene, however productivity of ethanol from xylose was low (0.01 g L^{-1} h^{-1} – 1.15 g L^{-1} h^{-1}) using normal inoculum size with ethanol yields varying between 0.09 g g^{-1} and 0.48 g g^{-1} (Ho and Tsao, 1998; Madhavan et al., 2009a; Walfridsson et al., 1996) (Figure 2.2a). The amount of XR protein and activity per gene copy in *S. cerevisiae* transformants was 20 times lower than in *P. stipitis* (Amore et al., 1991). Factors responsible for the poor performance of the recombinant *S. cerevisiae* are: (i) redox imbalance in the cell due to the lower affinity of XR for NADH compared to NADPH resulting in an accumulation of NADH produced by the NAD+ dependent XDH, and (ii) low utilization rate of pentose phosphate pathway metabolites especially by the enzymes transaldolase (TAL) and transketolase (TKL) (Dumsday et al., 1997). In order to overcome these metabolic restrictions, higher levels of XDH relative to XR are required to decrease the xylitol yield and increase fluxes towards ethanol formation (Hahn-Hägerdal et al., 2007). Additional vectors that overexpress yeast *TKL1* and *TAL1* genes were suggested (Dumsday et al., 1997). In order to improve performance, other yeast strains were investigated. Among those are the D-xylose-fermenting *Spathaspora* species (e.g. *Sp. passalidarum*, *Sp. arborariae*, *Sp. gorwiae* and *Sp. Hagerdaliae*), *Sp. passalidarum* as the best ethanol producer from pentoses under oxygen-limited or anaerobic conditions (Cadete and Rosa, 2018). Most xylose-fermenting microorganisms, such as *Pichia stipitis,* requires microaerobic conditions for ethanol production. However, *Sp. passalidarum* in both microaerobic (25 g L^{-1} ethanol after 24 h from 30 g L^{-1} glucose and 30 g L^{-1} xylose, 100% sugars conversion) and anaerobic condition (7.5 g L^{-1} ethanol after 24 h from 30 g L^{-1} glucose and 30 g L^{-1} xylose) gave high ethanol concentrations (Hou, 2012). As an example, the *Sp. passalidarum* CMUWF1–2 achieved ethanol yields of 0. 43, 0.40 and 0.20 g ethanol/g used xylose at 30 °C, 37 °C and 40 °C, respectively. In another student, under anaerobic xylose fermentation, *S. cerevisiae* TMB 3504, which expresses *XYL1.2* (both NADPH and NADH-dependent) from *Sp. passalidarum* revealed significantly higher ethanol yield and productivity based on cell dry weight (CDW) (0.40 g g_{CDW}^{-1} and 0.33 g g_{CDW}^{-1} h^{-1}) than *S. cerevisiae* TMB 3422, which expresses the *XYL1.1* (NADPH-dependent) gene from *P. stipitis* (0.34 g g_{CDW}^{-1} and 0.18 g g_{CDW}^{-1} h^{-1}) and eventually helped for the development of novel industrial pentose-fermenting strains (Cadete et al., 2016).

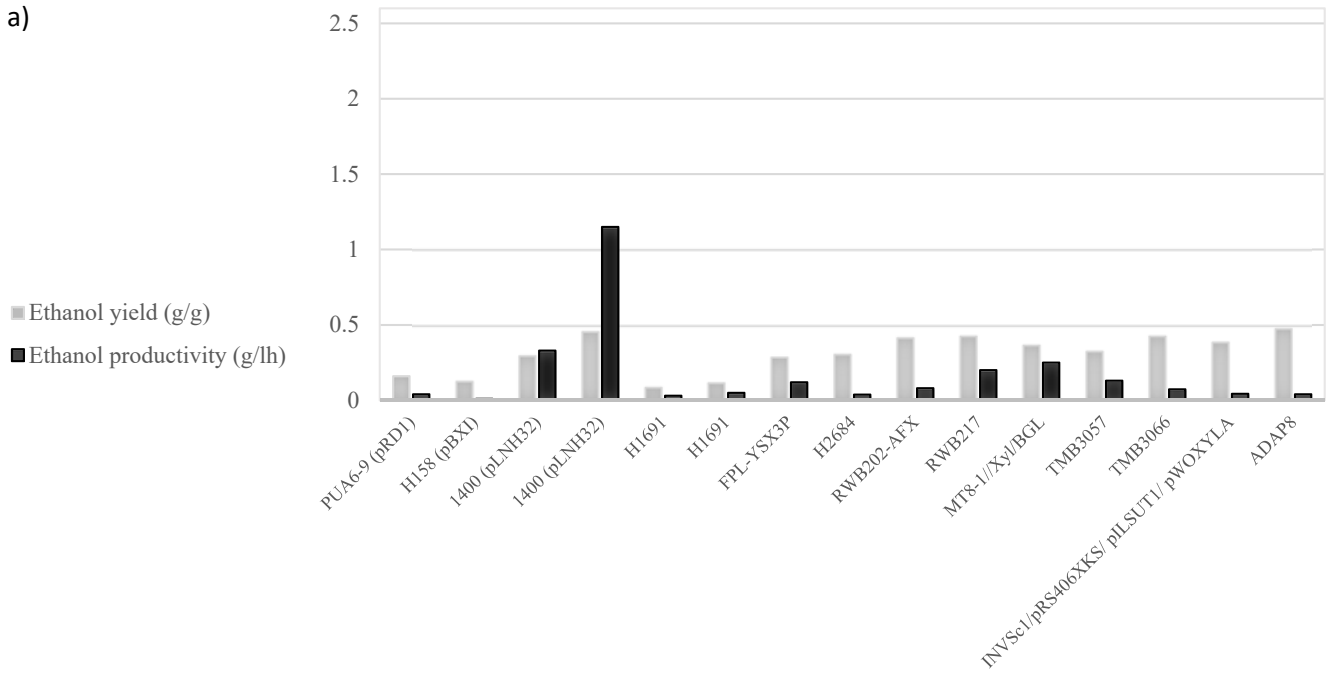
Another strategy reported is to engineer *S. cerevisiae* to produce xylose isomerase (XI or *xyl*A) as shown in Figure 2.1. XI is a bacterial enzyme in the pentose phosphate pathway that catalyses the reversible isomerization of D-xylose to D-xylulose without the need for cofactors. Researchers have sought to produce

a *S. cerevisiae* capable of converting xylose to ethanol (Amore et al., 1989; Walfridsson et al., 1996). This has met with limited success because of the differences in internal pH between bacteria and yeasts, incorrect folding of the enzyme and unsuitable post-translational modifications (Amore et al., 1989; Dumsday et al., 1997). The success of active XI expression in *S. cerevisiae* using *xylA* from *Thermus thermophiles* was attributed to the closer taxonomic similarity of *T. thermophilus* to *S. cerevisiae* (Walfridsson et al., 1996). Low activity of *xyl*A under fermentation conditions compatible with *S. cerevisiae* and poor transport of xylose across the yeast plasma membrane limited the utilization of xylose by the recombinant strains significantly (Madhavan et al., 2009a).

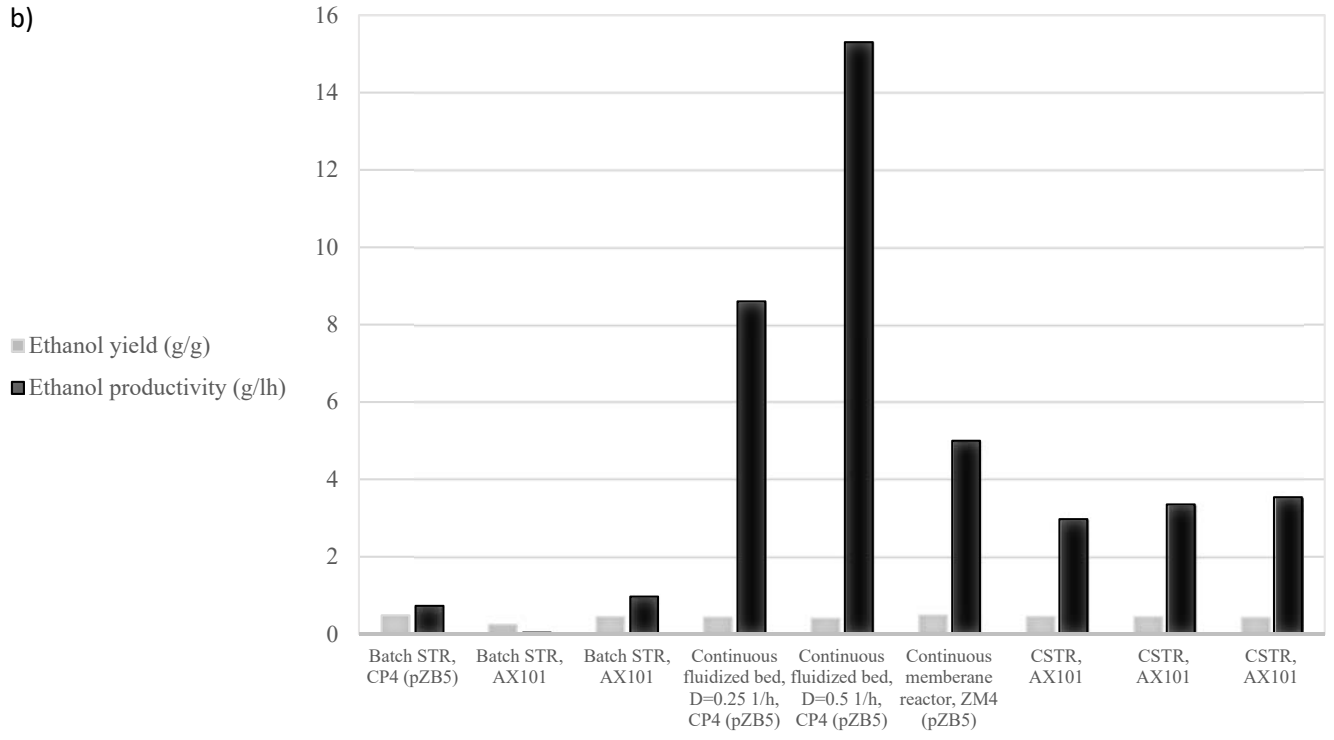
XI genes from the fungi *Orpinomyces* (Madhavan et al., 2009b) and *Piromyces* (Kuyper et al., 2003) have been expressed in *S. cerevisiae* at high levels, but the consumption of xylose remained slow. To overcome these issues, adaptation (Kuyper et al., 2005b; Madhavan et al., 2009a) or extensive genetic engineering (Kuyper et al., 2005a; Madhavan et al., 2009b) has been used to enhance the xylose utilization to ethanol. For example, XI genes from the anaerobic bacterium *Clostridium phytofermentans* (*CpXylA*) were successfully expressed in an industrial *S. cerevisiae* strain (Brat et al., 2009; Moysés et al., 2016) - low susceptibility to xylitol inhibition. Recombinant yeast expressing *Burkholderia cenocepacia xylA* does not accumulate xylitol and gave the best yield of 0.45 $g\ g^{-1}$ through XI genes using *S. cerevisiae* (BY4741) (de Figueiredo Vilela et al., 2015, 2013; Moysés et al., 2016). *S. cerevisiae* RWB218 overexpresses XI and non-oxidative pentose–phosphate pathway genes and has to date the fastest anaerobic xylose-fermenting engineered *S. cerevisiae* strain (Kuyper et al., 2005; van Maris et al., 2007). Performance of a batch fermentation of RWB 218 on wheat straw hydrolysate (50 $g\ L^{-1}$ glucose, 20 $g\ L^{-1}$ xylose, 6 $g\ L^{-1}$ arabinose and 6 $g\ L^{-1}$ of disaccharides) with 0.4 $g\ L^{-1}$ ammonium phosphate resulted in ethanol yield and productivity of 0.47 $g\ g^{-1}$, 1.5 $g\ L^{-1}\ h^{-1}$ (van Maris et al., 2007). Introducing the XI pathway rather than the XR and XDH pathways is key to eliminate xylitol production (Kuyper et al., 2003; Walfridsson et al., 1996).

Using an oxidative xylose catabolic pathway through Archae is a new strategy which it has not been used to engineer *S. cerevisiae* (Moysés et al., 2016). In this pathway, xylose is oxidized exclusively to the tricarboxylic acid cycle intermediate α-ketoglutarate through upon the action of xylose dehydrogenase, xylonate dehydratase, 2-keto-3-deoxyxylonate dehydratase, and α-ketoglutarate semialdehyde dehydrogenase (Johnsen et al., 2009; Moysés et al., 2016).

a)


2.5
2
1.5
1
0.5
0
Ethanol yield (g/g)
Ethanol productivity (g/lh)
PUA6-9 (pRD1)
H158 (pBXI)
1400 (pLNH32)
1400 (pLNH32)
H1691
H1691
FPL-YSX3P
H2684
RWB202-AFX
RWB217
MT8-1//Xyl/BGL
TMB3057
TMB3066
INVSc1/pRS406XKS/ pILSUT1/ pWOXYLA
ADAP8

b)


16
14
12
10
8
6
4
2
0
Ethanol yield (g/g)
Ethanol productivity (g/lh)
Batch STR, CP4 (pZB5)
Batch STR, AX101
Batch STR, AX101
Continuous fluidized bed, D=0.25 1/h, CP4 (pZB5)
Continuous fluidized bed, D=0.5 1/h, CP4 (pZB5)
Continuous memberane reactor, ZM4 (pZB5)
CSTR, AX101
CSTR, AX101
CSTR, AX101

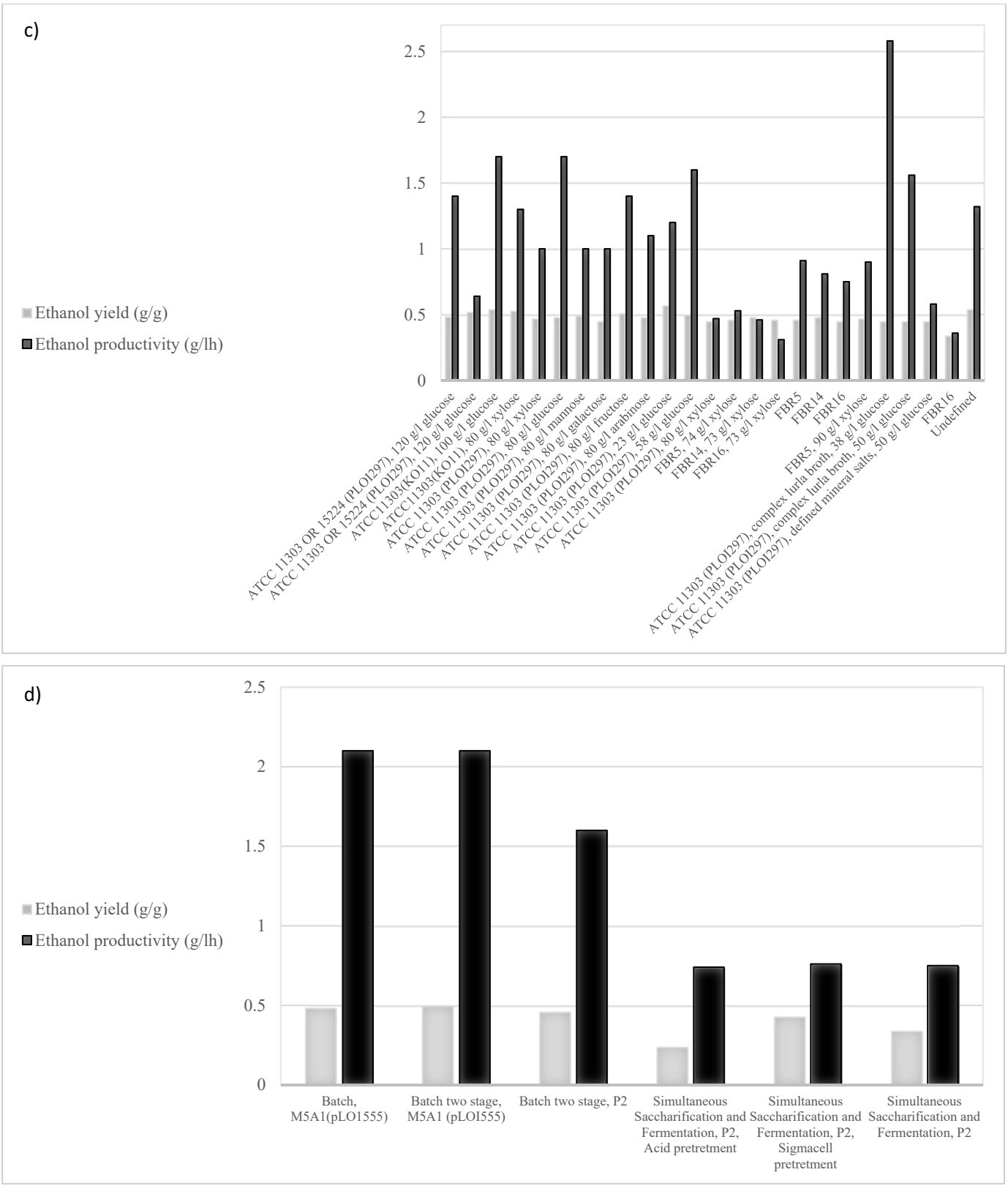


Figure 2.2.Ethanol productivity (g L^{-1} h^{-1}) and yield (g g^{-1}) of engineered (a) *S. cerevisiae* in batch anaerobic culture, (b) *Z. mobilis*, (c) *E. coli*, (d) *K. oxytoca* (Table I in appendix A)

2A.2.2. Recombinant *Z. mobilis*

To expand the substrate range, genes *xylA* (xylose isomerase) and *xylB* (xylulokinase) from either *Xanthomonas campestris* or *Klebsiella pneumoniae* were expressed in *Z. mobilis* (Feldmann et al., 1992); however, the strains remained unable to grow on xylose as the sole carbon source due to the absence of a complete pentose phosphate pathway (Feldmann et al., 1992). Zhang et al. (1995) inserted a functional xylose metabolic pathway into *Z. mobilis* strain ATCC 39676 through the introduction and expression of *E. coli* genes encoding xylose isomerase, xylulokinase, transaldolase and transketolase (Zhang et al., 1995). Xylose isomerase and xylulose kinase convert xylose into xylulose-5-phosphate, a significant intermediate in the pentose phosphate pathway (Dien et al., 2003). Xylulose-5-phosphate is converted to intermediates of the ED pathway by the transketolase and transaldolase (Figure 2.1). The genes were expressed on a plasmid using either the enolase or glyceraldhyde-3-phosphate dehydrogenase promoters from *Z. mobilis*. This pentose metabolism pathway converts xylose to central intermediates of the Enter-Doudoroff pathway and enables *Z. mobilis* to ferment xylose to ethanol. Deanda et al. (1996) reported the construction of a *Z. mobilis* strain capable of fermenting both xylose and arabinose (Deanda et al., 1996). The strain contains seven plasmid-borne genes encoding xylose-fermenting, arabinose-fermenting, and pentose phosphate pathway enzymes. Xylose was utilized much more slowly than glucose, and arabinose was utilized much more slowly than xylose due to the low affinity of the glucose permease transporter for both xylose and arabinose (Parker et al., 1995). Strain ATCC 39676 was identified as a good candidate for lignocellulosic biomass conversion based on its growth in hydrolysate prepared from yellow poplar wood in both Simultaneous Saccharification and Fermentation (SSF) and Separate Hydrolysis and Fermentation (SHF) (McMillan et al., 1999; Zhang et al., 1995) setups. This strain is, however, highly sensitive to microbial inhibitors commonly associated with hydrolysates, especially acetic acid (Lawford and Rousseau, 1999). Lawford et al. (1999) successfully adapted strain 39676 to tolerate higher concentrations of acetic acid, as well as other inhibitors, by adapting the strain in higher concentrations of hydrolysate through selective pressure (Lawford and Rousseau, 1999). The results for strain 39676 in McMillan et al. (1999) were classified as uneconomical in Simultaneous Saccharification and Fermentation (SSF) showing only 54 % xylose conversion in 7 days (McMillan et al., 1999).

Zhang and collaborators continued to make improvements to their *Z. mobilis* strain AX101 (parental strain ATCC 39676) in fermenting arabinose and xylose (Mohagheghi et al., 2002). The strain carries the seven essential recombinant genes integrated as part of its chromosomal DNA (Mohagheghi et al., 2002). Cultures of AX101, transferred for 160 generations on glucose, retained the ability to ferment both arabinose and xylose, demonstrating the genetic stability of the inserted genes (Dien et al., 2003). Despite improvements in stability, AX101 still ferments arabinose more slowly than xylose, with arabinose fermentations often incomplete (Lawford and Rousseau, 2002; Mohagheghi et al., 2002). The major shortcoming of AX101

cultures is their low tolerance for acetic acid, especially in the presence of ethanol and fermenting sugar mixtures. Acetic acid is commonly found in hydrolysates and originates from acetyl side-chain groups of the hemicellulose. Only 50% of the xylose (initial concentration 30 g L^{-1}) was consumed when both acetic acid (2.5 g L^{-1}, pH 5.5) and ethanol (30 g L^{-1}) were added to the medium (Lawford and Rousseau, 2002). Mohagheghi et al. (2002) examined acetic acid tolerance for AX101 growing in a continuous culture on a sugar mixture and the culture was challenged with increasing levels of acetic acid (Mohagheghi et al., 2002). Residual xylose began to accumulate once the acetic acid concentration exceeded 4.5 g L^{-1}. There are two ways to circumvent this problem: Adapting the strain to acetic acid, or removing acetic acid from the hydrolysate prior to fermentation (Mohagheghi et al., 2002).

Joachimsthal and Rogers (Joachimsthal and Rogers, 2000) transformed pZB5 into their best *Z. mobilis* ethanol-producing strain ZM4 (ATCC 31821). The resulting strain ZM4 (pZB5) demonstrated high ethanol yield (0.5 g g^{-1}), tolerance (67 g L^{-1}) and productivity (5 g L^{-1} h^{-1}) on glucose/xylose mixtures (Joachimsthal and Rogers, 2000) (Figure 2.2b).

2A.2.3. Recombinant *E. coli*

Producing ethanol from pyruvate using pyruvate formate lyase (*PFL*) is unbalanced in *E. coli* because one NADH, H+ is generated for each pyruvate made from sugars, and two NADH, H+ are required for converting pyruvate into ethanol. Hence, acetic and succinic acid are produced to balance this requirement. However, pyruvate decarboxylase (*PDC*) expression in *E. coli* would cause it to produce only ethanol like *Z. mobilis* and *S. cerevisiae* (Kazuyoshi Ohta et al., 1991). Early attempts to modify *E. coli* to enhance ethanol production were unsuccessful. Pyruvate resulting from glycolysis is converted to acetaldehyde and CO_2 through the ED or EMP pathway; acetaldehyde is then converted to ethanol by an alcohol dehydrogenase *(ADH)*. In *E. coli* the native *ADH* expression is low resulting in low ethanol production (Reynen and Sahm, 1988).

The construction of *E. coli* strains to produce ethanol selectively by Ingram et al. (1987) was an early successful application of metabolic engineering (Ingram et al., 1987). Ingram and co-workers modified the metabolism of *E. coli* and other bacteria by incorporating the *PDC* and *ADHII* genes from *Z. mobilis*. With *E. coli* TC4, production of ethanol was dominant and production of succinate, acetate and lactate were greatly reduced (Ingram et al., 1987).

The alcohol dehydrogenase (*ADHII*) and pyruvate decarboxylase (*PDC*) genes from *Z. mobilis* were cloned under the control of a single promoter from the *PET* operon to produce ethanol (Ingram et al., 1987). The *PET* operon was introduced into the chromosome of *E. coli*, and expression of the *PET* genes resulted in high ethanol production with good selectivity (K. Ohta et al., 1991; Kazuyoshi Ohta et al., 1991; Wood and Ingram, 1992). Expressing the *PET* operon on a higher copy number plasmid (pLOI297) gave higher

ethanol productivity than for the lower copy number plasmid (pLOI555) (K. Ohta et al., 1991). To improve the stability of ethanol production in *E. coli*, a series of novel ethanologenic *E. coli* strains were developed, in particular transforming the *PET* operon on pLOI297 (Alterthum and Ingram, 1989). After testing a number of *E. coli* strains as potential hosts for the *PET* plasmids, *E. coli* strain ATCC 11303 was chosen because of an acceptable level of environmental hardiness (e.g., ethanol tolerance), plasmid stability in non-selective medium, and optimal ethanol production characteristics (Alterthum and Ingram, 1989). Lawford and Rousseau (H G Lawford and Rousseau, 1993; Hugh G Lawford and Rousseau, 1993; Lawford and Rousseau, 1992; H.G. Lawford and Rousseau, 1991) tested the performance of *E. coli* ATCC 11303 (pLOI297) on synthetic and native hemicellulose hydrolysates. *E. coli* ATCC 11303 performed well when grown on hardwood hemicellulose hydrolysate (0.57 g g^{-1} using batch STR and 2.58 g L^{-1} h^{-1} using CSTR) (H.G. Lawford and Rousseau, 1991) (Figure 2.2c), but poorly in pulp mill softwood and hardwood spent sulphite liquor (0.47 g g^{-1} and 0.55 g L^{-1} h^{-1} using batch STR and $Ca(OH)_2$ treated) (H G Lawford and Rousseau, 1993), newsprint prehydrolysate (0.38 g g^{-1} and 0.25 g L^{-1} h^{-1} using batch STR) (Hugh G Lawford and Rousseau, 1993) and corn residue hydrolysate (Lawford and Rousseau, 1992). Increases in the ionic strength of the medium reduced the rate of ethanol production from xylose, with no effect on ethanol yield (Dumsday et al., 1997). Ohta et al. (1991) integrated the artificial operon containing the fermentative genes (*PDC*, *ADHII* and chloramphenicol resistance) into the chromosome of ATCC 11303 (pyruvate formate lyase (*PFL*)) to improve the genetic stability of the ethanologenic strain and prevent acetate formation from pyruvate (Kazuyoshi Ohta et al., 1991).

E. coli strain KO11, containing the same *Z. mobilis* pyruvate decarboxylase (*PDC*) and alcohol dehydrogenase II (*ADHII*) genes integrated into ATCC 11303, has been evaluated for fermentation of hemicellulose hydrolysates from Pinus wood, sugarcane bagasse and corn stover (Barbosa et al., 1992). *E. coli* KO11 grows faster on xylose-containing medium than its parent strain ATCC 11303 (Senthilkumar and Gunasekaran, 2005). *E. coli* KO11 is the only microorganism reported to ferment arabinose into ethanol efficiently (Dien et al., 1996). Beall and Ingram (Beall and Ingram, 1992) tested ethanol production by *E. coli* KO11 using hydrolysed corn residue as the substrate and achieved 100% conversion efficiency. Hahn-Hägerdal et al. (1994) tested the performance of KO11 with corn-cob hydrolysate and found that the strain required detoxification (over-liming with $Ca(OH)_2$) of the hydrolysate to achieve maximum productivity (B Hahn-Hägerdal et al., 1994). Lawford and Rousseau (Lawford and Rousseau, 1995) have raised concerns regarding the genetic stability of both the plasmid-harbouring and chromosomally integrated *E. coli* strains. When grown in serial batch culture for 12 generations (without added antibiotics) both ATCC 11303 (pLOI297) and KO11 exhibited a loss of ethanologenicity on glucose and mannose (Lawford and Rousseau, 1995). When the strains were grown in continuous culture, the apparent genetic instability again resulted in a reduction in ethanol production and increasing lactate yields, even using feed supplemented with

antibiotics (Lawford and Rousseau, 1996). The instability was not apparent in earlier continuous culture studies by the same investigators (Hugh G Lawford and Rousseau, 1991) using the plasmid-containing strain. In addition, researchers in several laboratories (Dien et al., 1997; Dumsday et al., 1997; B Hahn-Hägerdal et al., 1994) have been able to replicate Ingram's results (Alterthum and Ingram, 1989) showing that KO11 gave stable ethanol production with high yields in continuous culture when grown on glucose only, but not on xylose or glucose/xylose mixtures.

A problem identified with the recombinant *E. coli* strains was catabolite repression of other sugars in the presence of glucose (Padukone et al., 1995). Variants of these strains have been constructed with mutants not repressed by glucose, carrying a mutation in their phosphoenol pyruvate-glucose phosphor transferase system (ptsG) (Nichols et al., 2001). These strains can utilize arabinose, glucose and xylose simultaneously. However, ptsG- disables active glucose transport in *E. coli*.

2A.2.4. Recombinant *Klebsiella oxytoca*

K. oxytoca ferments glucose to a variety of organic acids, neutral products and ethanol through the pyruvate formate lyase (*PFL*) pathway (Senthilkumar and Gunasekaran, 2005).

Many studies have developed a series of recombinant *K. oxytoca* strains for efficient fermentation of pentose and hexose sugars to ethanol (Barbosa et al., 1992; Burchhardt and Ingram, 1992; Ingram et al., 1987; Wood and Ingram, 1992). In addition to *E. coli*, Ingram and his co-workers modified the *K. oxytoca* and *E. chrysanthemi* with the *PET* operon (Brooks and Ingram, 1995; Burchhardt and Ingram, 1992; Doran et al., 2000; K. Ohta et al., 1991). The resulting strains, however, had lower ethanol yields compared to *E. coli*. Expressing the *PET* operon on a lower copy number plasmid (pLOI555) gave higher ethanol productivity than for the higher copy number plasmid (pLOI297), in contrast to result observed for *E. coli* strains (K. Ohta et al., 1991). Strain M5A1 (pLOI555) fermented xylose as rapidly as glucose and twice as fast as *E. coli* strain KO11 (Senthilkumar and Gunasekaran, 2005). The *PET* operon was genetically stabilized in *K. oxytoca* M5A1 to create strain P2 by integrating the operon along with a chloramphenicol acetyl transferase (*cat*) marker at the site of the chromosomal pyruvate formate lyase (*PFL*) gene (Wood and Ingram, 1992). Strain P2 was tested successfully on various feedstocks including mixed office paper obtaining ethanol yields of 0.426 g g^{-1} (Brooks and Ingram, 1995), sugarcane bagasse with ethanol yields of 0.43 g g^{-1} (Doran et al., 2000), corn fiber with the yield of 0.35 g g^{-1} (Moniruzzaman et al., 1996), and sugar beet pulp with ethanol yields of 0.12 g g^{-1} (Doran et al., 2000) (Figure 2.2d). Golias et al. (2002) compared strain P2, *K. marxianus*, and *Z. mobilis* in an SSF process for fermenting microcrystalline cellulose (Sigmacell 50) (Golias et al., 2002). They determined that P2 fermentations were 25–50% faster than those with *K. marxianus* or *Z. mobilis*, but the final ethanol concentration was limited to 35.7 g L^{-1} owing to maximum ethanol tolerance, compared to *Z. mobilis* with 40.7 g L^{-1}, and *K. marxianus* with 37.5

g L^{-1}. Doran et al. (2000) compared strains *K. oxytoca* P2, *E. coli* KO11 and *E. chrysanthemi* EC16 (pLOI555) for the production of ethanol from sugar beet pulp (106 g L^{-1}) with simultaneous enzymatic hydrolysis of pectin and cellulose (Doran et al., 2000). The *E. coli* KO11 fermentations produced 40% more ethanol than the others.

2A.2.5. Fermentation performance of recombinant cells

Continuous fluidized beds gave the highest productivity using *Z. mobilis* CP4 (pZB5) (15.3 g L^{-1} h^{-1} with dilution rate of 0.5 h^{-1} and 8.6 g L^{-1} h^{-1} with dilution rate of 0.25 h^{-1}) (Krishnan et al., 2000), then membrane with cell recycle using *Z. mobilis* ZM4 (pZB5) (5 g L^{-1} h^{-1}) (Joachimsthal and Rogers, 2000), and after that CSTR using *Z. mobilis* AX101 (3.54 g L^{-1} h^{-1}) (Lawford and Rousseau, 2002) (Figure 2.2b). ATCC 11303 (KO11) (1.7 g L^{-1} h^{-1}) is one of the successful engineered *E. coli* that grew faster than ATCC 11303 (pLOI297) (1.2 g L^{-1} h^{-1}) and converted arabinose efficiently (Beall and Ingram, 1992; Senthilkumar and Gunasekaran, 2005); however, ATCC11303 (PLO1297) with 0.57 g g^{-1} (H.G. Lawford and Rousseau, 1991) gave higher ethanol yields than ATCC11303 (KO11) with 0.54 g g^{-1} (De Bari et al., 2004) using batch STR (Figure 2.2c). Ethanol yield (0.09 g g^{-1}-0.48 g g^{-1}) and productivity (0.01 g L^{-1} h^{-1} – 1.15 g L^{-1} h^{-1}) is still low with recombinant *S. cerevisiae* (Chen and Ho, 1993; Ho and Tsao, 1998) (Figure 2.2a), and further developments will be required for this approach to reach commercial reality. Engineered *K. oxytoca* (M5A1 or P2) with average productivity of 2.1 g L^{-1} h^{-1}, fermented xylose faster than *E. coli* with average productivity of 1.7 g L^{-1} h^{-1} (Figures 2.2c and 2.2d), but the final ethanol concentration was less than *E. coli* (Senthilkumar and Gunasekaran, 2005). A major shortcoming of each of these engineered strains is that the genes for xylose utilization are carried on a plasmid. Therefore, the feed medium for continuous culture was supplemented with antibiotic (tetracycline) to ensure plasmid maintenance (Bothast et al., 1999).

For glucose conversion to ethanol production, there are industrial strains that account for 70% of all ethanol production in Brazil such as PE2, CAT1, FT858L and Fermel (selected by Fermentec), BG1 and SA1 (selected by CTC) (Lopes et al., 2016). While, there are some progress for fermenting both glucose and xylose to bioethanol in pilot plant size, less has been accomplished in industrial scale. Currently, engineered *S. cerevisiae* strains were suggested for industrial application such as F12 (*S. cerevisiae* F HIS3: YIploxZEO overexpressing XR, XDH, and XK) with ethanol yield of 0.26 g g^{-1} from 50 g L^{-1} xylose (Sonderegger et al., 2004), A4 (*S. cerevisiae* A HIS3: YIploxZEO (industrial, polyploid strain) overexpressing XR, XDH, and XK) with ethanol yield of 0.27 g g^{-1} from 50 g L^{-1} xylose and 50 g L^{-1} glucose (Zaldivar et al., 2002), evolved A4 population (evolved population obtained through evolutionary engineering of A4) with ethanol yield of 0.24 g g^{-1} from 50 g L^{-1} xylose (Sonderegger et al., 2004), TMB 3399 (*S. cerevisiae* USM21 HIS3: YIpXR/XDH/XK (industrial, polyploid strain) overexpressing XR, XDH, and XK) with ethanol yield of 0.21 g g^{-1} from 50 g L^{-1} xylose, and TMB 3400 (isolated after

mutagenesis and selection of TMB 3399) with ethanol yield of 0.25 g g^{-1} from 50 g L^{-1} xylose (Wahlbom et al., 2003) and ethanol yield of 0.34 g g^{-1} from 50 g L^{-1} xylose (Karhumaa et al., 2007). However, the performances of these strains are yet to be reached in industrial scale. The engineered ethanologenic bacteria that currently show the most promise for industrial exploitation are *E. coli*, *K. oxytoca* and *Z. mobilis* (Senthilkumar and Gunasekaran, 2005). However, industrial strains are generally selected for optimal performance under industrial conditions, i.e. to show tolerance to hydrolysates, to have higher inhibitor tolerance and to show better stability than laboratory strains (Matsushika et al., 2009). However, industrial strains tend to be polyploids in nature and therefore have limitations in the regulation of metabolic pathways. Further intensive studies are thus required to develop engineered strains that are capable of efficiently fermenting all sugars including D-xylose found in lignocellulosic hydrolysates to ethanol at an industrial scale (Matsushika et al., 2009).

2A.3. Co-culturing

The other approach is to co-culture strains with complementary metabolic pathways, creating a mixed metabolism. Among major studies of co-culture, most important are co-culture of *P. stipitis* & *S. cerevisiae*, *P. stipitis* & *S. diastaticus*, *P. stipitis* & *K. marxianus*, *P. stipitis* & *Z. mobilis*, and *K. marxianus* & *S. cerevisiae* which are considered here. It can be seen (Figure 2.3; Table II in appendix A) that the overall ethanol yield of different co-culture systems ranged from 0.25 g g^{-1} to 0.5 g g^{-1} (Fu et al., 2009; M. Taniguchi et al., 1997; Taniguchi and Tanaka, 2004), with ethanol productivity from 0.11 g L^{-1} h^{-1} (Wan et al., 2012) to 4.3 g L^{-1} h^{-1} (J.M. Laplace et al., 1993).

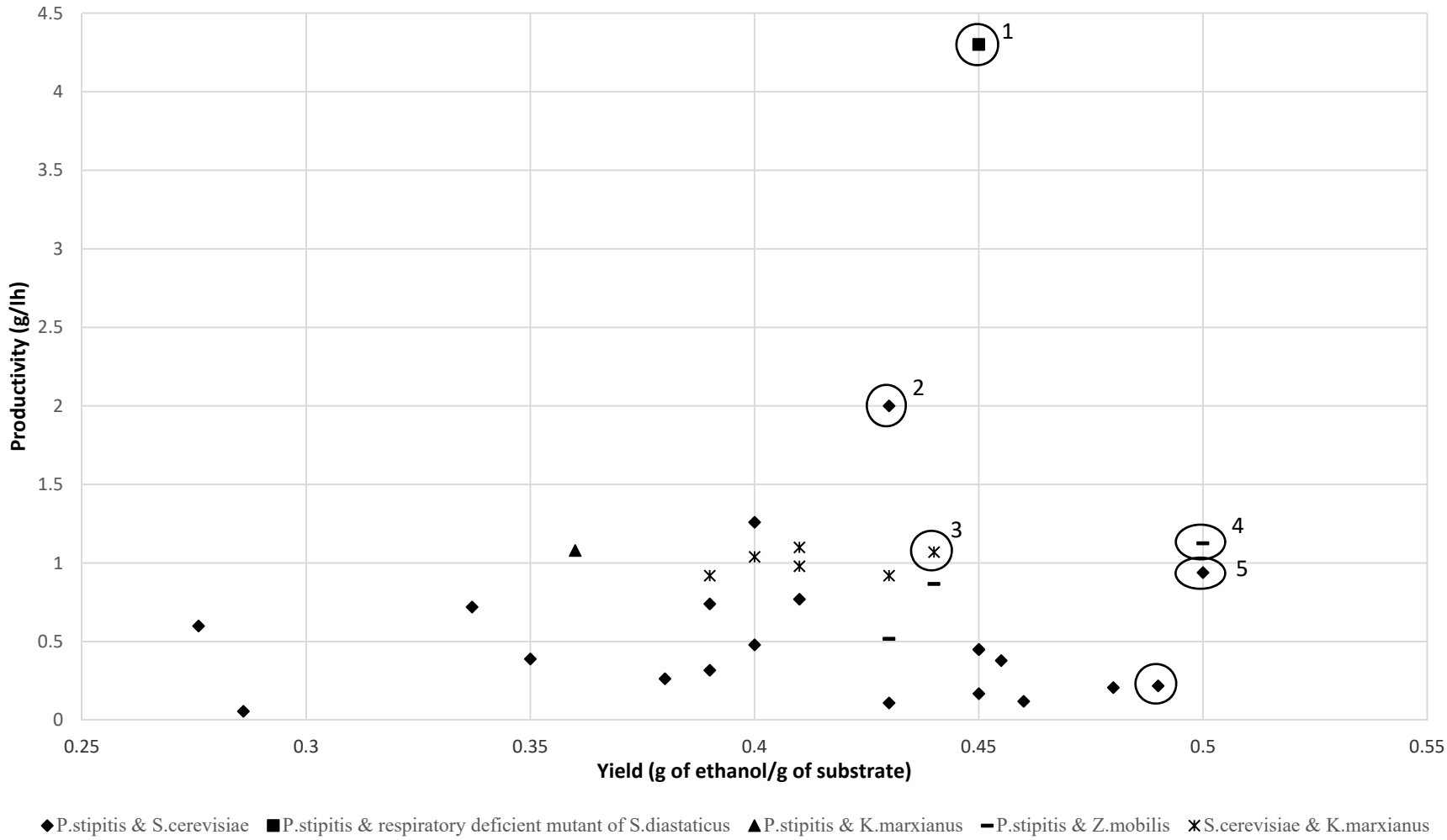


Figure 2.3.Ethanol productivity (g L^{-1} h^{-1}) of different co-culture vs yield (g g^{-1}) (Table II in appendix A)

2A.3.1. Co-culture of *P. stipitis* & *S. cerevisiae*

The co-culture process of *P. stipitis* mutants with native strains of *S. cerevisiae* using glucose and xylose for bioethanol production yields 0.28 to 0.40 g-ethanol/g-substrate compared to growth in glucose alone of 0.38 to 0.45 g-ethanol/g-substrate (Kordowska-Wiater and Targoński, 2002). In co-consumption, *S. cerevisiae* and *P. stipitis* showed a five-fold increase in the volumetric ethanol productivity compared to the respective mono-cultures (Gutiérrez-Rivera et al., 2011). In a co-culture of *S. cerevisiae* and *P. stipitis*, it was difficult to control q_{O2} at an optimum value for xylose fermentation by *P. stipitis* because oxygen was consumed primarily by *S. cerevisiae* (M. Taniguchi et al., 1997). However, in a co-culture of *P. stipitis* and the respiratory-deficient mutant of *S. cerevisiae*, the optimum q_{O2} for xylose fermentation by *P. stipitis* was successfully maintained (14.3 mg g-cell^{-1} h^{-1}) due to the lower oxygen affinity and utilization from the mutant strain (M. Taniguchi et al., 1997). They achieved complete sugar utilization with ethanol productivity of 0.94 g L^{-1} h^{-1} and ethanol yield of 0.50 g g^{-1}. Taniguchi et al. (1997) controlled the average specific oxygen uptake rate at 66.7 mg g-cell^{-1} h^{-1} for glucose consumption and then controlled at 14.3 mg g-cell^{-1} h^{-1} for xylose consumption (Masayuki Taniguchi et al., 1997). The co-culture of *P. stipitis* and respiratory deficient mutant of *S. cerevisiae* was tested at two different temperatures, obtaining ethanol yields (and volumetric productivity) of 0.5 g g^{-1} (0.94 g L^{-1} h^{-1}) at 30 °C compared to 0.38 g g^{-1} (0.264 g L^{-1} h^{-1}) at 28 °C (Kordowska-Wiater and Targonski, 2001; M. Taniguchi et al., 1997). Chandel and his co-workers (Chandel et al., 2011) were able to obtain noticeable levels of ethanol in mono and co-culture fermentation using *P. stipitis* and *S. cerevisiae* after detoxification of substrate to remove acidic hydrolysate (1.5%(v/v) H_2SO_4) with $Ca(OH)_2$ overliming. Gutiérrez-Rivera et al. (2011) showed that the oxygen present in the glucose/xylose mixture promotes complete sugar consumption by *P. stipitis* resulting in improved ethanol production (Gutiérrez-Rivera et al., 2011). However, in co-cultures with *S. cerevisiae* under aerobic conditions, a 20.4 % xylose residue was caused by oxygen limitation and ethanol inhibition by *P. stipitis.* Wan et al. (2012) tested consumption of glucose and xylose for bioethanol production from non-detoxified lignocellulosic hydrolysates to eliminate costs from detoxification (Wan et al., 2012). They successfully fermented xylose/glucose mixtures to ethanol concentrations of 27.4 g L^{-1}, with ethanol productivity of 0.29 g L^{-1} h^{-1}, and an ethanol yield of 0.43 g g^{-1}, in 96 h fermentation with furfural and HMF. Dhabhai et al. (2013) reported a co-culture of immobilized *S. cerevisiae* and suspended *P. stipitis* exhibiting a tenfold increase in reaction rates compared with a co-culture of free *S. cerevisiae* and free *P. stipitis* (Dhabhai et al., 2013). Grootjen et al. (1991) proved that for complete conversion of a glucose/ xylose mixture, co-immobilization is the most promising solution (Grootjen et al., 1991a). With co-immobilized cells, the glucose concentration in the centre of the beads approaches zero due to limitations in diffusion making xylose conversion possible. However, for complete conversion of the sugars, a high initial *P. stipitis*

concentration must be used because *P. stipitis* does not grow when co-immobilized with *S. cerevisiae*. De Bari et al. (2004) utilized 99.5 % of the 42 g L^{-1} glucose and 12 g L^{-1} xylose in the lignocellulosic hydrolysate within 24 h using co-immobilized of *S. cerevisiae* and *P. stipitis* giving ethanol yields of 0.396 g g^{-1} and productivity of 0.89 g L^{-1} h^{-1} (De Bari et al., 2004). De Bari et al. (2013) later indicated that the use of *P. stipitis* and *S. cerevisiae* in free co-culture resulted in higher volumetric ethanol productivity (0.6 g L^{-1} h^{-1}) than single cultures of *P. stipitis* with either free (0.3 g L^{-1} h^{-1}) or immobilized (0.25 g L^{-1} h^{-1}) from 60 g L^{-1} substrate (30 g L^{-1} xylose and 30 g L^{-1} glucose) (De Bari et al., 2013).

2A.3.2. Co-culture of *P. stipitis* & *S. diastaticus*

Laplace et al. (1993) tested *P. stipitis* and respiratory deficient mutant of *S. diastaticus* in a continuous process achieving 94% conversion of a glucose/xylose mixture (50 g L^{-1}), high ethanol productivity (4.3 g L^{-1} h^{-1}) and yield (0.45 g g^{-1}) (J.M. Laplace et al., 1993). Hanly and Henson (2013) also identified this co-culture as a promising system for microaerobic ethanol production (Hanly and Henson, 2013). They developed a dynamic flux balance model describing the co-culture metabolism and validated the model predictions with batch experiments. This demonstrates the suitability of the dynamic co-culture metabolic model for guiding the process and metabolic engineering aimed at increasing microaerobic ethanol production from glucose/xylose mixtures.

2A.3.3. Co-culture of *P. stipitis* & *K. marxianus*

Hamidimotlagh et al. (2007) studied the co-culture of *K. maxianus* and *P. stipitis* along with co-culture of *P. stipitis* and *S. cerevisiae* for bioethanol production from mixed sugars (Hamidimotlagh et al., 2007). The *P. stipitis* and *K. marxianus* co-culture showed a high ethanol productivity of 1.08 g L^{-1} h^{-1} compared to 0.77 g L^{-1} h^{-1} in a co-culture of *P. stipitis* and *S. cerevisiae*. However, the ethanol yield was 0.36 g g^{-1} compared to 0.41 g g^{-1} in the co-culture of *P. stipitis* and *S. cerevisiae*. This is attributed to the higher ethanol tolerance and productivity of *K. marxianus* to ferment hexoses to ethanol than with *P. stipitis*. *P. stipitis,* however, showed higher metabolic flux towards ethanol and resulted in higher ethanol yields than *K. marxianus*.

2A.3.4. Co-culture of *P. stipitis* & *Z. mobilis*

Fu et al. (2009) studied different fermentation schemes of free co-culture and immobilized *Z. mobilis* with suspended *P. stipitis* in batches (Fu et al., 2009). Free cell co-culture of *P. stipitis* and *Z. mobilis* failed to entirely convert xylose. Consumption of sugar varied between 87% and 91% based on changing aeration levels between 50 and 100 cm^3/min. In batch fermentations and shake flask experiments *P. stipitis* had low ethanol tolerance and *Z. mobilis* had rapid production (0.518 g L^{-1} h^{-1}, 0.43 g g^{-1}) of ethanol, leading to the suppression of xylose fermentation using *P. stipitis*. None of the free cell co-culture fermentations

completed xylose consumption. The entrapment of *Z. mobilis* cells in calcium alginate gel beads resulted in complete xylose fermentation (100% sugar consumption) when co-cultured with 50% (v_{cells}/v_{ferm}) inoculum sized of *P. stipitis* free cells (ethanol productivity: 0.868 g L^{-1} h^{-1}; ethanol yield: 0.44 g g^{-1}) (Fu et al., 2009). Fu and his co-workers then tested immobilized *Z. mobilis* with suspended *P. stipitis* in a modified batch fermentor fitted with a draining sieve to remove the immobilized *Z. mobilis* beads from the medium after the completion of glucose consumption (Fu et al., 2009). This showed high ethanol yield (0.50 g g^{-1}) and high productivity (1.126 g L^{-1} h^{-1}).

2A.3.5. Co-culture of *K. marxianus* & *S. cerevisiae*

Eiadpum et al. (2012) studied free-suspension, ALM (alginate-loofa-matrix-immobilized cells) and TSC (thin-shell silk cocoon-immobilized cells) in co-culturing of *K. marxianus* and *S. cerevisiae* (Eiadpum et al., 2012). This indicated that ethanol production improved between temperatures of 33°C and 45°C for co-culture of free cells, ALM co-immobilized cells and TSC co-immobilized cells. The co-culture of *S. cerevisiae* and *K. marxianus* in thin-shell silk cocoon-immobilized cells offered best results in ethanol yield and productivity when grown at 40 °C (0.44 g g^{-1}, 1.07 g L^{-1} h^{-1}).

2A.3.6. Fermentation performance of co-culture

Employing the combination of *P. stipitis* and respiratory-deficient mutant *S. cerevisiae* in batch fermentation, Taniguchi et al. (M. Taniguchi et al., 1997) successfully achieved complete sugar utilization, with ethanol yield of 0.50 g g^{-1} and ethanol productivity of 0.94 g L^{-1} h^{-1} (Figure 2.3). Fu et al. (Fu et al., 2009) converted glucose and xylose to ethanol using co-culture of immobilized *Z. mobilis* and suspended *P. stipitis* with high ethanol yield (0.50 g g^{-1}) and high productivity (1.126 g L^{-1} h^{-1}) in their modified batch reactor (Figure 2.3). Fu and his colleagues (Fu et al., 2009) also compared free cells and immobilized *Z. mobilis* with suspended *P. stipitis* in normal batch reactors. The productivity and yield of immobilized *Z. mobilis* with suspended *P. stipitis* (0.868 g L^{-1} h^{-1}, 0.44 g g^{-1}) were better than for free cells (0.518 g L^{-1} h^{-1}, 0.43 g g^{-1}). Laplace et al. (J.M. Laplace et al., 1993) tested *P. stipitis* and respiratory deficient mutant of *S. diastaticus* in a continuous process with high ethanol productivity (4.3 g L^{-1} h^{-1}) and yield (0.45 g g^{-1}), but these two microorganisms have not been studied much. Respiratory-deficient mutant of *S. cerevisiae* paired with yeast is the most common combination in co-culture systems because of better fermentation performance arising from the low oxygen consumption of the mutant yeast strain (Hanly and Henson, 2013; Kordowska-Wiater and Targonski, 2001; Kordowska-Wiater and Targoński, 2002; M. Taniguchi et al., 1997; Taniguchi and Tanaka, 2004).

To date, dilute sulfuric acid hydrolysis is one of the most promising pretreatment method and is extensively employed in industry (Srilekha Yadav et al., 2011). The concentrated and detoxified hydrolysate was

fermented with a co-culture of *S. cerevisiae* and *P. stipitis,* leading to the conversion of both hexoses and pentoses in the hydrolysate with higher ethanol yields (0.49 g g^{-1}) than that achieved with monocultures of *S. cerevisiae* (0.3 g g^{-1}) (Srilekha Yadav et al., 2011). Co-culture technique proves to be a useful technology such as the co-culture of *Candida shehatae* and *Saccharomyces cerevisiae* by which a mixture of hexose and pentose was converted with yields of 0.45 g g^{-1} from autohydrolysate and 0.37 g g^{-1} from acid hydrolysate (Saini et al., 2015). Several lab and pilot scale as well as demonstration studies for ethanol production from agro-wastes have been reported successfully however still there exists a huge gap between the projected and actual bioethanol production at industrial levels (Saini et al., 2015).

2A.4. Sequential batch culture

Limitations in co-culture are: catabolite repression of xylose by glucose, diauxic shift with consumption of xylose and ethanol after glucose depletion, low xylose conversion by xylose-fermenting microorganisms, low ethanol tolerance of xylose-fermenting microorganisms in batch and fed-batch cultures, oxygen competition between strains, and little rigorous modelling to describe the dynamics of the system quantitatively. To remove the limitations of co-culture, sequential culture was tested. Sequential batch processes have been tested most to date, with a best ethanol productivity of 2.32 g L^{-1} h^{-1} (Fu and Peiris, 2007) and yield of 0.47 g g^{-1} (Fu et al., 2009) (Figure 2.4; Table III in appendix A).

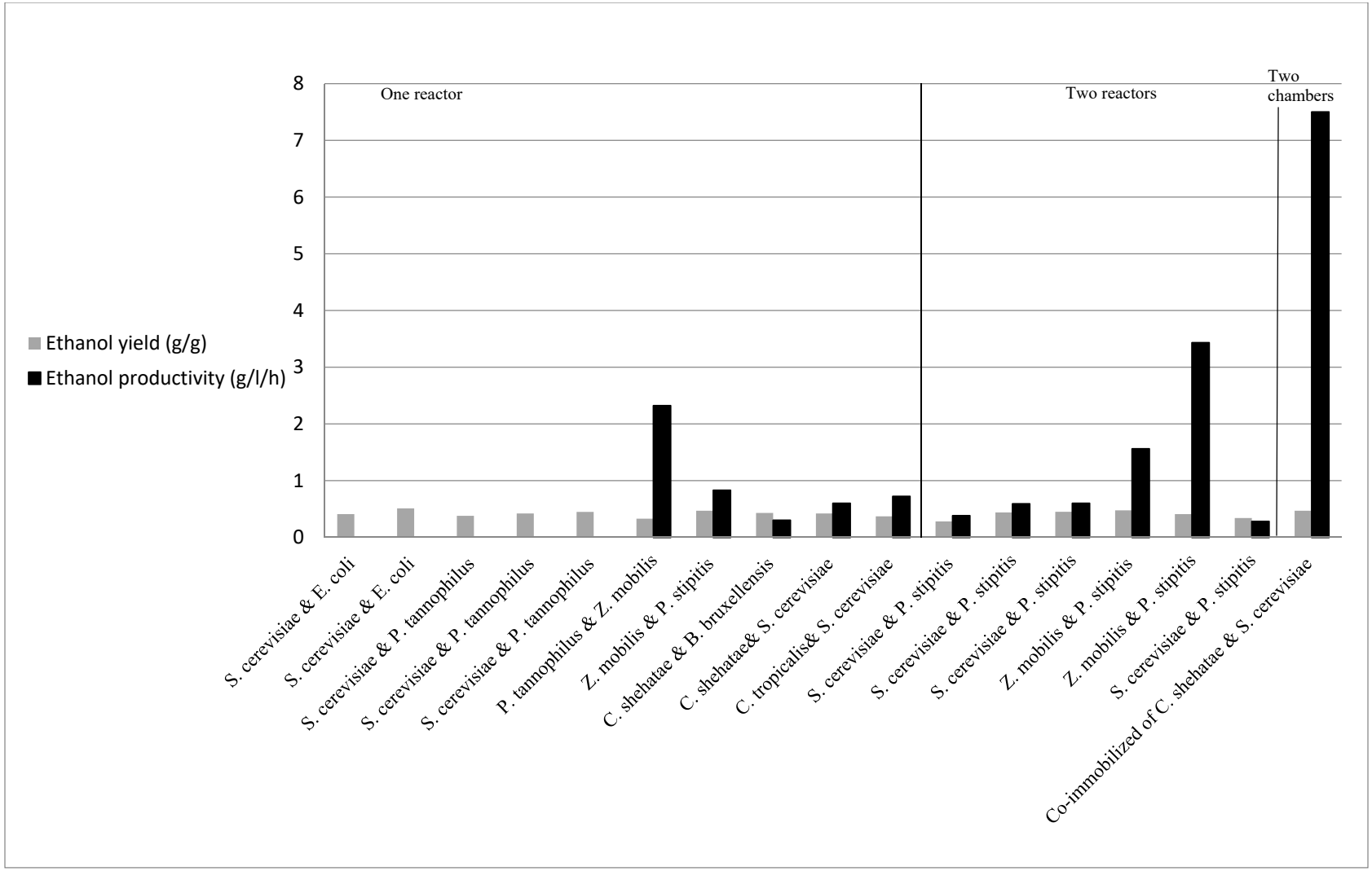


Figure 2.4.Ethanol yield (g g^{-1}) and productivity (g L^{-1} h^{-1}) of two-stage fermentation in one and two bioreactors (Table III in appendix A)

2A.4.1. Sequential batch culture: single bioreactor

Glucose-fermenting microorganisms also metabolize ethanol as a carbon source. The risk of diauxic shift is minimized in a sequential process because cells are removed immediately after glucose exhaustion, avoiding ethanol consumption (Guan et al., 2013). For example, when glucose is consumed completely, the glucose-fermenting microorganism (e.g. *S. cerevisiae*) is removed from the culture by centrifugation and the xylose-fermenting microorganism (e.g. *P. stipitis*) is added to the supernatant. Kocher and Uppal (Kocher and Uppal, 2013) optimized the fermentation variables of temperature (30 °C), pH (6)and stirring rate (92 rpm), leading to improving ethanol concentration from 20.61 to 22.24 g L^{-1}. Fu and Peiris (Fu and Peiris, 2007) achieved the best ethanol productivity (2.32 g L^{-1} h^{-1}) with reasonable yield (0.33 g g^{-1}) using sequential *Z. mobilis* and *P. tannophilus* (Figure 2.4). The initial fermentation was carried out with *Z. mobilis* with no aeration, followed by the inactivation of the bacterium after the utilization of glucose, and second fermentation with *P. tannophilus* under limited aeration (Fu and Peiris, 2007). Similarly, Fu et al. (2009) performed fermentation with sequential cultivation of *Z. mobilis* and *P. stipitis* obtaining yield of 0.47 g g^{-1}, productivity of 0.83 g L^{-1} h^{-1}, with complete glucose and xylose fermentation in 2.5 hours and 26 hours respectively (Figure 2.4) (Fu et al., 2009). The separation of *Z. mobilis* cells from *P. stipitis* cells is a prerequisite for successful co-utilization of sugars by these two strains (Fu et al., 2009). It seems that inefficiency of xylose fermentation could be caused by oxygenation competition between *Z. mobilis* and *P. stipitis* or inhibition of *Z. mobilis* cells on the xylose fermentation using *P. stipitis*. Thus, a sequential culture of *Z. mobilis* and *P. stipitis* for pure sugars using two reactors in series with *Z. mobilis* in the first (glucose consumption); and *P. stipitis* in the second (xylose fermentation) was suggested. This gave an ethanol productivity of 1.56 g L^{-1} h^{-1} (Chaudhary and Ghosh, 2014) (Figure 2.4). The maximum ethanol yield for the first and second reactors 0.49 g g^{-1} and 0.46 g g^{-1}, with glucose, completely consumed and 40% consumption of xylose (Chaudhary and Ghosh, 2014).

Based on the concept that xylose fermentation should be completed at low ethanol concentration, Guan et al. (2013) developed a process for fermentation of cellulose and xylan with sequential batch fermentation of two microbial strain combinations (Guan et al., 2013). The two experiments were: (1) *C. shehatae* followed by *S. cerevisiae* (ethanol yield of 0.42 g g^{-1} after 92.5 h), and (2) *C. shehatae* followed by *Brettanomyces bruxellensis* (ethanol yield of 0.43 g g^{-1} after 192 h) (Guan et al., 2013). *C. shehatae* was initially used for glucose and xylose fermentation before adding a high-ethanol-tolerant yeast, either *S. cerevisiae* or *B. bruxellensis*, for cellobiose fermentation (Guan et al., 2013). Castañón-Rodríguez et al. (2014) studied sequential batch culture using *C. tropicalis* and *S. cerevisiae* in simulated sugarcane bagasse (Castañón-Rodríguez et al., 2014). This showed that *C. tropicalis* was sensitive to ethanol concentrations above 30 g L^{-1}. They found the addition of *S. cerevisiae* at the beginning of the fermentation and addition

of *C. tropicalis* inoculation after 24 hours of the start of the fermentation gave the best ethanol yield (0.37 g g^{-1}) and productivity (0.72 g L^{-1} h^{-1}).

2A.4.2. Sequential batch culture: two chambered bioreactor

Lebeau et al. (1997) found, using a two-compartment batch bioreactor, that co-immobilized *C. shehatae* and *S. cerevisiae* (ethanol yield 0.47 g g^{-1}; volumetric productivity 7.5 g L^{-1} h^{-1}) performance was close that of immobilized mono-cultures of *C. shehatae* (0.48 g L^{-1}, 6 g L^{-1} h^{-1}) and *S. cerevisiae* (0.46 g g^{-1}, 7 g L^{-1} h^{-1}) (Lebeau et al., 1997). These immobilized cells were separated with one chamber of the bioreactor filled with 280 ml of medium containing both glucose (35 g L^{-1}) and xylose (15 g L^{-1}) whereas the second compartment received an equal volume of a substrate-free mineral medium (280 ml of phosphate buffer (KH_2PO_4: 10 g L^{-1})). Batch incubation of the immobilized cell structure was performed at 30°C. Asymmetrical oxygenations by continuous gas bubbling with anaerobiosis in the substrate chamber (N_2) and microaerobiosis in the buffer chamber (O_2/N_2 mixture at low O_2 content) and symmetrical oxygenation with oxygen supply by transfer through the air/liquid interface in both chambers were tested. Asymmetrical oxygenation negatively affected the fermentation performance of immobilized cultures. Grootjen et al. (1991) reached a similar conclusion with a continuous process using co-immobilized yeast cells (Grootjen et al., 1991a).

2A.4.3. Fermentation performance of sequential batch culture

Fu and Peiris (Fu and Peiris, 2007) reported the best ethanol productivity (2.32 g L^{-1} h^{-1}) using sequential *P. tannophilus* and *Z. mobilis.* Two years later, Fu and coworkers (Fu et al., 2009) used sequential *Z. mobilis* and *P. stipitis,* the highest yield of 0.47 g g^{-1} in one reactor (Figure 2.4). Lebeau et al. (1997) tested co-immobilized of *C. shehatae* & *S. cerevisiae* in two-chambered bioreactors, with ethanol yield at 0.47 g g^{-1} and ethanol productivity at 7.5 g L^{-1} h^{-1} (Lebeau et al., 1997).

2A.5. Conclusions

The use of lignocellulosic substrates as feedstocks for biocommodities, particularly the liquid biofuel ethanol, provides an opportunity for ample supply of carbon source, but presents the challenge of the hydrolysates containing both glucose and xylose. To date, no suitable wild type organisms concomitantly ferment both sugars efficiently to ethanol, owing to catabolite repression by glucose and product toxicity. To meet the technoeconomic demands of the process, effective substrate conversion is essential, particularly owing to the high cost-contribution of carbon source in ethanol production. This review highlights potential routes overcoming this challenge.

Three approaches were discussed: genetic modification of the desired microorganism to include the pathway for the metabolism of the second sugar, co-culture of compatible species each utilizing a different sugar, and sequential batch culture of the two microorganisms selected using a two-phase inoculation.

The highest ethanol yield (0.57 g g^{-1}) was achieved using engineered *E. coli* ATCC 11303 in a batch stirred tank reactor configuration (H.G. Lawford and Rousseau, 1991). The highest ethanol productivity (15.3 g L^{-1} h^{-1}) was found with engineered *Z. mobilis* -pZB5 (CP4) using a continuous fluidized bed (Krishnan et al., 2000). Success has been seen in metabolic engineering for combined glucose and xylose utilization (Altintas et al., 2006; Feng, 2013) where limitations owing to the regulation of metabolic pathways have been overcome. Most importantly, re-engineering of these species to achieve robust production strains is time-consuming and frequently too long for commercial reality.

An alternative is the use of two wild type microorganisms in co-culture; one is using glucose and the other xylose. Here, the two species operate under the same culture conditions. This restricts the use of optimum conditions, rather using a common operating window. Key challenges include competition for nutrients, including oxygen, differing tolerance for oxygen and ethanol, and differing kinetic parameters. Further, the interacting dynamic growth and product formation of the co-culture lacks in-depth understanding, limiting optimization and modelling potential.

In place of co-culture, sequential batch culture can be used, allowing individual optimization of conditions. To date, this has been explored in a single batch reactor by eliminating the first microorganism on depletion of its carbon substrate and re-inoculating with the second microbe. However, in batch processes, ethanol productivity is limited by ethanol inhibition.

To further increase productivity, a continuous reactor configuration is preferred. Using engineered *E. coli*, productivity increased from 1.2 g L^{-1} h^{-1} in batch culture to 2.58 g L^{-1} h^{-1} in continuous culture (H.G. Lawford and Rousseau, 1991); similarly, in co-culture of *P. stipitis* and respiratory deficient mutant of *S. cerevisiae*, productivity increased from 0.94 g L^{-1} h^{-1} in batch culture to 2 g L^{-1} h^{-1} in continuous culture (Delgenes et al., 1998; M. Taniguchi et al., 1997; Taniguchi and Tanaka, 2004).

In investigations into improved process engineering, medium design, oxygen availability, inoculum size, a range of microbial species and immobilization, are significant factors that are easily manipulated and implemented to improve ethanol yield and productivity. As an illustration, the efficient fermentation of xylose by Fu et al. (2009) (0.5 g g^{-1} and 1.126 g L^{-1} h^{-1}) (Fu et al., 2009) and Moniruzzaman et al. (1997) (0.44 g g^{-1} and 3.44 g L^{-1} h^{-1}) (Moniruzzaman et al., 1997) is directly attributed to the large inoculum size (50% v_{cells}/v_{ferm}) and type of microorganisms selected. De Bari et al. (2013) indicated that immobilization increased the relative consumption rate of xylose-to-glucose from 2 to 6 times depending on the fermentation medium composition (De Bari et al., 2013). Micro-encapsulation proved to be an effective approach for entrapping the facultative aerobes into the gel beads, thereby avoiding undesirable oxygen

competition with the xylose-fermenters. With a more durable immobilization matrix than calcium alginate, the production of ethanol could be further improved (Fu et al., 2009).

In conclusion, to optimize ethanol production from woody biomass hydrolysate, it is essential to ensure efficient conversion of both glucose and xylose to ethanol for high ethanol yields, best achieved by the complementary use of both a xylose- and glucose-utilizing ethanol producers. To enhance productivity, these organisms need to operate in the continuous culture that facilitates removal of ethanol and limits substrate and other inhibition. The combined optimization of yield and productivity is enhanced by high biomass concentration that may be achieved through immobilization for biomass retention, also enabling protection of the biomass from environmental factors. Moving forward, a combination of metabolic reprogramming and efficient fermenter design or bioprocessing setup for the removal of product, by-product inhibition, and catabolite repression will increase technological readiness for a more cost effective and profitable bioethanol process.

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2A.7. Declaration of interest

The authors declare no competing financial interest.

2A.8. Supporting Information

Supporting information is available in Tables I-III of appendix A: current studies of engineered culture, co-culture, and sequential culture, including culture system, fermentation mode, fermentation condition, and performance for bioethanol production from glucose and xylose.

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Paper title:

Mathematical modelling of bioethanol fermentation processes from glucose or/and xylose – a review

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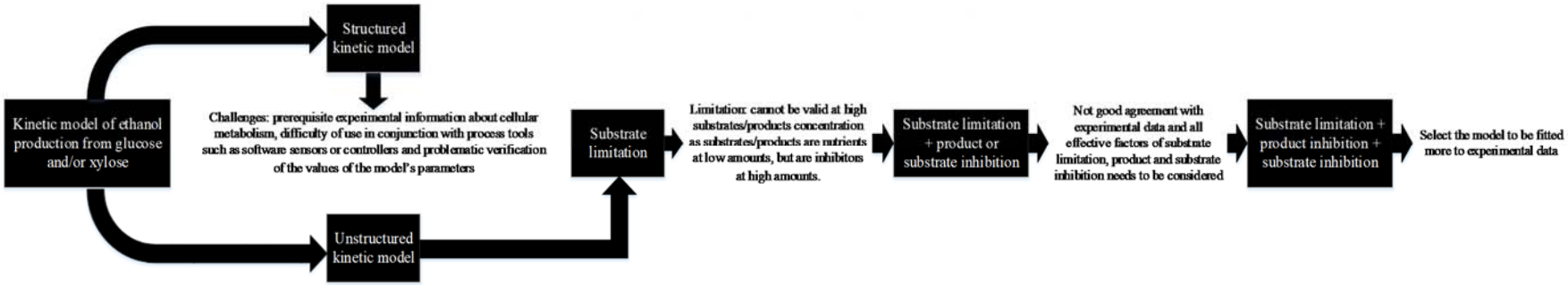
ChemBioEng Reviews (prepared for submission)

Abstract

Current bioethanol fermentation kinetic studies are solved with effective factors of, but not limited to, substrate limitation, oxygen limitation, substrate inhibition, product inhibition, and cell death. Majority of these studies have been done using *Saccharomyces cerevisiae* and regular mathematical modelling – unstructured unsegregated kinetic modelling. The aim of this paper is to review the bioethanol fermentation kinetics from pentoses and/or hexoses. Modes of culture (e.g. batch, continuous), strains of microorganisms used, process conditions, and equations with variety of effective factors are discussed. The best kinetic models that gave the best fit to the experimental data were proposed by the "Ghaly and EL-Taweel" or "Hjersted and Henson" equations. The results showed there was better agreement between modelling and experiment in simpler equation considering three important effective factors of substrate limitation, substrate inhibition and product inhibition when grown in standard growth medium. However, more complex equations show better fit when the optimum temperature is unclear, co-culturing is employed, or when growth conditions deviate from standard media and process conditions.

Keywords: bioethanol, fermentation, glucose and/or xylose, kinetics, mathematical modelling


Kinetic model of ethanol production from glucose and/or xylose
Structured kinetic model
Unstructured kinetic model
Challenges: prerequisite experimental information about cellular metabolism, difficulty of use in conjunction with process tools such as software sensors or controllers and problematic verification of the values of the model's parameters
Substrate limitation
Limitation: cannot be valid at high substrates/products concentration as substrates/products are nutrients at low amounts, but are inhibitors at high amounts.
Substrate limitation + product or substrate inhibition
Not good agreement with experimental data and all effective factors of substrate limitation, product and substrate inhibition needs to be considered
Substrate limitation + product inhibition + substrate inhibition
Select the model to be fitted more to experimental data

2B.1. Introduction

The use of suitable kinetic models to predict the fermentation performance of bioethanol production efficiency is essential because this can decrease experimentation work required, resulting in lower cost and quicker time for process improvement (Torres et al., 1997). Reaction kinetics are prescriptive as to how fast a reaction takes place in relation to the effects of various process conditions, i.e. pressure, temperature, pH, chemical composition and the presence of catalysts. Correspondingly, a kinetic model depicts the uptake of the substrate or the formation of products and microorganisms by means of the rates of reaction (Fogler, 2010).

The steps of kinetic modelling involve: analysing the process, defining objectives, considering assumptions for simplification, establishing a draft model, identifying kinetic parameters through literature or experimental work, finding the optimal parameters based on objectives through modelling and experimental work, and validating the model's accuracy. Kinetic modelling is an iterative process between experimental work and modelling itself. If, for instance, the model does not predict the experimental results of the reaction, the assumptions or structure of effective factors should be re-evaluated. In this case, the model grows in intricacy and accuracy without converting to complex equations. Following this, a model established in this way can predict performance under different process conditions, including design, optimization, and control of processes (Dunn et al., 2003).

Kinetic models are divided into two types; structured and unstructured models. Unstructured (black box) models are those without physical interpretation or internal structure, and cannot be extrapolated, interpreted, or analysed in terms of fermentor behaviour (Mantovaneli and Filho, 1992). Structured (metabolic pathway, white box) models do, however, allow for physical interpretation and provide a more detailed description of the intracellular environmental parameters of the fermentor system. This then allows for a qualification of the degree to which cells may change with time regarding variances in metabolic reactions or cellular processes. Structured models, however, have drawbacks that include prerequisite experimental information about cellular metabolism, the difficulty of use in conjunction with process tools such as software sensors or controllers and problematic verification of the values of the model's parameters (Dunn et al., 2003; Schügerl and Bellgardt, 2000). As a result, most of the models used are of the unstructured type (Kovárová-Kovar and Egli, 1998).

Unstructured models are divided into two types: The segregated unstructured model and the unsegregated unstructured model (Schügerl and Bellgardt, 2000). In a segregated model, the heterogeneity of the population is taken into account. For instance, a model may take the cell cycle into consideration or distinguish among different cell states (Uchiyama and Shioya, 1999). Unsegregated models, however, assume that cells are homogenous, so that all cells are assumed to be at the same point in the cell cycle, and

behave in isolation without interaction. Under these assumptions, a biological reaction's behaviour is directly correlated to the internal environment of the bioreactor or shake flask. Therefore, these models are a combination of kinetics describing the influence on the process such as pH and temperature (Nosrati-ghods et al., 2018).

It is very difficult to predict what model will fit with which process, as the suitability of each model is dependent on the objective of the model, specific microorganism and process conditions (Tan et al., 1996). In this review, attention is focused on unstructured and unsegregated models. Indeed, such models are more often used as engineering tools for control and state observations as these models depend on the limited number of state variables and parameters, thus avoiding numerous expensive sensors and expensive analytical tools. Unstructured models can also provide a general understanding of the metabolic processes involved as well as the basis for process optimization. Fermentation kinetic models are concerned with production rates along with the effects of different parameters, for example, temperature, pH, initial substrate concentration, dilution rate, dissolved oxygen concentration, substrate inhibition, product inhibition, substrate limitation, oxygen limitation and inoculum size. Most fermentation studies focus only on two or three effective parameters in its rate equations. This paper discusses the biomass and ethanol formation rates. Furthermore, it summarizes fundamental concepts and theories associated with modelling for bioethanol fermentation from C5 and/or C6 sugars to find the effects of key factors on ethanol yield and productivity, therefore proposing a suitable kinetic model for bioethanol fermentation.

2B.2. Unsegregated Kinetic Model

The most renowned unsegregated unstructured kinetic model is the Monod model for cell growth. It was developed in 1942 by Jacques Monod as seen in Equation 2 of Table 2.4 (Monod, 1949, 1942). This French scientist played a substantial role in the advancement of the theory of microbial growth kinetics and did as much as Michaelis and Menten did for enzymology (Panikov, 1995). He presented distinct and measured cultivation conditions and introduced simple and reproducible experimental methods. For many years the Monod equation was acknowledged as "fundamentally true", and in the 1950s work was devoted to the experimental testing of this equation (Contois, 1959; Moser, 1957; Tessier, 1942). The Monod model is used as a basis for most developed models, and as a result, Monod-type models control the field. However, researchers found that not all experimental data could reasonably fit the Monod model. Hence, they included additional constants into the original Monod model to describe additional aspects present in the biological system. For example, the inclusion of a third parameter into the Monod model, like the parameter *n* in the Moser model (Table 2.4, Equation 4), or presentation of the cell population density term in the Contois model (Table 2.4, Equation 5), led to an improvement in estimate competence. Some of the models

that use the original Monod model are the well-known modified Monod models for cell growth. They are shown in Table 2.4.

The differences in the result of various researchers are due not only to the microorganisms used, but also the conditions of the experiments and the manner in which the data was obtained. For example, Roca and his co-workers who used immobilized *Saccharomyces cerevisiae* and glucose in a packed column, found that the original Monod model fitted their experimental data more closely than other models (Roca et al., 1996). Another example where the simple Monod model also gave an acceptable representation of the experimental data was from the data of Birol et al. (1998) (Birol et al., 1998). Birol et al. (1998) fermented glucose using immobilized *S. cerevisiae* and studied a variety of different kinetic models related to biomass, ethanol production, and glucose utilization (Birol et al., 1998). Tan and his co-workers (Tan et al., 1996) compared experimental data from literature (Boopathy and Daniels, 1992; Grant, 1967; Tam and Finn, 1977) to the Aiba-Edwards model (Table 2.4, Equation 18) as well as two models designed by them (Table 2.4, Equation 33). Of the three sets of experimental data tested in above-mentioned models, the Aiba-Edwards model represented the better agreement than others with no unique model for all experiments.

Table 2.4.Summary of some modified Monod models for biomass and ethanol production rate

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
		Limitary effects			
1	Blackman (Blackman, 1905)	$\mu = \frac{\mu_{max} S}{2K_{xb}}$ for $S < 2K_{xb}$ $\mu = \mu_{max}$ for $S > 2K_{xb}$	NA*	Batch and continuous	Carbon dioxide, light intensity
2	Monod (Birol et al., 1998; Blanco et al., 2006; Buehler and Mesbah, 2016; Monod, 1949, 1942)	$\mu = \frac{\mu_{max} S}{K_x + S}$	$v = \frac{v_{max} S}{K_p + S}$	Continuous/ *E. coli*	Nitrogen source: ammonium, Temp: 37 °C, Suboptimal partial pressure of carbon dioxide / Glucose or mixture of glucose and xylose
3	Tessier (Tessier, 1942)	$\mu = \mu_{max}\left(1 - e^{-\frac{S}{Kr_x}}\right)$	NA*	NA*	NA*
4	Moser (Moser, 1957)	$\mu = \frac{\mu_{max} S^n}{K_x + S^n}$	NA*	NA*	NA*
5	Contois (Contois, 1959; Fujimoto, 1963)	$\mu = \frac{\mu_{max} S}{K_x X + S}$	NA*	Continuous/ *Aerobacter aerogenes*	Nitrogen source: ammonium, pH: 6.8, temp: 27 °C/ Glucose or succinate
				Batch/ *S. cerevisiae*	4<pH<5.5, Temp: 30 °C, Stirring rate: 250 rpm, Aeration level: 1 vvm/ Glucose
				Batch/ *Saccharomyces formosensis*	Nitrogen source: ammonium, pH: 4.5, Temp: 30 °C, Stirring rate: 50 rpm / Glucose
				Batch/ *E. coli*	pH: 7.2, Temp: 37 °C, various aeration level, various inoculum size/ Glucose

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
6	Kono and Asai (Kono, 1968; Kono and Asai, 1969a, 1969b)	$\mu = K_x \emptyset$ (Kono, 1968)	$v = K_{p1}\emptyset + K_{p2}(1 - \emptyset)$ (Kono and Asai, 1969a, 1969b)	Batch/ *Lactobacillus delbrueckii, S. cerevisiae, Pseudomonas ovalis, E. coli, L. fibroblasts, hamster kidney cells*	NA*
				Continuous/ *Torula utilis*	NA*
				CSTR, tubular	NA*
				Batch and continuous	
				Lactobacillus delbrueckii	pH: 6.0, Temp:45 °C/ Glucose
				Acetobacter suboxydans	Sorbitol
				Aspergillus Niger	pH: 4.5, Temp:25 °C/ Sucrose
				Streptomyces niveus	Various agitation/ Novobiocin
7	Dabes (Dabes et al., 1973)	$S = \mu K_{xb} + \frac{\mu K_x}{\mu_{max} - \mu}$	NA*	Batch/ *E. coli*	Temp: 37 °C/ Glucose
				Batch/ *lactis aerogenes*	Phosphate

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
		Inhibitory effects			
8	Hinshelwood (Hinshelwood, 1946)	$\mu = \frac{\mu_{max} S}{K_x + S}(1 - K_{ipx} P)$	$v = v_{max}\left(\frac{S}{K_p + S}\right)(1 - K_{ipp} P)$	Continuous/ *Bacterium lactis aerogenes*	Various temperature/ Alcohols
9	Webb (Webb, 1963)	$\mu = \frac{\mu_{max} S}{S + K_x + \left(1 + \frac{\sigma}{K_{isx}}\right)} e^{1.17\sigma}$ with σ = ionic strength	NA*	NA*	NA*
10	Webb (Edwards, 1970; Webb, 1963)	$\mu = \frac{\mu_{max} S}{K_x + S + \frac{S^2}{K_{isx}}}\left(\frac{K_{isx} + (\alpha * S)}{K_{isx}}\right)$	NA*	NA*	NA*
				Continuous/ *Klebsiella aerogenes*	pH: 7.12, Aerobic/ Benzenoid compound
11	Monod-Jerusalimsky (Jerusalimsky and Neronova, 1965)	$\mu = \frac{\mu_{max} S}{K_x + S}\left(\frac{K_{ipx}}{K_{ipx} + P}\right)$	$v = v_{max}\left(\frac{S}{K_p + S}\right)\left(\frac{K_{ipp}}{K_{ipp} + P}\right)$	Continuous/ *Propionic bacteria*	Lactate
12	Yano (Edwards, 1970; Yano et al., 1966)	$\mu = \frac{\mu_{max}}{\frac{K_x}{S} + 1 + \sum_j \left(\frac{S}{K_{isx}}\right)^j}$	NA*	Batch and continuous/ *Pseudomonas ovalis*	pH: 6.8, Temp: 30 °C/ Glucose, galactose, xylose, fructose, sucrose
				Continuous/ *Klebsiella aerogenes*	pH: 7.12, Aerobic/ Benzenoid compound
13	Holzberg (Holzberg et al., 1967)	$\mu = \mu_{max} - K_{ipx1}(P - K_{ipx2})$	$v = v_{max} - K_{ipp1}(P - K_{ipp2})$	*S.cerevisiae var. ellipsodeus*	Grape juice

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
14	Powell (Powell, 1967)	$\mu = \frac{\mu_{max} S}{K_x + K_D + S}$	NA*	NA*	NA*
				Batch and Continuous	NA*
15	Andrews (Haldane) (Andrews, 1968; Bailey and Ollis, 1986; Doble et al., 2004; Edwards, 1970; Galaction et al., 2010)	$\mu = \frac{\mu_{max} S}{K_x + S + \frac{S^2}{K_{isx}}} = \frac{\mu_{max}}{\frac{K_x}{S} + 1 + \frac{S}{K_{isx}}}$	$v = \frac{v_{max} S}{K_p + S + \frac{S^2}{K_{isp}}}$	Continuous/ *Klebsiella aerogenes, Candida utilis, Nitrosomonas, Nitrobacter*	pH: 7.12, Aerobic/ Benzenoid compound
				NA*	NA*
				NA*	NA*
				Bioreactor with stirred beds of biocatalysts/ *S. cerevisiae*	Temp: 28 °C, Stirring rate: 250 rpm/ Glucose
16	Andrews to/and Noack (Andrews, 1968; Noack, 1968)	$\mu = \mu_{max} \frac{S}{K_x + S} \left(\frac{1}{1 + \frac{S}{K_{isx}}} \right)$	NA*	Batch and continuous	NA*
				NA*	NA*
17	Aiba (Aiba et al., 1968)	$\mu = \mu_{max} \frac{S}{K_x + S} e^{-K'_{ipx} P}$	$v = v_{max} \frac{S}{K_p + S} e^{-K'_{ipp} P}$	Chemostat/ *S. cerevisiae*	pH: 4.0, Temp: 30 °C/ Glucose
18	Edwards/Aiba-Edwards (Edwards, 1970)	$\mu = \mu_{max} \frac{S}{K_x + S} e^{-\frac{S}{K'_{isx}}}$	NA*	Continuous/ *Klebsiella aerogenes*	pH: 7.12, Aerobic/ Benzenoid compound

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
19	Tessier-type OR Tessier-Edward (Edwards, 1970)	$\mu = \mu_{max}\left(e^{-\frac{S}{K'_{isx}}} - e^{-\frac{S}{K'_{x}}}\right)$	NA*	Continuous/ *Klebsiella aerogenes, Nitrosomonas*	pH: 7.12, Aerobic/ Benzenoid compound
20	Reuss and Wagner (Reuss and Wagner, 1973)	$\mu = \frac{\mu_{max}\,S}{K_x + S}\left(\frac{S}{K_{isx} + S}\right)$	NA*	NA*	NA*
21	Tseng and Wayman (Tseng and Wayman, 1975; Wayman and Tseng, 1976)	$\mu = \frac{\mu_{max}S}{K_x + S} - K_{isx}(S - S_c)$ when $S > Sc$ $\mu = \frac{\mu_{max}S}{K_x + S}$ when $S < Sc$	NA*	Shake flasks/ Two strains of *Candida utilis, C. lipolytica, S. cerevisiae*	pH: 4.5, Temp: 30 °C, Stirring rate: 250 rpm/ Ethanol, acetic acid, Ethyl acetate, n-butanol
				Pseudomonas methanica, Arthrobacter	Methanol, and n-butanol
22	Bazua and Wilke (Bazua and Wilke, 1977)	$\mu = \mu_{max} - K_{ipx1}\bar{P}(K_{ipx2} - \bar{P})$ (a) $\mu = \mu_{max}\left(1 + \frac{\bar{P}}{P_{x,\,max}}\right)^{0.5}$ (b)	$v = v_{max} - K_{ipp1}\bar{P}(K_{ipp2} - \bar{P})$ $v = v_{max}\left(1 + \frac{\bar{P}}{P_{p,\,max}}\right)^{0.5}$	Batch and continuous/ *S. cerevisiae*	pH: 4, Temp: 30 °C/ Glucose
23	Levenspiel (Levenspiel, 1972; Melick et al., 1987)	$\mu = \frac{\mu_{max}S}{K_x + S}\left(1 - \frac{P}{P_{x,\,max}}\right)^{\beta x}$	$v = \frac{v_{max}S}{K_p + S}\left(1 - \frac{P}{P_{p,\,max}}\right)^{\beta p}$	Plug-flow, batch, and mixed flow	NA*
				Packed bed/ *Z. mobilis*	pH: 6, Temp: 33 °C/ Glucose

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
24	Hoppe and Hansford (Hoppe and Hansford, 1982)	$\mu = \frac{\mu_{max} S}{K_x + S}\left(\frac{K_{ipx}}{K_{ipx} + Y_{sp}/(S_0 - S)}\right)$	$v = \frac{v_{max} S}{K_p + S}\left(\frac{K_{ipp}}{K_{ipp} + Y_{sp}/(S_0 - S)}\right)$	Continuous/ *S. cerevisiae*	pH: 4, Temp: 30 °C, Stirring rate: 300 rpm/ Glucose
25	Luong (Luong, 1987)	$\mu = \frac{\mu_{max} S}{K_x + S}\left(1 - \frac{S}{S_{x\ max}}\right)^{\gamma x}$	NA*	Batch/ *Candida utilis*	pH: 4.5, Temp:30 °C, Aeration level:1 vvm/ n-Butanol
26	Han and Levenspiel (Han and Levenspiel, 1988)	$\mu = \frac{\mu_{max} S}{K_x\left(1 - \frac{X}{X_{x,\ max}}\right)^{\alpha' x} + S}\left(1 - \frac{X}{X_{x\ max}}\right)^{\alpha x}$	$v = \frac{v_{max} S}{K_p\left(1 - \frac{P}{P_{p,\ max}}\right)^{\beta' p} + S}\left(1 - \frac{P}{P_{p\ max}}\right)^{\beta p}$	*Pentose-consuming bacterium, Arthrobacter*	n-pentane, n-butanol
27	Grootjen (Grootjen et al., 1991a)	$\mu_{glu} = \frac{\mu_{glu,\ max} S_{glu}}{K_{x,\ glu} + S_{glu}}$ $\mu_{xyl} = \frac{\mu_{xyl,\ max} S_{xyl}}{K_{x,\ xyl}\left(1 + \frac{S_{glu}}{K_{ixg}}\right) + S_{xyl}}$	NA*	Continuous/ Co-cultures of *P. stipitis* & *S. cerevisiae*	pH: 5, Temp: 30 °C, Anaerobic/ Glucose and xylose
28	Grootjen (Grootjen et al., 1991b)	$\mu_{glu} = \frac{\mu_{glu,\ max} S_{glu}}{K_{x,\ glu} + S_{glu}}\left(\frac{C_o}{K_{o,\ glu} + C_o}\right)$ $\mu_{xyl} = \frac{\mu_{xyl,\ max} S_{xyl}}{K_{x,\ xyl}\left(1 + \frac{S_{glu}}{K_{ixg}}\right) + S_{xyl}}\left(\frac{C_o}{K_{o,\ xyl} +}\right.$	NA*	Batch and Continuous/ *P. stipitis*	Temp: 30 °C, Oxygen-limited condition/ Glucose and xylose
29	Hanly and Henson (Hanly and Henson, 2013, 2011)	$\mu_{glu} = \frac{\mu_{glu,\ max} S}{K_{x,\ glu} + S}\left(\frac{K_{ipx,\ glu}}{K_{ipx,\ glu} + P}\right)$ $\mu_{xyl} = \frac{\mu_{xyl,\ max} S}{K_{x,\ xyl} + S}\left(\frac{K_{ipx,\ xyl}}{K_{ipx,\ xyl} + P}\right)\left(\frac{K_{ixg}}{K_{ixg} + S_{glu}}\right)$	$v_{glu} = \frac{v_{glu,\ max} S}{K_{p,\ glu} + S}\left(\frac{K_{ipp,\ glu}}{K_{ipp,\ glu} + P}\right)$ $v_{xyl} = \frac{v_{xyl,\ max} S}{K_{p,\ xyl} + S}\left(\frac{K_{ipp,\ xyl}}{K_{ipp,\ xyl} + P}\right)\left(\frac{K_{ipg}}{K_{ipg} + S_{glu}}\right)$	Batch/ Co-cultures of *E. coli* & *S. cerevisiae*	Common pH and temperature, aerobic and anaerobic/ Glucose and xylose
				Batch/ Co-cultures of *P. stipitis* & *S. cerevisiae*	pH: 5, Temp:30 °C, Stirring rate: 500 rpm, Microaeobic/ Glucose and xylose

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
		Advanced inhibitory effects			
30	Ghose and Tyagi (Ghose and Tyagi, 1979; Sulieman et al., 2018)	$\mu = \mu_{max}\left(1 - \frac{P}{P_{x,\,max}}\right)$ $\mu = \frac{\mu_{max}S}{K_x + S + \frac{S^2}{K_{isx}\omega}}\left(1 - \frac{P}{P_{x,\,max}}\right)$	$v = v_{max}\left(1 - \frac{P}{P_{p,\,max}}\right)$ (a) $v = \frac{v_{max}S}{K_p + S + \frac{S^2}{K_{isp}\omega'}}\left(1 - \frac{P}{P_{p,\,max}}\right)$ (b)	Batch and continuous with cell recycle/ *S. cerevisiae*	pH: 4.0, Temp:30 °C, Aeration level: 0.127 vvm/ Glucose
31	Rogers (Lee and Rogers, 1983; Leksawasdi et al., 2001; Mokomele et al., 2013)	$\mu = \frac{\mu_{max}S}{K_x + S}\left(1 - \frac{P - P_{ix}}{P_{x,\,max} - P_{ix}}\right)\left(\frac{K_{isx}}{K_{isx} + S}\right)$	$v = \frac{v_{max}S}{K_p + S}\left(1 - \frac{P - P_{ip}}{P_{p,\,max} - P_{ip}}\right)\left(\frac{K_{isp}}{K_{isp} + S}\right)$	Batch and continuous/ *Z. mobilis*	pH: 5, Temp: 30 °C, Stirring rate: 200 rpm, anaerobic/ Glucose and xylose
32	Huang and Chen (Huang and Chen, 1988)	$\mu = \mu_{max}\frac{S}{K_x + S}F_p(P,T)G_p(S,T)$ $G_p(S,T) = \frac{K_{isx}}{K_{isx} + (S - S_{ix})}$ for $S > S_{ix}$ $G_p(S,T) = 1$ for $S \le S_{ix}$ $F_p(P,T) = 1 - \frac{P\left[1 - \exp\left(-\frac{t}{\tau}\right)\right]}{P_{x\,max}}$ for $P\left[1 - \exp\left(-\frac{t}{\tau}\right)\right] < P_m$ $G_p(S,T) = 0$ for $P\left[1 - \exp\left(-\frac{t}{\tau}\right)\right] \ge P_{x,\,max}$	$v = v_{max}\frac{S}{K_p + S}F_p(P,T)G_p(S,T)$ $G_p(S,T) = \frac{K_{isp}}{K_{isp} + (S - S_{ip})}$ for $S > S_{ip}$ $G_p(S,T) = 1$ for $S \le S_{ip}$ $F_p(P,T) = 1 - \frac{P\left[1 - \exp\left(-\frac{t}{\tau}\right)\right]}{P_{p,\,max}}$ for $P\left[1 - \exp\left(-\frac{t}{\tau}\right)\right] < P_m$ $G_p(S,T) = 0$ for $P\left[1 - \exp\left(-\frac{t}{\tau}\right)\right] \ge P_m$	Batch/ *Z. mobilis*	pH: 5, 30° C ≤Temp≤ 40 °C, Stirring rate: 120 rpm, Anaerobic/ Glucose

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
33	Tan (Tan et al., 1996)	$\mu = \frac{\mu_{max} S}{K_x + S + \frac{S^2}{K_{isx}}}$ $\mu = \frac{\mu_{max} S}{K_x + S + \frac{S^2}{K_{isx1}} + \frac{S^3}{K_{isx1} K_{isx2}}}$	NA*	Marine methanogenic bacterium (*Methanococcus sp.*)	Sulfide
				Klebsiella aerogenes,	Sodium p-hydroxybenzoate
				Methylomonas mucosa	Methanol
34	Ghaly and EL-Taweel (Ghaly and El-Taweel, 1997)	$\mu = \frac{\mu_{max} S}{K_x + S}\left(\frac{K_{ipx}}{K_{ipx} + P}\right)\left(\frac{K_{isx}}{K_{isx} + S}\right)$	$v = \frac{v_{max} S}{K_p + S}\left(\frac{K_{ipp}}{K_{ipp} + P}\right)\left(\frac{K_{isp}}{K_{isp} + S}\right)$	Continuous/ *Candida seudotropicalis*	4.1≤pH≤4.7, 29.2 °C ≤Temp≤31.6 °C, Stirring rate: 300 rpm, Anaerobic/ Cheese whey
35	Krishnan (Krishnan et al., 1999, 1995)	$\mu = \frac{\mu_{max} S}{K_x + S + \frac{S^2}{K_{isx}}}\left(1 - \left(\frac{P}{P_{x,\,max}}\right)^{\beta' x}\right)$	$v = \frac{v_{max} S}{K_p + S + \frac{S^2}{K_{isp}}}\left(1 - \left(\frac{P}{P_{p,\,max}}\right)^{\beta' p}\right)$	Batch/ *Saccharomyces 1400 yeasts*	Temp: 30 °C, Stirring rate: 150-200 rpm/ Glucose and xylose
				Co-cultures (*P. stipitis* & *S. cerevisiae*)	Glucose, Xylose, Glucose and xylose
36	Hjersted and Henson (Hjersted and Henson, 2006)	$\mu = \frac{\mu_{max} S}{K_x + S + \frac{S^2}{K_{isx}}}\left(\frac{K_{ipx}}{K_{ipx} + P}\right)$	$v = \frac{v_{max} S}{K_p + S + \frac{S^2}{K_{isp}}}\left(\frac{K_{ipp}}{K_{ipp} + P}\right)$	Fed-batch/ *S. cerevisiae*	Temp: 30 °C, Microaerobic/ Glucose

No	Model name	Equation of biomass growth rate	Equation of ethanol production rate	Mode/ Species	Condition/ Feedstock
37	de-Andrade or Rivera (de Andrade et al., 2012; Rivera et al., 2013)	$\mu = \frac{\mu_{max} S}{K_x + S}\left(e^{-K'_{isx} S}\right)\left(1 - \frac{P}{P_{x,max}}\right)^{\beta x}$	$v = Y_{sp}\,\mu + m_p$ $Y_{sp} = \frac{Pf - P0}{S0 - Sf}$	Batch/ *S. cerevisiae*	Temp: 30,32,34 °C, 300rpm/ Glucose, Xylose, Arabinose, 5-hydroxymethylfurfural, and Acetic acid
				Fed-batch with cell recycle/ *S. cerevisiae*	Low pH, Temp: 33 °C, Stirring rate: 250rpm/ Glucose
38	Andrews and Levenspiel (Farias et al., 2014)	$\mu = \frac{\mu_{max} S}{K_x + S + \frac{S^2}{K_{isx}}}\left(1 - \frac{P}{P_{x,max}}\right)^{\beta x}$	NA*	Batch and continuous	NA*
				Plug-flow, batch, and mixed flow	NA*
				Batch and Continuous/ *P. stipitis*	pH: 4.5, Temp: 28 °C, Stirring rate: 150 rpm, Microaerated at 0.05 vvm/ Xylose
39	de-Andrade or Rivera in combination with Logistic and Gompertz (Ccopa Rivera et al., 2017; Rivera et al., 2013)	$\mu = \frac{\mu_{max} S}{K_x + S}\left(e^{-K'_{isx} S}\right)\left(1 - \frac{P}{P_{x,max}}\right)^{\beta x}\left(1 - \frac{C_{x0}}{C_{x,max}}\right)^{\gamma x,}$	$v = Y_{sp}\,\mu + m_p$ $Y_{sp} = \frac{Pf - P0}{S0 - Sf}$	Batch and VHG fed-batch fermentation with cell recycling/ *S. cerevisiae*	Temp: 28-34 °C, Stirring rate: 200 rpm under very-high-gravity fermentation conditions

* NA: Not available

2B.3. Effective aspects

Some models in Table 2.4 are able to model limitation by a substrate (Blackman, 1905; Dabes et al., 1973; Monod, 1949; Moser, 1957; Powell, 1967; Tessier, 1942) better, while some other researchers (Aiba and Shoda, 1969; Andrews, 1968; Edwards, 1970; Jerusalimsky and Neronova, 1965; Noack, 1968; Tseng and Wayman, 1975) have modelled inhibition by substrate or product and are renowned for its inhibitory effects. There are some studies that considered collectively substrate limitation, substrate and product inhibition in their models and are called advanced inhibitory effects equations in this paper (de Andrade et al., 2012; Farias et al., 2014; Ghaly and El-Taweel, 1997; Ghose and Tyagi, 1979; Hjersted and Henson, 2006; Huang and Chen, 1988; Krishnan et al., 1999, 1995; Lee and Rogers, 1983; Leksawasdi et al., 2001; Mokomele et al., 2013; Rivera et al., 2013). These limitation and inhibition terms are the effective factors in bioethanol production from sugars. In some cases, researchers improved their model's fit to experimental work by incorporating parameters such as biomass, substrate, and product inhibition (Farias et al., 2014; Ghose and Tyagi, 1979; Huang and Chen, 1988; Leksawasdi et al., 2001). However, these models still require more improvement by considering more effective parameters.

2B.3.1. Limitary effects

Several mathematical models have been studied for quantifying the limitary effect of substrate on the biomass/ethanol formation rate that is given in Table 2.4, Equations 1-7. The appearance of microbial kinetics can be dated back to the publication of a paper by Blackman (Blackman, 1905). The rate of photosynthesis was investigated as a function of carbon dioxide concentration or light intensity without any mathematical justification (Blackman, 1905). Blackman (Blackman, 1905) supposed a linear relationship between the growth rate of phototrophs and the availability of a growth-limiting nutrient (e.g. the CO_2 concentration in the air) (Table 2.4, Equation 1) (Blackman, 1905). When the CO_2 concentration exceeds a certain threshold value, there was no further increase in the rate of the process (Blackman, 1905). Blackman also assumed that the growth rate of a cell was on the whole determined by a single enzymatic reaction, which can be identified kinetically as the slowest one or ‘bottle-neck’ (Blackman, 1905). Monod emphasized that agreement with Blackman's ‘bottle-neck’ principle is unlikely (Panikov, 1995). The constraint which was proposed by Monod was stronger than that implied by the original Blackman formulation (Panikov, 1995). The Monod model regards the specific growth rate of a microorganism as a function of the limiting substrate concentration. However, Contois (Contois, 1959) proposed that the cell population density also affects the growth rate(s) of cells and therefore, attempted to improve the Monod model by developing a model for cell growth incorporating the effect of population density on the growth rate (Table 2.4, Equation 5).

Conversely, Kono (Kono, 1968) found that the Monod equation could not fully correspond to experimental data of microbial cell growth in many batch cultivations, and the predicted values based on the Monod equation do not agree with observed values in many of the continuous cultivation examined. Therefore, the Monod equation by introducing new concepts of critical concentration was adjusted and coefficient of consumption activity was derived from the growth rate equation (Table 2.4, Equation 6 of biomass growth rate) that corresponded to the whole growth curve including the lag, exponential and stationary phase of cell growth in both batch and continuous cultivations (Kono, 1968). A year later, Kono and Asai (Kono and Asai, 1969a, 1969b) derived mathematical equations to describe product concentration in fermentation time courses by developing the general equation of production rate and the clarified characteristic of the fermentation process (Table 2.4, Equation 6 of ethanol production rate). K_{P1} and K_{P2} represent the characteristic of fermentation process as follows: 1) product formation is associated with growth and non-growth when both K_{P1} and K_{P2} have a positive value, 2) product formation is associated with growth when K_{P1} has a positive value and K_{P2} is zero, 3) product formation is associated with non-growth when K_{P1} is zero and K_{P2} has a positive value, 4) product formation is associated with growth and decreased with non-growth when K_{P1} has a positive value and K_{P2} has a negative value. The modelling result of cell and product concentration based on the new theory showed good agreement with the observed values in both batch and continuous cultivation. Dabes et al. (1973) proposed the 'three parameter equation' with two conditions for the general cell growth model (Table 2.4 2.4, Equation 7): i) only the upper limit of the growth rate by a single enzymatic step should be fixed, and ii) more than one step in a series of enzyme reactions influences the growth rate at low substrate concentrations (Dabes et al., 1973). In comparing the 'three parameter equation', Blackman's kinetics and original Monod data for glucose-limited growth of *Escherichia coli*, experimental results were best matched to the Dabes' model (Dabes et al., 1973) as it includes both Monod and Blackman form.

2B.3.2. Inhibitory effects

Modified Monod equations in part of limitary effects in Table 2.4 (Blackman, 1905; Dabes et al., 1973; Monod, 1949; Moser, 1957; Powell, 1967; Tessier, 1942) like Monod's model accounts only for substrate limitations and not for product or substrate inhibition, hence are not valid at high substrate/product concentrations as substrates/products are nutrients at low concentrations, but are inhibitors at high concentrations. Several mathematical models, which are shown in Table 2.4 (Equations 8 to 29), have been developed for quantifying the inhibitory effect of biomass/ethanol formation rates, and are generally adapted from equations for substrate or product inhibition of enzymatic reactions. Hinshelwood (Dagley and Hinshelwood, 1938; Hinshelwood, 1946) observed the reduction of the growth rate of *Bacterium lactis aerogenes* by alcohols. A kinetic model that exhibits a linear relationship between the growth of *Klebsiella aerogenes* (mean generation times) and ethanol (benzenoid compound) at high inhibitor concentrations was

proposed. The combined effects on the growth of *propionibacterium* on lactate as the substrate and propionate or acetate as products were described in the Monod-Jerusalimsky equation. Jerusalimsky and Neronova (Jerusalimsky and Neronova, 1965) showed the dependence of μ on P by hyperbolic or sigmoidal curves. Aiba and his co-workers proposed a model of ethanol inhibitory effect on yeast cell growth and ethanol production with glucose (Equation 17 of Table 2.4) (Aiba et al., 1968; Aiba and Shoda, 1969). Bai (2007) mentioned two major problems with Aiba models: i) infinite ethanol concentration cannot be approached when cell growth and ethanol production are inhibited completely by ethanol concentration; ii) cell growth and ethanol production cannot be zero since yeast cells and ethanol are continuously produced in practice (Bai, 2007). After the Aiba model in 1968 (Aiba et al., 1968), Edwards (Edwards, 1970) proposed product inhibition correlation which may be used to estimate substrate inhibition. In fact, a modification of the Aiba model is the Edwards model, also known as the Aiba-Edwards model as presented in equation 18 of Table 2.4. However, the equation of Aiba-Edwards failed to predict the maximum substrate concentration at which growth will be completely inhibited. It is worth noting that when $S/K_{isx} \leq 1$, the equation of Aiba-Edward becomes equivalent to the following equation by a Taylor series analysis:

$$\mu = \frac{\mu_{max} S}{K_X + S}\left(1 - \frac{S}{K_{isx}}\right) \qquad (A)$$

Based on the Hinshelwood (Hinshelwood, 1946) equation, Tseng and Wayman (Tseng and Wayman, 1975; Wayman and Tseng, 1976) proposed a correlation with the growth data of *Candida utilis*, *Candida lipolytica, Arthrobacter*, and *Pseudomonas methanica* on ethyl alcohol, acetic acid, ethyl acetate, and 1-butanol. The study reported growth rates at a series of concentrations of each substrate. Literature data also indicated that the relationship between μ and S is not always a linear one. Tseng and Wayman (Tseng and Wayman, 1975) revealed that substrates which act as inhibitors at high concentrations may not be inhibitors at all at low concentrations and found that at lower concentrations 1-butanol is a nutrient and at higher concentrations, an inhibitor. Therefore, substrate concentrations above a characteristic threshold concentration -S_c- would inhibit growth.

To improve the kinetic model, combining the Tessier model with inhibitory concentrations led to Edwards proposing the Tessier-type (Tessier-Edwards) model for correlating growth data. The model takes into account the behaviour that substrates will act as inhibitors at higher concentrations and as nutrients at lower levels (Edwards, 1970; Tessier, 1942). The Tessier-type equation, however, failed to predict the total inhibition concentration, the level at which growth cannot occur, since μ approaches zero only when S approaches infinity.

Haldane is one of the simple, effective and significant models after Monod (Haldane, 1930). Andrews (Andrews, 1968) proposed an inhibition function based on a relationship presented by Haldane for the inhibition of enzymes by high substrate concentrations to batch and continuous cultures of microorganisms.

Unfortunately, validating of the model with experimental data was not done. Haldane (Haldane, 1930) described enzyme inhibition by the formation of an inactive complex of the enzyme with two substrates molecules. Boon and Laudelout (Boon and Laudelout, 1962) later found that Haldane's equation was fitted to their experimental results using *Nitrobacter winogradskyi.* Mechanisms and kinetics of substrate, nitrite inhibition, product (nitrate) inhibition, pH effect and oxygen limitation were analysed; showed that the rate of nitrite oxidation could be related to nitrite concentration by the proposed inhibition function (Boon and Laudelout, 1962).

Yano et al. (1966) generalized Haldane's equation by assuming the formation of multiple inactive enzyme-substrate complexes *(Yano et al., 1966)*. The decrease of respiration rates in the presence of excess sugars, substrates and non-substrates on free cells of *Pseudomonas ovalis* were investigated and good correlation with the experimental data was shown *(Yano et al., 1966)*. Three years later, Yano and Koga (Yano and Koga, 1969) extended their work on substrate inhibition and suggested the use of a respiration kinetics approach. However, no verification was given with experimental data. A theoretical study was done on the dynamic behaviour of a single vessel using continuous process subjected to growth inhibition at high concentrations of the rate limiting substrate.

Subsequently, Edwards (Edwards, 1970) pointed out that many other inhibitory models could be borrowed from enzyme kinetics to fit kinetic data taken from the literature, so five different equations – Haldane (Equation 15 of Table 2.4), Webb (non-linear one, Equation 10 of Table 2.4), Yano (Equation 12 of Table 2.4), Aiba, and Tessier-type (Equation 19 of Table 2.4) – were compared to experimental results. Edwards (Edwards, 1970) recommended that the simplest and most accurate of these equations is the Haldane equation. As an illustration for the recent studies on Haldane equation (Haldane, 1930), Galaction et al.(2010) studied the alcoholic fermentation using a bioreactor with stirred/mobile beds of immobilized *S. cerevisiae* cells on alginate (Galaction et al., 2010). Using the mathematical model of Haldane, the kinetic parameters were estimated and compared to the kinetic constants (Aiba et al., 1968) for alcoholic fermentation with free *S. cerevisiae* cells with and without ethanol inhibition conditions (Galaction et al., 2010). It was found that the variation of ethanol production during fermentation was related to the glucose concentration and the initial ethanol concentrations. It was noted that the fermentation duration was controlled by the size and concentration of the biocatalyst particles with a certain initial glucose concentration (Galaction et al., 2010).

Levenspiel (1972) proposed a model which is a more general form of the modified Monod kinetics for ethanol production (Levenspiel, 1972). Listed in Table 2.4 (Equation 23), the product inhibition term reduces to one when there is no product inhibition. The effects of both substrate and inhibitors were considered in the Levenspiel model (Levenspiel, 1972). Levenspiel (Levenspiel, 1972) derived an expression of ethanol inhibition performance for various reactor types including plug flow, batch and

packed bed reactor. Interestingly, Luong (Luong, 1987) then proposed substrate inhibition based on product inhibition of the Levenspiel model. Melick et al. (1987)(Melick et al., 1987), through the Levenspiel model, described ethanol production in a packed bed fermentor containing *Zymomonas mobilis* entrapped in small spheres of calcium alginate (Melick et al., 1987). A diffusivity value for glucose and ethanol in a cell-loaded calcium alginate was determined.

Hoppe and Hansford (Hoppe and Hansford, 1982) reported studies in a continuous culture where high concentrations of ethanol were produced in the fermentor using yeast by feeding a high concentration of glucose, and the chosen model was similar to the studies used for non-competitive inhibition of enzyme kinetics. Table 2.5 shows the values of kinetic constants evaluated from the data of some research (Aiba and Shoda, 1969; Bazua and Wilke, 1977; Cysewski and Wilke, 1976; Egamberdiev and Jerusalimsky, 1968; Hoppe and Hansford, 1982; Pironti, 1971). The data of Cysewski and Wilke (Cysewski and Wilke, 1976) and Pironti (Pironti, 1971) were obtained with exogenous ethanol and gave low values for the product inhibition constant. High values of K_{ipx} having endogenous ethanol show that exogenous ethanol is not as strongly inhibitory as endogenous ethanol (Ghose and Tyagi, 1979; Nagodawithana and Steinkraus, 1976; Navarro and Durand, 1978; Novak et al., 1981; Strehaiano et al., 1978). Hoppe and Hansford (Hoppe and Hansford, 1982) eventually validated their model on the yeast *S. cerevisiae* and showed good fit to the experimental data.

Table 2.5.Kinetic constants and yield in terms of inhibitory effect of exogenous and endogenous ethanol (Hoppe and Hansford, 1982)

S	μ_{max} (h^{-1})	K_x (g L^{-1})	K_{ipx} (g L^{-1})	$Y_{p/s}$	Reference
30, endogenous	0.64	3.3	5.2	0.43	(Hoppe and Hansford, 1982)
28, endogenous	0.31	-	20.6	0.39	(Egamberdiev and Jerusalimsky, 1968)
30, endogenous	0.43	-	55	0.35	(Aiba and Shoda, 1969)
35, exogenous	0.58	4.9	5.0	0.44	(Cysewski and Wilke, 1976)
30, exogenous	0.26	15.5	13.7	0.47	(Pironti, 1971)
35, endogenous	0.64	0.24	40	0.52	(Bazua and Wilke, 1977)

Some models such as Holzberg et al. (1967) and Bazua and Wilke (1977) considered only product inhibition without considering other effective factors such as substrate limitation. Holzberg et al. (Holzberg et al., 1967) used enriched grape juice as the carbohydrate source, and the yeast employed was *S. cerevisiae var.*

ellipsodeus. The study discovered a threshold concentration of ethanol for yeast *S. cerevisiae* below which there was no inhibition and above which inhibition followed a linear pattern (Holzberg et al., 1967). They found that an equation of a linear type could represent their data (Holzberg et al., 1967).

Bazua and Wilke (1977) proposed experimental equations to correlate the growth of *S. cerevisiae* and ethanol production with respect to product concentrations (Bazua and Wilke, 1977). Bazua and Wilke (1977) proposed a two- and a three-parameter equation, where $\bar{P}$ is the average value of product concentration, and k_{ipp1}, k_{ipx1}, k_{ipp2}, k_{ipx2} are empirical constants (Bazua and Wilke, 1977). Their equations in Table 2.4 (Equation 22) show that there is a limiting concentration of P, beyond which the cells will not grow (Bazua and Wilke, 1977).

Most substrates are competitive for utilization; for instance, glucose and xylose are competitive substrates when present together for utilization as a carbon source. (Grootjen et al., 1991a, 1991b, Hanly and Henson, 2013, 2011; Leksawasdi et al., 2001). In this case, inhibition of xylose utilization by glucose should be considered along with other effective factors such as temperature, pressure, pH, and stirring rate. Grootjen and his group using *P. stipitis* (Grootjen et al., 1991b), Hanly and Henson using co-cultures of *E. coli* and *S. cerevisiae* (Hanly and Henson, 2011), Hanly and Henson using co-culture of respiratory-deficient *S. cerevisiae* and wild-type *P. stipitis* (Hanly and Henson, 2013) considered both glucose and xylose as the substrate and added one factor for inhibition of xylose utilization with glucose (catabolite repression) in their model for bioethanol production. Grootjen et al. (1991) validated their model in both batch and continuous culture through experiments under oxygen-limited conditions (Grootjen et al., 1991b). Good agreement was found for glucose and xylose concentration, but not for ethanol and biomass concentration (Grootjen et al., 1991b). Under the simplifying assumption that both microbes in co-cultures grow optimally under common environmental conditions, optimization of the strain inoculum and the aerobic to anaerobic switching time produced an almost twofold increase in ethanol productivity over the pure cultures (Hanly and Henson, 2011). Two years later, Hanly and Henson (Hanly and Henson, 2013) identified the co-culture of respiratory-deficient *S. cerevisiae* and wild-type *P. stipitis* as a promising system for microaerobic ethanol production because *S. cerevisiae* only consumes glucose while *P. stipitis* efficiently converts xylose to ethanol. Hanly and Henson (Hanly and Henson, 2013) used a dynamic co-culture model to predict: i) the inoculum concentration and aeration level that maximized batch ethanol productivity, and ii) how engineered strain improvements to the *P. stipitis* xylose transport system could improve co-culture ethanol production (Hanly and Henson, 2013). The data was validated in a batch co-culture with experiments (Hanly and Henson, 2013).

2B.3.3. Advanced inhibitory effects

During alcoholic fermentation, both ethanol and substrate concentration can prevent the production of ethanol and cells (as it is a growth-associated product), therefore both terms should be considered in a kinetic model. Ghose and Tyagi (Ghose and Tyagi, 1979) observed a linear kinetic pattern for growth and product formation. However, the substrate inhibition term was considered in the final rate expression because the degree of substrate inhibition was higher than ethanol inhibition when grown on pure sugars.

Rogers and his co-workers (Lee and Rogers, 1983) modelled glucose using wild type of *Z. mobilis* and 18 years later in 2001 (Leksawasdi et al., 2001) modelled and validated fermentations on a mixture of glucose/xylose using engineered strain of *Z. mobilis* based on substrate limitation, substrate inhibition, and product (ethanol) inhibition. The model provided good predictions of experimental batch culture data. Huang and Chen (Huang and Chen, 1988) proposed a model of ethanol fermentation subjected to temperature variation and inhibition of substrate and product using *Z. mobilis* ATCC29191. This model is a good model when an optimum temperature is not clear in the process.

It is noteworthy that the model of Ghaly and El-Taweel (Ghaly and El-Taweel, 1997) predicted the cell, lactose, and ethanol concentrations with high accuracy when the model was fitted to experimental data (5 liter continuous bioreactor at 55 ºC using cheese whey as a substrate). The cell concentration, lactose utilization and ethanol production were significantly affected by the hydraulic retention time and initial substrate concentration (Ghaly and El-Taweel, 1997). Krishnan et al. (1995,1999) used kinetic studies to develop a *S. cerevisiae* fermentation model from glucose and mixture of glucose and xylose incorporating the effects of substrate inhibition, product inhibition, and inoculum size (Krishnan et al., 1999, 1995). Using single-substrate kinetics, it was noticed that the specific growth rate of the yeast and the specific ethanol productivity on glucose were greater than what was obtained on xylose as a substrate, but the effect of ethanol inhibition was more pronounced for xylose fermentation than for glucose fermentation (Krishnan et al., 1999). Good agreement was obtained between model predictions and experimental data from the batch fermentation of glucose, xylose, and their mixtures (Krishnan et al., 1999). In addition, Dhabhai et al. (2013) tested equations developed by Krishnan et al. (1995, 1999) using mono- and co-culture of *S. cerevisiae* and *P. stipitis* and found that the ethanol formation and substrate consumption were very close to their experimental data (Dhabhai et al., 2013).

The models of Krishnan and Rogers gave good correlation, however, different microorganisms of *S. cerevisiae* and *Z. mobilis* were used. Both of them showed good agreement between their models and experimental results. There is a significant difference in the product inhibition term between these two models. Glycolysis pathway of *Z. mobilis* ZM4 is based on the Entner–Doudoroff (ED) pathway and *S. cerevisiae* CEN.PK 113-7D metabolisms is based on the Embden–Meyerhof–Parnas (EMP) pathway. The

ED pathway has a faster glucose conversion to ethanol and therefore has smaller effects from the ethanol inhibition term.

Hjersted and Henson (2006), and Rivera et al. (2013) developed a dynamic flux balance model for a fed-batch process using *S. cerevisiae* (Hjersted and Henson, 2006; Rivera et al., 2013). A concise model for a fed-batch process was obtained (Hjersted and Henson, 2006; Rivera et al., 2013). Hjersted and Henson (2006) considered substrate limitation, substrate and product inhibitions (Table 2.4, Equation 36), whereas Rivera et al. (2013) considered cell inhibitions along with substrate limitation, substrate and product inhibitions in their model to describe the fermentation (Table 2.4, Equation 37) and their model provided a good prediction for cell, substrate and ethanol concentrations. Hjersted and Henson (Hjersted and Henson, 2006) performed optimization test to examine the impact of the objective function (weighted sum of ethanol yield and ethanol productivity on glucose), model parameters, and modelling errors on the accuracy and sensitivity of the model.

Most kinetic models are based on the Monod model introducing growth-controlling/limiting substrate terms. However, classical growth models such as Logistic, Gompertz and modified Gompertz models differ by describing kinetics of cell growth. Gompertz model has been used to describe mortality law, medicine for tumor and biology (Gompertz et al., 1825; Jukić et al., 2004), Logistic model describes the kinetics of cell growth in exponential and stationary phase, while the modified Gompertz equation includes the lag time of cell growth as well (Fan et al., 2015; Olaoye and Kolawole, 2013). Although there are some progress for "classical" model and had found a good agreement with experimental data, substrate limitary effect and inhibitory effects of all substrate, ethanol and cell were not considered (Erkmen, 2003; Fan et al., 2015; Ganucci et al., 2018; Olaoye and Kolawole, 2013; Phukoetphim et al., 2017; Puligundla et al., 2018; Sulieman et al., 2018). Also, specifying maximum ethanol rate and lag time for modified Gompertz model was necessary. In Equation 39 of Table 2.4, to have an advanced kinetic model and better agreement with experimental data, the effects of temperature on *S. cerevisiae* growth with combination of Rivera or de Andrade model with Logistic model, and ethanol production from sugarcane under very-high-gravity (VHG) ethanol fermentation by Luedeking-piret model were simulated and good agreement was found in validation with experimental data (Ccopa Rivera et al., 2017; Puligundla et al., 2018).

Logistic model:

$$C_x = \frac{C_{x0}\exp(\mu_{max}\,t)}{1-\left[\left(\frac{C_{x0}}{C_{x,max}}\right)\left(1-\exp(\mu_{max}t)\right)\right]} \qquad (B)$$

Modified Gompertz model:

$$P = P_{p,max}\exp\left[-\exp\left(\frac{r_{e\_m}\exp(1)}{P_{p,max}}\right)(t_L - t) + 1\right] \qquad (C)$$

Lastly, Farias et al. (2014, 2017) focused on the kinetics of ethanol production by *Pichia stipitis* on xylose with the development of a mathematical model (combined Andrews' and Levenspiel's models) considering the effect of substrate and product concentrations on growth rate (Farias et al., 2017, 2014). Experiments were carried out in batch and continuous modes. The kinetic parameters through linear and non-linear regression methods were determined (Farias et al., 2014). The kinetic model fitted satisfactorily with experimental data and by far is one of the better models tested (Farias et al., 2014).

2B.4. Modelling from literature experimental values

Most experiments were done using *S. cerevisiae* (Aiba et al., 1968; Bazua and Wilke, 1977; de Andrade et al., 2012; Galaction et al., 2010; Ghose and Tyagi, 1979; Hjersted and Henson, 2006; Hoppe and Hansford, 1982; Krishnan et al., 1999, 1995) as listed in Table 2.4. *Saccharomyces yeast* 1400 kinetic data for glucose conversion was selected in this study to find the best modified Monod equation among the equations which had good agreement with experimental results in literature (Andrews, 1968; Farias et al., 2017, 2014; Ghaly and El-Taweel, 1997; Ghose and Tyagi, 1979; Grootjen et al., 1991a, 1991b, Hanly and Henson, 2013, 2011; Hjersted and Henson, 2006; Krishnan et al., 1999, 1995; Leksawasdi et al., 2001; Levenspiel, 1972). In this paper, the models are compared to the results of experiments (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995) which were carried out as a batch with a stirring rate of 200 rpm, at 30 °C, and at pH 5.0, incorporating substrate limitation, substrate inhibition, and product inhibition functions. To model the fermentation process of bioethanol production using *Saccharomyces yeast* 1400, the values of the kinetic parameters given in Table 2.6 (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1999, 1995) were used.

Table 2.6.Kinetic constants of *Saccharomyces yeast* 1400 (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1999, 1995)

	Value		Value		Value
μ_{max}	0.524	βx	3.4	K_{iss}	4882.8
K_x	0.572	K_{ipx}	12.25	K'_{iss}	0.0048828
$P_{x,max}$	95.4	P_{ix}	22.9	$\beta's$	1.42
K_{isx}	1127.8	q_{max}	4.34	βs	3.4
K'_{isx}	0.0011278	K_s	1.34	K_{ips}	12.4
$\beta' x$	1.29	$P_{s,max}$	103.03	P_{is}	22.6
				$Y_{p/s}$	0.46

The equations which had good correlation with experimental results in literature, given in Equations 27-38 of Table 2.4, were used to find the best mathematical model for bioethanol production and these results were compared to experimental results where the initial glucose concentration was 80 g L^{-1} (Davis et al.,

2006; Ho et al., 1998) and 100 g L^{-1} (Krishnan et al., 1995) respectively. The glucose concentration, ethanol production and biomass concentration for initial glucose concentration of 80 g L^{-1} are shown in Figure 2.5, Figure 2.6 and Figure 2.7 respectively. The experimental data together with the majority of the models showed a curve typical to a batch fermentation ranging from a lag, exponential/logarithmic and a stationary phase.

Table 2.7.Correlation coefficient results (modelling and experiment)

	Glucose consumption		**Ethanol production**		**Biomass production**	
	Initial glucose concentration (g L^{-1})		**Initial glucose concentration (g L^{-1})**		**Initial glucose concentration (g L^{-1})**	
	80	**100**	**80**	**100**	**80**	**100**
Krishnan (Krishnan et al., 1995)	0.99	0.95	0.94	0.998	-	0.85
Leksawasdi (Lee and Rogers, 1983; Leksawasdi et al., 2001)	0.86	0.81	0.41	0.84	-	0.91
Ghose and Tyagi (Ghose and Tyagi, 1979)	0.38	0.33	0.62	0.19	0.88	0.67
Andrews and Levenspiel (Andrews, 1968; Farias et al., 2014; Levenspiel, 1972)	0.85	0.45	0.96	0.68	0.95	0.13
Grootjen (Grootjen et al., 1991a, 1991b)	0.13	0.49	0.45	0.04	0.69	0.76
Ghaly and EL-Taweel (Ghaly and El-Taweel, 1997)	0.98	0.85	0.99	0.98	0.97	0.18
Hjersted and Henson (Hjersted and Henson, 2006)	0.98	0.85	0.99	0.96	0.75	0.18
Hanly and Henson (Hanly and Henson, 2013, 2011)	0.97	0.84	0.99	0.96	0.83	0.55
de-Andrade or Rivera (de Andrade et al., 2012; Rivera et al., 2013)	0.95	0.85	0.99	0.96	0.86	0.05

There is a good agreement with equations showing an exponential nature for glucose conversion, given by the correlation coefficient or regression coefficient (statistical measure of how close the data in the modelling are to the data in experiment) of 0.9 or higher as defined by Equation D. Similarly, models that predicted glucose conversion well also predicted ethanol production sufficiently when grown with initial glucose concentration of 80 g L^{-1}. However, in modelling biomass production, the models were less accurate and showed larger deviation from experimental data. Ghaly and EL-Taweel (Ghaly and El-Taweel, 1997) or Hjersted and Henson (Hjersted and Henson, 2006) had the best agreement with experiments of 80 g L^{-1} initial glucose concentration using *Saccharomyces yeast* 1400 (Davis et al., 2006; Ho et al., 1998) for substrate consumption with a correlation coefficient value of 0.985, ethanol production with a correlation coefficient value of 0.993, and biomass production with a correlation coefficient value of 0.94 which are given in Figure 2.5, Figure 2.6 and Figure 2.7, respectively and also in Table 2.7.

$$\text{Correlation coefficient (regression coefficient)} = 1 - \frac{\left(\text{Residual sum of squares } \sum_{p=1}^{np}(dp-xp)^2\right)}{\left(\text{Total sum of squares } \sum_{p=1}^{np}(dp-\overline{dp})^2\right)} \quad \text{(D)}$$

Among the selected equations (Andrews, 1968; Farias et al., 2017, 2014; Ghaly and El-Taweel, 1997; Ghose and Tyagi, 1979; Grootjen et al., 1991a, 1991b, Hanly and Henson, 2013, 2011; Hjersted and Henson, 2006; Krishnan et al., 1999, 1995; Leksawasdi et al., 2001; Levenspiel, 1972), Krishnan et al. (1995) (correlation coefficient of 0.95 for substrate consumption, 0.998 for ethanol production), Ghaly and El-Taweel (1997) (correlation coefficient of 0.85 for substrate consumption, 0.98 for ethanol production) or Hjersted and Henson (2006) (correlation coefficient of 0.85 for substrate consumption and 0.96 for ethanol production) had the best agreement with experiment data (Krishnan et al., 1995) for ethanol production and substrate consumption with an initial glucose concentration of 100 g L^{-1} based on the correlation coefficient value, as it is shown in Table 2.7.

In this case, Ghaly and EL-Taweel (Ghaly and El-Taweel, 1997) or Hjersted and Henson (Hjersted and Henson, 2006) showed the closest agreement with experimental data from literature (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995).

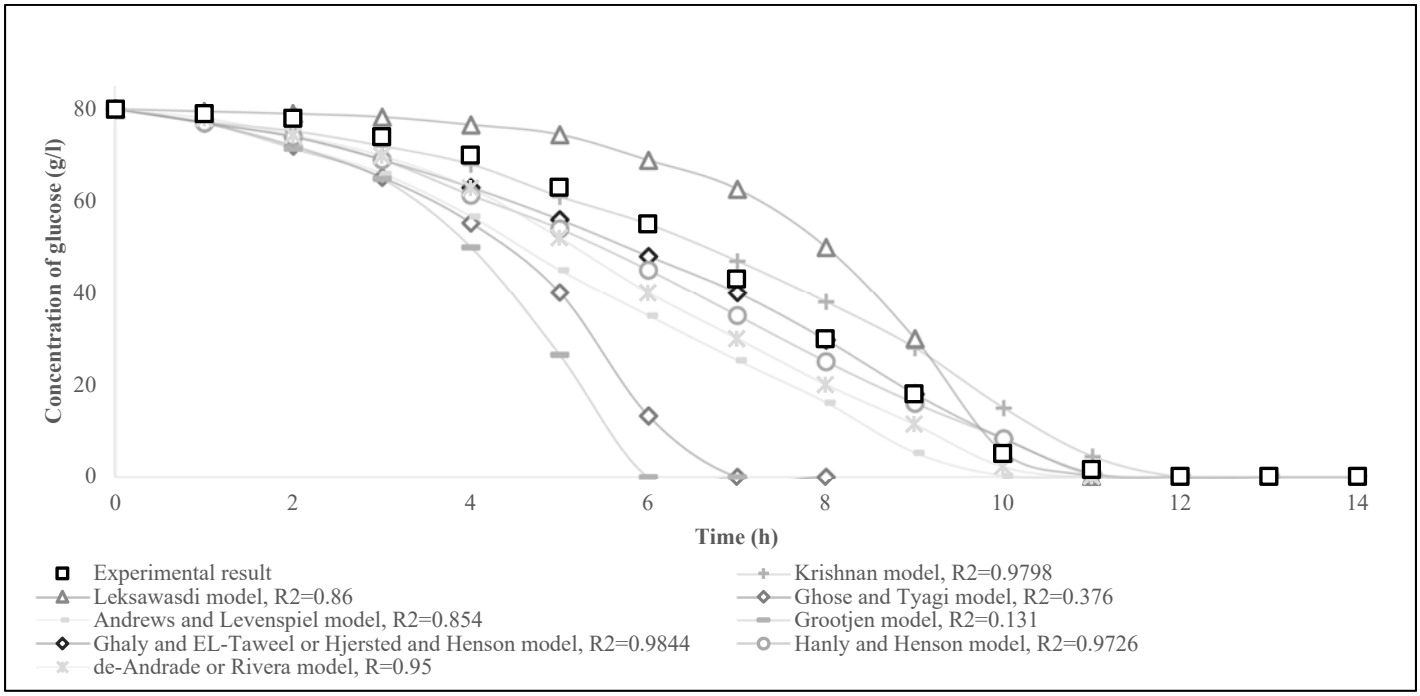


Figure 2.5.Glucose concentration vs time for the initial glucose concentration of 80 g L^{-1} using *Saccharomyces yeast* 1400. The R2 represents correlation coefficient denoted in Equation D. The open square symbol denotes the experimental data (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995). Labels with grey symbols and lines denote the respective models used in the comparison.

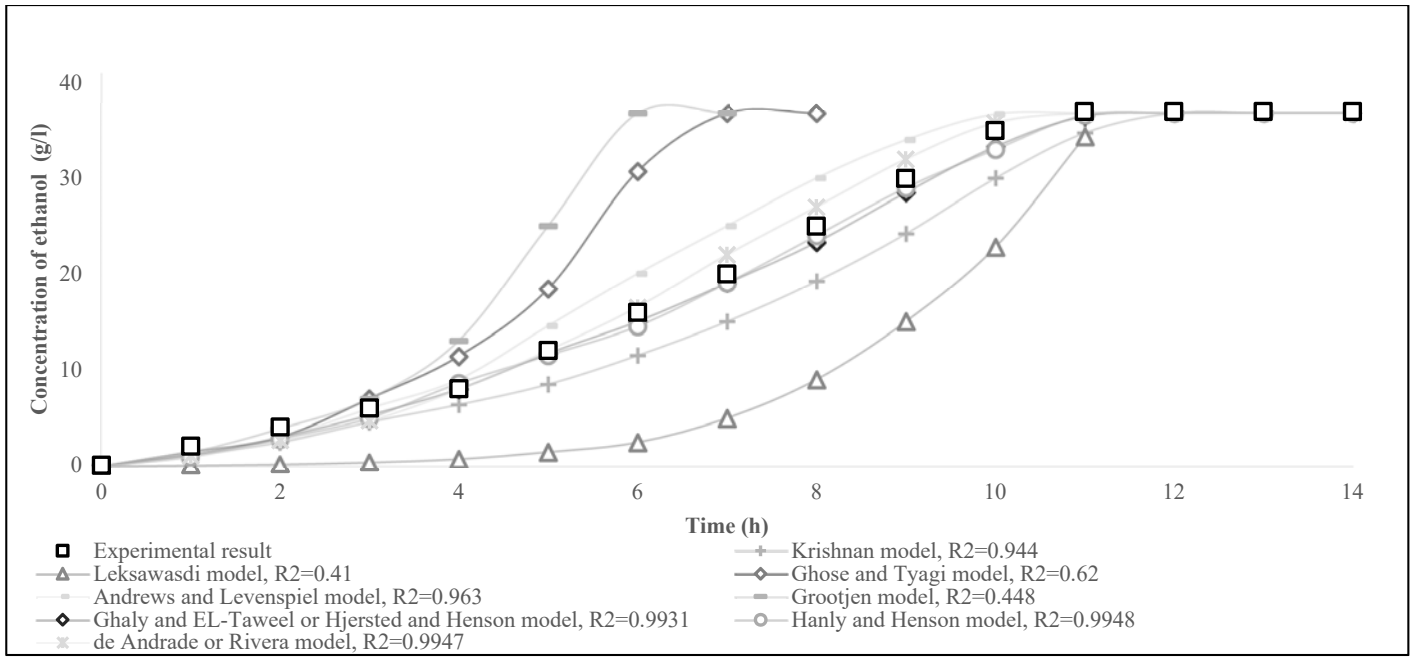


Figure 2.6.Ethanol production vs time for the initial glucose concentration of 80 g L^{-1} using *Saccharomyces yeast* 1400. The R2 represents correlation coefficient denoted in Equation D. The open square symbol denotes the experimental data (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995). Labels with grey symbols and lines denote the respective models used in the comparison.

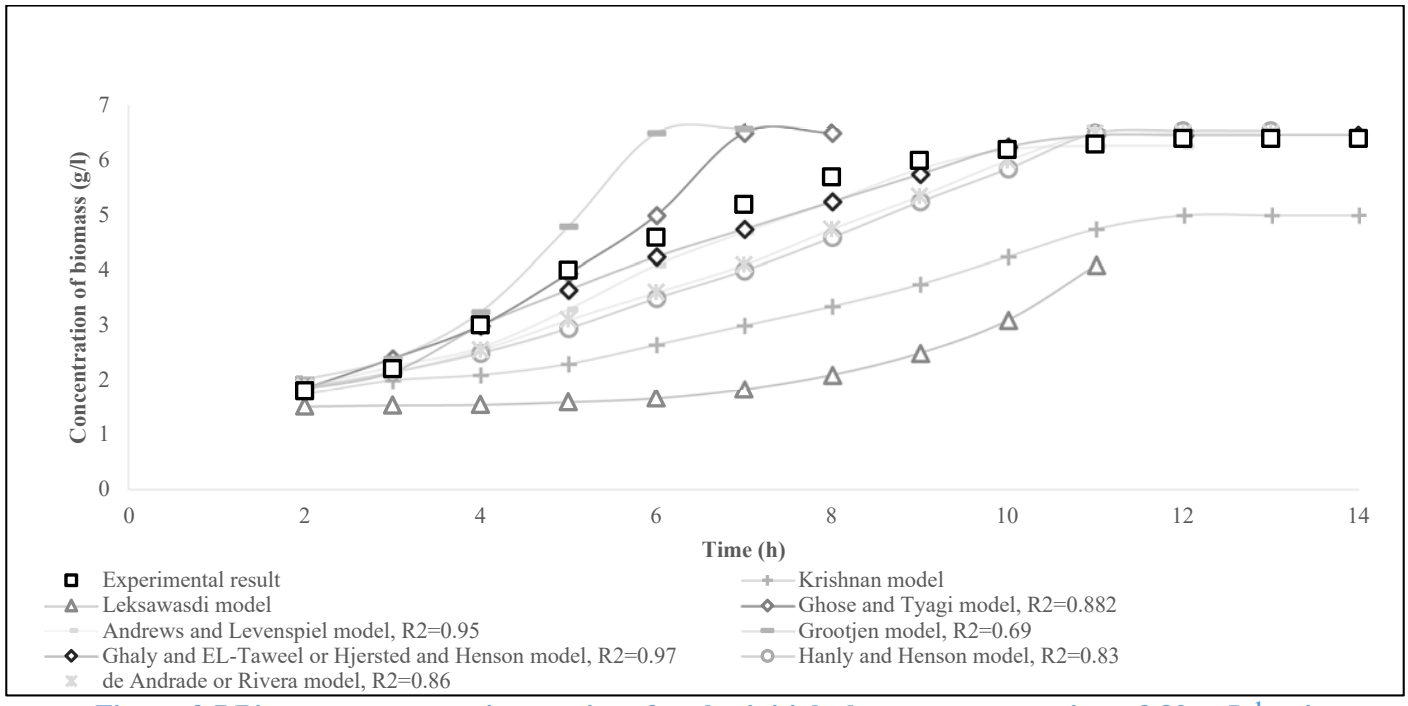


Figure 2.7.Biomass concentration vs time for the initial glucose concentration of 80 g L^{-1} using *Saccharomyces yeast* 1400. The R2 represents correlation coefficient denoted in Equation D. The open square symbol denotes the experimental data (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995). Labels with grey symbols and lines denote the respective models used in the comparison.

2B.5. Conclusion

Among the modified Monod kinetic models that have been studied, considering only one substrate as limiting without considering any inhibition parameter, Contois, Tessier, and Blackman found better agreement with experimental results when looking at the effects of the limiting substrate. However, the Tessier, Contois, Blackman, and Moser models failed to achieve any recognition because these models lack substrate/product inhibition and therefore unable to show good agreement when the substrate/product concentration is higher than its concentration tolerance.

At high substrates/products concentration, substrates/products are inhibitors. Substrate and product inhibitory parameters should therefore be considered, but most studies have only considered either substrate or product inhibition. When looking at limiting and inhibitory effects, Haldane is the simplest model that showed relatively close agreement with experimental data in comparison to Webb, Tessier-type, Aiba, and Yano (Edwards, 1970). Galaction et al. (2010) and Andrews (1968) also used the Haldane equation in their studies and had good agreement with their experimental data (Andrews, 1968; Galaction et al., 2010). The Haldane model eventually assisted in explaining the long lag phases sometimes experienced in batch cultures from inhibitory substrates and demonstrated the possible instability of steady state processes with similar growth conditions (Andrews, 1968).

In order to improve the sensitivity and accuracy of the kinetic models, effective factors of substrate limitation, product inhibition and substrate inhibition were considered. The kinetic models of advanced inhibitory effects (Andrews, 1968; Farias et al., 2017, 2014; Ghaly and El-Taweel, 1997; Ghose and Tyagi, 1979; Grootjen et al., 1991a, 1991b, Hanly and Henson, 2013, 2011; Hjersted and Henson, 2006; Krishnan et al., 1999, 1995; Leksawasdi et al., 2001; Levenspiel, 1972; Sulieman et al., 2018) were compared to experimental data (Davis et al., 2006; Ho et al., 1998; Krishnan et al., 1995) resulting in Ghaly and El-Taweel (Ghaly and El-Taweel, 1997) or Hjersted and Henson (Hjersted and Henson, 2006) showing better agreement with these experimental data (Krishnan et al., 1995). Overall, Ghaly and EL-Taweel (Ghaly and El-Taweel, 1997) or Hjersted and Henson (Hjersted and Henson, 2006) is a useful equation that can be used with varying conditions to obtain an acceptable result when fermentation conditions are unavailable in literature. However better agreement between modelled and experimental results can be achieved in specific conditions in terms of the objective of the model, specific microorganism and process conditions with further studies on additional factors that may affect fermentation processes such as substrate utilization for maintenance energy and factoring in time-delay-response constants to changes in substrate and product concentrations.

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2B.7. Declaration of interest

The authors declare no competing financial interest.

2B.8. Nomenclature:

C_o: Oxygen concentration (mass/unit volume)

$C_{AC,max}$: Maximum Acetic Acid concentration in substrate above which cells do not grow (mass/unit volume)

C_{Ac}: Acetic acid concentration (mass/unit volume)

C_x: Cell concentration in medium (mass/unit volume)

C_{x0}: Initial cell concentration in medium (mass/unit volume)

$C_{x,max}$: Maximum cell concentration in medium (mass/unit volume)

dp: Predicted values of experimental data

$\overline{dp}$: Average of experimental values

J: Order of inhibition by substrate (dimensionless)

$K_{x:}$ Substrate limitation constant for biomass production rate (mass/unit volume)

$K_{xb:}$ Substrate limitation constant for biomass production rate based on Blackman model (mass/unit volume)

$K_{x,glu:}$ Glucose limitation constant for biomass production rate (mass/unit volume)

$K_{x,xyl:}$ Xylose limitation constant for biomass production rate (mass/unit volume)

K_p: Substrate limitation constant for product formation rate (mass/unit volume)

$K_{p,glu}$: Glucose limitation constant for product formation rate (mass/unit volume)

$K_{p,xyl:}$ Xylose limitation constant for product formation rate (mass/unit volume)

$K_{o:}$ Oxygen limitation constant of substrate for biomass production rate (mass/unit volume)

$K_{o,glu:}$ Oxygen limitation constant of glucose for biomass production rate (mass/unit volume)

$K_{o,xyl}$: Oxygen limitation constant of xylose for biomass production rate (mass/unit volume)

$K'_{isx,}$ K_{isx}, K_{isx1}, $K_{isx2:}$ Substrate inhibition constant for biomass production rate (mass/unit volume)

$K'_{isp,}$ K_{isp}: Substrate inhibition constant for product formation rate (mass/unit volume)

$K'_{iss,}$ K_{iss}: Substrate inhibition constant for substrate uptake rate (mass/unit volume)

$K_{ipx:}$ Product inhibition constant of substrate for biomass production rate (mass/unit volume)

$K_{ipx,glu:}$ Product inhibition constant of glucose for biomass production rate (mass/unit volume)

$K_{ipx,xyl}$: Product inhibition constant of xylose for biomass production rate (mass/unit volume)

K_{ipp}: Product inhibition constant of substrate for product formation rate (mass/unit volume)

$K_{ipp,glu}$: Product inhibition constant of glucose for product formation rate (mass/unit volume)

$K_{ipp,xyl}$: Product inhibition constant of xylose for product formation rate (mass/unit volume)

k_{ipp1}, k_{ipx1}, k_{ipp2}, $k_{ipx2:}$ Empirical constants

$K_{isg:}$ Inhibition constant of xylose by glucose for substrate uptake rate (mass/unit volume)

K_{ixg}: Inhibition constant of xylose by glucose for biomass production rate (mass/unit volume)

K_{ipg}: Inhibition constant of xylose by glucose for product formation rate (mass/unit volume)

K_D: Empirical constants (cell wall permeability, substrate diffusion, and cell size) (mass/unit volume)

K_{P1}, K_{P2}: Production rate constants (mass/unit volume)

m_p: Product maintenance coefficient (1/unit time)

n: Kinetic constant in Moser model (dimensionless)

P: Product concentration (mass/unit volume)

$\bar{P}$: Average of product concentration (mass/unit volume)

P_{ip}: Inhibition of product to growth for product formation from substrate (mass/unit volume)

P_{ix}: Inhibition of product to growth of biomass from substrate (mass/unit volume)

P_0: Input product concentration (mass/unit volume)

P_f: Output product concentration (mass/unit volume)

$P_{x,max}$: Maximum product concentration in substrate above which cells do not grow (mass/unit volume)

$P_{p,max}$: Maximum product concentration in substrate above which cells do not produce product (mass/unit volume)

$r_{e,m}$: Maximum ethanol production rate (mass/unit volume/unit time)

S_{xyl}: Xylose concentration (mass/unit volume)

S_{glu}: Glucose concentration (mass/unit volume)

S_c: Threshold substrate concentration below which the organism grows apparently without inhibition (mass/unit volume)

S: Substrate concentration (mass/unit volume)

S_0: Input substrate concentration (mass/unit volume)

S_f: Output substrate concentration (mass/unit volume)

S_{ip}: Inhibition of substrate for product formation from substrate (mass/unit volume)

S_{ix}: Inhibition of substrate to growth of biomass from substrate (mass/unit volume)

$S_{x,maxl}$: Maximum substrate concentration in substrate above which cells do not grow (mass/unit volume)

t: Time

t_L: Lag time (time)

τ: Residence time (time)

X: Cell concentration in medium (mass/unit volume)

$X_{x,max}$: Maximum biomass concentration in substrate above which cells do not grow (mass/unit volume)

X_p: Predicted values of model

Y_{sp},$Y_{p/s}$: Product yield constant from substrate (g-product/g-substrate)

$Y_{sx:}$ Cell yield constant from substrate (g-cells/g-substrate)

μ_{max}: Maximum specific growth rate in substrate (1/unit time)

$\mu_{max,xyl}$: Maximum specific growth rate in xylose (1/unit time)

$\mu_{max,glu}$: Maximum specific growth rate in glucose (1/unit time)

$\mu_{:}$ Specific growth rate in substrate (1/unit time),

$\mu_{xyl:}$ Specific growth rate in xylose (1/unit time),

μ_{glu}: Specific growth rate in glucose (1/unit time)

$\nu_{max:}$ Maximum specific rate of product formation in substrate (1/unit time)

$\nu_{max,xyl:}$ Maximum specific rate of product formation in xylose (1/unit time)

$\nu_{max,glu}$: Maximum specific rate of product formation in glucose (1/unit time)

ν: Specific rate of product formation in substrate (1/unit time)

ν_{xyl}: Specific rate of product formation in xylose (1/unit time)

ν_{glu}: Specific rate of product formation in glucose (1/unit time)

α: Correction factor (optional, dimensionless)

γx: Substrate inhibition constant for growth of biomass (dimensionless)

β'x, βx: Product inhibition constant in substrate for growth of biomass (dimensionless)

γx: Biomass inhibition constant in substrate for growth of biomass (dimensionless)

mx: Acetic acid inhibition constant in substrate for growth of biomass (dimensionless)

np: Number of experimental points

β'p, βp: Product inhibition constant in substrate for product formation (dimensionless)

β's, βs: Product inhibition constant in substrate for substrate uptake (dimensionless)

αx: Biomass inhibition constant in substrate for growth of biomass (dimensionless)

γp: Substrate inhibition constant for substrate uptake (dimensionless)

ω: Degree of substrate inhibition for growth (dimensionless)

ω': Degree of substrate inhibition for product formation (dimensionless)

ϕ: Coefficient of consumption activity (dimensionless)

2B.9. References

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3. Research approach

Top-fermenting microorganisms such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* are the benchmark microorganisms for bioethanol production (Harun et. al., 2010). Both of these microorganisms have a high ethanol tolerance and yield, but are unable to ferment xylose to ethanol (Harun et. al., 2010; Kotter and Ciriacy, 1993). Some yeast such as *Pichia stipitis* and *Kluyveromyces marxianu*s on the other hand, can ferment both xylose and glucose sugars, but the ethanol yields are substantially lower than those seen in *S. cerevisiae* or *Z. mobilis*. This is due to varying complexity in metabolism, for instance, pentoses having to be taken through an additional pathway - the pentose phosphate pathway prior to glycolysis (Slininger et al., 1987), the sensitivity of these microorganisms to low concentrations of inhibitors commonly found in hydrolysates (i.e., acetate), the inability to grow without oxygen, the ability to produce ethanol only in an optimum oxygen level, and a fairly low ethanol tolerance. Since no suitable microorganism is available to convert both xylose and glucose to ethanol efficiently, co-culturing and sequential culturing have been explored, in tandem with construction of genetically engineered microorganisms.

In co-culture experiments, the combined utilization of two microorganisms may show advantages due to synergistic action of their metabolic pathways (Bader et al., 2010). Co-culture has shown slow xylose fermentation compared to glucose because of catabolite repression, low ethanol tolerance of the xylose-fermenters and varying oxygen requirements between the combinations of microorganisms (Grootjen et al., 1991; Hamidimotlagh et al., 2007; Kordowska-Wiater and Targoński, 2002; Laplace et al., 1993; Lebeau et al., 1997). Genetic modifications have therefore been considered and have enabled increased substrate range or modified metabolism for xylose fermentation. Recombinant *E. coli* and *Z. mobilis* are the most suitable strains for use with hemicellulosic hydrolysates (Lawford and Rousseau, 1991). Despite developments using engineered strains, limitations remain with limited research on the metabolic network model. Alternatively, sequential fermentation, where microorganisms are cultured in series rather than simultaneously, allows for efficient conversion of pentoses and hexoses to ethanol in a simple and powerful approach (Grootjen et al., 1991) by permitting hexose to be utilized first followed by pentose conversion. However, sequential cultures are mostly batch processes with optimization constraints.

Manipulation and optimizing process conditions such as initial total sugar concentrations, ratio of glucose to xylose, aeration conditions, and immobilization technique offer an alternative or extension to improving yields and productivity of bioethanol through enhancing microbial capacity in sequential cultures.

3.1. Objective

The purpose of this research is to optimize the co-fermentation process for the maximum production of bioethanol from mixed glucose and xylose using free and immobilized cultures through experimental work and modelling.

The objectives of this project are divided into five stages.

1) To determine the suitable bio-kinetic models, considering required kinetic parameters of sugar utilization, oxygen utilization (where applicable), ethanol production, and biomass growth for the microbial cultures of interest,

2) To determine optimal values for key process parameters that result in the closest fit with the experimental observations to develop and test a modelling approach

3) To model the batch and continuous fermentation system for fermentation of glucose only, xylose only, and a mixture of glucose and xylose,

4) To evaluate the batch and continuous fermentation process experimentally using planktonic cultures and glucose only, xylose only, and mixture of glucose and xylose as a carbon source,

5) To manipulate process conditions such as immobilization technique, high inoculum sizes, aeration rates and stirrer speed experimentally and to compare ethanol yields and productivities of these conditions tested.

3.2. Hypothesis

This study was therefore defined by the following central hypothesis:

3.2.1. Segregation of free or immobilized *Z. mobilis* for glucose conversion from free or immobilized *P. stipitis* for xylose conversion into bioethanol in two stage batch fermentation in one reactor (sequential batch culture) will result in better ethanol yield, productivity and tolerance than existing co-fermentation results.

3.2.2. Segregation of immobilized *Z. mobilis* for glucose conversion from free *P. stipitis* for xylose conversion into bioethanol in two stage continuous fermentation in two reactors in series will result in better ethanol yield, productivity and tolerance than existing co-fermentation results and of sequential batch cultures.

3.2.3. Modelling using kinetic data with optimization from free and immobilized culture experimental work will allow for a working model to determine the optimal dilution rate, oxygenation level, and initial sugar concentration for bioethanol production.

3.2.4. The model and optimization solved in Aspen Plus with Aspen Custom Modeller (ACM) and MATLAB will describe and predict the concentration of glucose, xylose, ethanol, biomass and oxygen in a batch and continuous process by free cultures correctly compared to experimental results.

3.3. Key Questions

The following key questions will be addressed by investigating the hypotheses outlined above.

3.3.1. How do immobilized cultures affect ethanol fermentation performance in comparison to free cultures?

3.3.2. How does sparging oxygen in free and immobilized *P. stipitis* reactor affect ethanol fermentation performance?

3.3.3. What are the optimal conditions for the oxygenation level, initial sugar concentration, dilution rate in maximizing bioethanol production from xylose and glucose using free and immobilized cultures in batch and continuous process?

3.3.4. What are the ethanol concentration, biomass concentration, and substrate concentration in batch and continuous process under optimum performance in experimental work and modelling?

3.3.5. What are the conversion rates of glucose and xylose to ethanol and ethanol yields under optimum performance in experimental work and modelling?

3.4. Approach

The approach of this Ph.D. is to address the above-mentioned objectives and is divided into three parts - the preliminary set-up, modelling set-up and experimental set-up.

3.4.1. Preliminary set-up

To improve the sensitivity and accuracy of the kinetic models for better agreement between modelled and experimental results, effective factors of substrate limitation (e.g. sugars and oxygen), product inhibition, and substrate inhibition (e.g. sugars and oxygen) were considered. Finding the suitable kinetic model and the missing kinetic parameters for mathematical modelling for *P. stipitis and Z. mobilis* based on literature or, where necessary, through culture experiments is necessary. Suitable kinetic models for both *Z. mobilis* (Rogers model, Equation 31 in Table 2.4) and *P. stipitis* (Andrews and Levenspiel model, Equation 38 in Table 2.4) were found from literature and are available in Chapter 2B as major available kinetic models for fermentation of bioethanol production from glucose and/or xylose were reviewed in this chapter. Some literature experimental data (e.g. on *Z. mobilis* ZM4 (pZB5), *S. cerevisiae* CEN.PK 113-7D, *S. cerevisiae* RWB217) were used in Chapter 4.3 to find the kinetic parameters of bioethanol fermentation from glucose using *Z. mobilis*. The necessary information to set up a bio-kinetic model (e.g. kinetic parameters) and mass transfer considering oxygen transfer of bioethanol fermentation from xylose using *P. stipitis* which were not available from literature were found from experiments and are mentioned in detailed in Chapter 5, Table 5.1.

3.4.2. Modelling set-up

The batch and continuous fermentation systems for bioethanol production from pure glucose, pure xylose, and the mixture of glucose and xylose using *Z. mobilis* for glucose conversion and *P. stipitis* for xylose conversion were modelled. A modelling approach to determine optimal values for key process parameters that resulted in the closest fit to experimental observations were developed and tested. These are addressed in Chapter 2, (select the best model to be fitted with experimental data when the process conditions are not specified in fermentation for bioethanol production from C5 or/and C6), Chapter 4 (modelling the ethanol, biomass and substrate concentration over time in batch process for bioethanol fermentation from glucose using *S. cerevisiae* and *Z. mobilis* to find kinetic parameters dependency between wild and engineered strains of the same culture), Chapter 5 (Novel model to find the ethanol, biomass and substrate concentration over time in batch process for bioethanol fermentation from xylose using *P. stipitis*), and Chapter 6 (novel custom kinetic models of bioethanol fermentation, developed in Aspen Custom Modeller within Aspen Plus instead of a stoichiometry model in Aspen Plus).

3.4.3. Experimental set-up

The batch fermentation model from glucose for bioethanol production using *Z. mobilis* were evaluated and validated experimentally in Chapter 4. The new batch fermentation model using *P. stipitis* was validated experimentally in Chapter 5. Process conditions such as using immobilization techniques, process configuration and high inoculum sizes were manipulated to increase ethanol yield and productivity in Chapter 7. The biomass, ethanol and substrate concentration over time for fermentation of bioethanol production from the mixture of glucose and xylose were found based on the experimental work of this study. Experimental data of this study for fermentation of bioethanol production with two reactors in series using immobilized *Z. mobilis* in CSTR/fluidized-bed-reactor in the first reactor and free *P. stipitis* in CSTR in the second reactor were compared with experimental data of other co-fermentation methods for bioethanol production. Optimized process conditions were determined from the available literature experimental results, experimental study of this study and mathematical model. Experiments were carried out in this study to validate the model for ethanol yields and productivities.

3.5. Limitations of the study

Fermentation limitations include:

- Using pure glucose/xylose in media instead of pre-treated/hydrolysed substrate
- Considering idealized condition for simplicity (e.g. homogeneity in fermentor)
- Fragile immobilized cells, bead damage, bead breakage with stirrer,

- Effects of heat on ethanol evaporation during removal of glucose-fermenting microorganisms in sequential batch culture process,
- Effect of adding $CaSO_4.2H_2O$ to prevent bead damage on product, handling of activating and adaptability of immobilized cell.

Modelling limitations include:

- Handling of solid components in Aspen Custom Modeller (ACM),
- Availability of bio-components such as microorganisms in ACM,
- Handling of converting dynamic processes from ACM to Aspen Plus.

3.6. Novel & substantial contribution

There is novelty in the intra (wild type vs engineered) and inter species (*S. cerevisiae* vs *Z. mobilis*) kinetic parameters dependency for bioethanol production with glucose and/or xylose as a carbon source when the same xylose utilization pathway is engineered in these strains (Chapter 4). There is novelty in modelling of effective factors of substrate limitation, substrate inhibition, ethanol inhibition, oxygen limitation and oxygen inhibition on growth rate simultaneously for bioethanol production using *P. stipitis* from xylose (Chapter 5). There was also novelty in finding the optimum oxygenation aeration level for xylose conversion using *P. stipitis* through experimental observation and considering the oxygen term in the model (Chapter 5).

From the development of the mathematical kinetic model, there are new findings with the custom kinetic models of bioethanol fermentation developed in Aspen Custom Modeller, within Aspen Plus, instead of a stoichiometry model in Aspen Plus alone (Chapter 6).

Co-fermentation for bioethanol production with immobilized *Z. mobilis* and free *P. stipitis* in a continuous process in two reactors (immobilized *Z. mobilis* in first CSTR/fluidized-bed-reactor and free *P. stipitis* in second CSTR) presents a novel approach in maximizing productivity for ethanol (Chapter 7).

3.7. References:

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4. Kinetic model and kinetic parameters of *Z. mobilis* for bioethanol fermentation using wild and engineered strains from glucose and xylose

Kinetic parameters of a specific process are frequently not available in literature, leading to tedious experimental work to establish these parameters. In this study, relationships in substrate and product kinetics between two different genera of microorganisms were explored. The idea behind this study was to determine if a correction factor exist when a pathway is genetically engineered into a microorganism, and if this correction factor is persistent across different microorganisms under such modification. Here, the intra (wild type vs engineered) and inter species (*S. cerevisiae* vs *Z. mobilis*) kinetic parameters relationship for bioethanol production with glucose and/or xylose as a carbon source when the same xylose utilization pathway is engineered in these strains were examined.

The two types of microorganisms with an engineered pentose metabolism pathway were selected: 1) *Saccharomyces cerevisiae* CEN.PK 113-7D vs RWB 217 (xylose isomerase gene from the fungus *Piromyces sp.* E2 expressed), 2) *Zymomonas mobilis* ZM4 vs ZM4 (pZB5) (xylose isomerase gene from the bacteria *Escherichia coli* expressed).

The following publication presents the novel approach in relating the changes in kinetic constant values between wild type and engineered strains of the same genus and eventually using these set of correction factors generated to estimate kinetic constant values of other genera addressing objectives (1), (2) and (3) of this Ph.D. thesis project. This paper is under preparation to be published in the Biochemical Engineering Journal.

Author contribution:

Supervisor Dr. Siew L. Tai (SLT) put forward the idea of this study. The manuscript was written by Nosaibeh Nosrati Ghods (NNG) and edited by Prof. Susan T.L. Harrison (STLH), Associate Prof. Adeniyi J. Isafiade (AJI) and SLT. STLH and SLT guided in design of research approach and in interpretation of findings. STLH funded the entire cost of the experimental work. Technical assistance during the commissioning stage was provided by STLH and SLT. The primary author designed the study, performed the experiments, developed the model and statistically analysed the data used to validate the model. NNG encoded the model in Matlab and provided the model output. The financial support of the National Research Foundation of South Africa Competitive Programme for Rated Researchers (CPRR: 87744), kindly prepared by Prof. Duncan M. Fraser and Associate Prof. Adeniyi J. Isafiade; the financial support of the research development grant from the University Research

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Paper title:

Kinetic data analysis and mathematical modelling of intra (wild type vs engineered) and inter species (*Saccharomyces cerevisiae* vs *Zymomonas mobilis*) dependency for bioethanol production from glucose or/and xylose

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Abstract

The kinetic data for bioethanol production using wild type and engineered strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis* with glucose, and glucose-xylose mixtures were investigated. Linear and non-linear regression methods were used to obtain predicted rates and kinetic parameters in MATLAB. The purpose of this study is to investigate intra (wild type vs engineered) and inter (*S. cerevisiae* vs *Z. mobilis*) species kinetic parameters relationship for bioethanol production with glucose and/or xylose as a carbon source when the same xylose utilization pathway is engineered in these strains. Here, kinetic constants of wild type and engineered strains of *S. cerevisiae* obtained were compared and resulted in a set of correction factors due to the introduction of the xylose pathway in the engineered strain (intra species dependency). These correction factors were then used to determine process parameters *in silico* (e.g. biomass and product rates) of wild type *Z. mobilis* from experimental kinetic parameters obtained from an engineered *Z. mobilis* strain presented in the literature (inter species dependency). The estimated process parameters for wild type *Z. mobilis* were validated using experimental data generated by the authors and showed good correlation. Results showed a relationship between wild type and engineered strains of the same microorganism. For example, the substrate limitation constants in the engineered strain of xylose ($K_{s,xyl}$, $K_{p,xyl}$, $K_{x,xyl}$) and of glucose ($K_{s,glu}$, $K_{p,glu}$, $K_{x,glu}$) were nine and three times higher in the engineered strain compared with that of the wild type ($K_{s,glu}$, $K_{p,glu}$, $K_{x,glu}$) respectively. This study suggest that these correction factors are consistent and can be used as a guide to estimate kinetic constant values for species or strains for which they are not available.

Keywords: bioethanol, *Saccharomyces cerevisiae, Zymomonas mobilis*, fermentation, kinetics

4.1. Introduction

The use of suitable mathematical models, for example, kinetic models, to define the performance of bioethanol fermentation has been accepted to be essential because it can lessen the number of experiments required and quantitatively define *in silico* optimization, design, and control (Torres et al., 1997). Furthermore, mathematical models can be used to forecast the outcome of complex biochemical networks (Torres and Voit, 2002). Various kinetic models have been proposed to describe the dynamic behaviour of bioethanol production from experimental data quantitatively using mono-sugar with different type of microorganisms (Aiba et al., 1968; Andrews, 1968; Bailey and Ollis, 1986; Bazua and Wilke, 1977; Birol et al., 1998; Blanco et al., 2006; Doble et al., 2004; Edwards, 1970; Galaction et al., 2010; Han and Levenspiel, 1988; Hinshelwood, 1946; Holzberg et al., 1967; Hoppe and Hansford, 1982; Jerusalimsky and Neronova, 1965; Levenspiel, 1972; Melick et al., 1987; Monod, 1949, 1942). Several researchers carried out kinetic studies for bioethanol production from mixed sugars using co-culture, sequential culture or engineered strains (Ghaly and El-Taweel, 1997; Ghose and Tyagi, 1979; Hanly and Henson, 2013, 2011; Hjersted and Henson, 2006; Huang and Chen, 1988; Krishnan et al., 1999; Leksawasdi et al., 2001; Rivera et al., 2013). None of the proposed models thus far have analysed differences of kinetic constants between the wild type and engineered strains of the same species.

Kinetic modelling is an iterative process between experimental work and *in silico* modelling. If the model does not predict the experimental results, the assumptions or structure of effective factors should be adjusted. Hence, finding a suitable model and kinetic constants through experimental work is a time-consuming and costly process. In this study, the relationship between kinetic constants of one species of the microorganism with different constructs such as a wild type or an engineered strain was examined. Sets of correction factors were generated from the difference in metabolism due to the engineered pathway. It is postulated that this set of correction factors can be used to estimate kinetic parameters intra and cross species.

A kinetic study on bioethanol production using glucose as the sole carbon source in wild type *Z. mobilis* ZM4 (ATCC 31821) and *S. cerevisiae* CEN.PK 113-7D; and a mixture of glucose and xylose in engineered strains of *Z. mobilis* ZM4 (pZB5) and *S. cerevisiae* RWB 217 were carried out. It is worth noting that *Z. mobilis* ZM4 uses the Entner-Doudoroff (ED) pathway, fermenting glucose more efficiently and rapidly than *S. cerevisiae* CEN.PK 113-7D in which glucose is assimilated through the Embden-Meyerhof-Parnas (EMP) pathway (Figure 4.1) (Bothast et al., 1999; Dien et al., 2003; Kuyper et al., 2005a, 2005b; Matsushika et al., 2009; Senthilkumar and Gunasekaran, 2005; Zhang et al., 1995). The ED pathway yields half the ATP per mole of glucose compared to the EMP pathway (Zhang et al., 1995). Hence, *Z. mobilis*

produces less biomass than *S. cerevisiae* but maintains a high glucose flux through the ED pathway, channelling more carbon towards fermentation products (Zhang et al., 1995). This difference between the glycolysis pathway of *Z. mobilis* and *S. cerevisiae* results in different product inhibition terms in their mathematical models which may increase the complexity in developing and comparing kinetic models between these two microorganisms.

There are mostly two different strategies in pentose pathway to convert D-xylose to D-xylulose (Moysés et al., 2016; Stephens et al., 2007; Wang et al., 1980):

i) incorporating the xylose reductase (XR or *XYL1*) and xylitol dehydrogenase (XDH or *XYL2*) (Hahn-Hägerdal et al., 1994; Rizzi et al., 1989; Verduyn et al., 1985),

ii) producing the xylose isomerase (XI or *xyl*A) as shown in Figure 4.1 (Amore et al., 1989; Hahn-Hägerdal et al., 2007; Walfridsson et al., 1996).

In *S. cerevisiae* RWB 217 the xylose isomerase gene from the fungus *Piromyces sp.* E2 was expressed. The native genes for the conversion of xylulose to the glycolytic intermediates as shown in Figure 4.1 were overexpressed (Kuyper et al., 2005b). The overexpressed enzymes were xylulokinase, ribulose 5-phosphate isomerase, ribulose 5-phosphate epimerase, transketolase and transaldolase (Kuyper et al., 2005a). In *Z. mobilis* ZM4 (pZB5), the *Escherichia coli* genes for production of xylose isomerase, xylulokinase, transketolase, and transaldolase were introduced into the pZB5 plasmid as shown in Figure 4.1 (Leksawasdi et al., 2001).

Initially, the kinetic parameters of *S. cerevisiae* RWB 217 and *S. cerevisiae* CEN.PK 113-7D were determined from literature experimental data using non-linear regression. The differences in the magnitude of the kinetic parameters of wild type versus the engineered strain were determined from these estimated kinetic constants and resulted in a set of correction factors. The kinetic parameters of engineered strain *Z. mobilis* ZM4 (pZB5) were then determined from literature experimental data using non-linear regression. The calculated correction factors from the difference between wild type *S. cerevisiae* CEN.PK 113-7D & engineered strain *S. cerevisiae* RWB 217 were then used to estimate kinetic parameters of *Z. mobilis* ZM4 from the estimated kinetic parameters of *Z. mobilis* ZM4 (pZB5). The estimated kinetic constants of wild *Z. mobilis* ZM4 were finally validated with experimental work carried out in this study and with experimental data from literature.

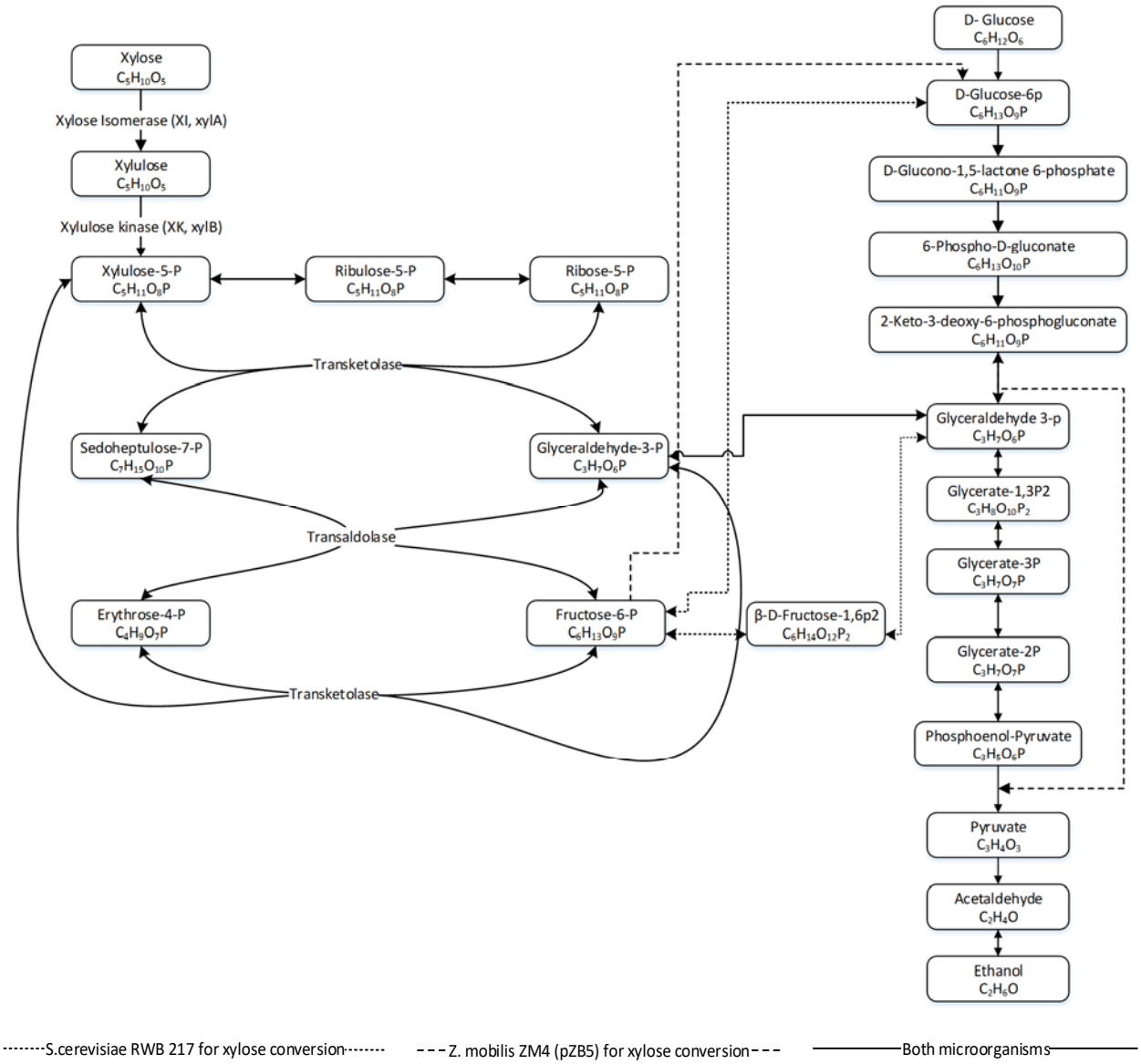

Figure 4.1.Metabolism in selected recombinant and wild type *S. cerevisiae*, and *Z. mobilis* (Bothast et al., 1999; Dien et al., 2003; Kuyper et al., 2005a, 2005b; Matsushika et al., 2009; Senthilkumar and Gunasekaran, 2005; Zhang et al., 1995). Dashed lines denote *Z. mobilis*, dotted lines denote *S. cerevisiae* and solid lines denote both microorganisms.

4.2. Materials and Methods

4.2.1. Kinetic models

The kinetic models of Rogers and his groups (Lee and Rogers, 1983; Leksawasdi et al., 2001) were used for *Z. mobilis* and kinetic model of Krishnan and his groups (Krishnan et al., 1999, 1995) were used for *S. cerevisiae.* The initial concentration values of sugars, biomass and ethanol using wild type and engineered strain of *S. cerevisiae* and *Z. mobilis* are given in Table 4.1.

Table 4.1.Initial concentrations of *S. cerevisiae* and *Z. mobilis* strains from literature experimental results which was used for modelling of this study

	Wild type *S. cerevisiae* CEN.PK 113-7D (g L^{-1}) [1]	Engineered strain *S. cerevisiae* RWB 217 (g L^{-1}) [2]		
C_{glu0}/C_{xyl0}	20.0/0.0	20.0/20.0		
P_0	0.01	0.01		
C_{x0}	0.10	0.20		
	Wild type *Z. mobilis* ZM4 (g L^{-1}) [3]	**Engineered strain *Z. mobilis* ZM4 (pZB5) (g L^{-1}) [4]**		
C_{glu0}/C_{xyl0}	100.0, 150.0, 200.0/0.0, 0.0, 0.0	25.1/27.7	51.1/51.0	59.3/63.2
P_0	0.010	1.410	2.840	3.830
C_{x0}	0.250	0.028	0.017	0.003

[1](Kuyper et al., 2005a, 2005b); [2](Kuyper et al., 2005a, 2004, 2003); [3](Lee and Rogers, 1983); [4](Leksawasdi et al., 2001)

4.2.1.1. Biomass production rate

The specific biomass growth rate for microorganisms of *Z. mobilis* and *S. cerevisiae* for glucose and xylose are represented by equations (1), (2), (3), and (4). In wild type, glucose conversion equation was used, but in engineered strain, both equations of glucose and xylose that lead to equation (5) to find specific biomass growth rate for mixed glucose and xylose were used. Biomass growth rate was then calculated from equation (6).

Specific biomass growth rate, *Z. mobilis* (Entner–Doudoroff (ED) pathway):

$$\mu_{glu} = \left(\frac{\mu_{m,glu} C_{glu}}{K_{x,glu} + C_{glu}}\right)\left(\frac{K_{ix,glu}}{K_{ix,glu} + C_{glu}}\right)\left(1 - \frac{P - P_{ix,glu}}{P_{m,x,glu} - P_{ix,glu}}\right) \quad (1)$$

$$\mu_{xyl} = \left(\frac{\mu_{m,xyl} C_{xyl}}{K_{x,xyl} + C_{xyl}}\right)\left(\frac{K_{ix,xyl}}{K_{ix,xyl} + C_{xyl}}\right)\left(1 - \frac{P - P_{ix,xyl}}{P_{m,x,xyl} - P_{ix,xyl}}\right) \quad (2)$$

Specific biomass growth rate, *S. cerevisiae* (Embden–Meyerhof–Parnas (EMP) pathway):

$$\mu_{glu} = \left(\frac{\mu_{m,glu} C_{glu}}{K_{x,glu} + C_{glu}}\right)\left(\frac{K_{ix,glu}}{K_{ix,glu} + C_{glu}}\right)\left(1 - \left(\frac{P}{P_{m,x,glu}}\right)^{\beta x}\right) \quad (3)$$

$$\mu_{xyl} = \left(\frac{\mu_{m,xyl} C_{xyl}}{K_{x,xyl} + C_{xyl}}\right)\left(\frac{K_{ix,xyl}}{K_{ix,xyl} + C_{xyl}}\right)\left(1 - \left(\frac{P}{P_{m,x,xyl}}\right)^{\beta x'}\right) \quad (4)$$

Specific biomass growth rate for mixed glucose and xylose:

$$\mu = \left(\frac{C_{xyl}}{C_{glu} + C_{xyl}}\right)\mu_{xyl} + \left(\frac{C_{glu}}{C_{glu} + C_{xyl}}\right)\mu_{glu} \quad (5)$$

Biomass growth rate:

$$r_x = \mu \times C_x \quad (6)$$

4.2.1.2. Substrate uptake rate

The specific glucose uptake rate for both microorganisms are represented by equations (7), (8), (9), and (10). Equation (11) illustrates substrate consumption rate that was used in this paper.

Specific substrate consumption rate, *Z. mobilis* (Entner–Doudoroff (ED) pathway):

$$q_{glu} = \left(\frac{q_{m,glu}\, C_{glu}}{K_{s,glu} + C_{glu}}\right)\left(\frac{K_{is,glu}}{K_{is,glu} + C_{glu}}\right)\left(1 - \frac{P - P_{is,glu}}{P_{m,s,glu} - P_{is,glu}}\right) \quad (7)$$

$$q_{xyl} = \left(\frac{q_{m,xyl}\, C_{xyl}}{K_{s,xyl} + C_{xyl}}\right)\left(\frac{K_{is,xyl}}{K_{is,xyl} + C_{xyl}}\right)\left(1 - \frac{P - P_{is,xyl}}{P_{m,s,xyl} - P_{is,xyl}}\right) \quad (8)$$

Specific substrate consumption rate, *S. cerevisiae* (Embden–Meyerhof–Parnas (EMP) pathway):

$$q_{glu} = \left(\frac{q_{m,glu}\, C_{glu}}{K_{s,glu} + C_{glu}}\right)\left(\frac{K_{is,glu}}{K_{is,glu} + C_{glu}}\right)\left(1 - \left(\frac{P}{P_{m,s,glu}}\right)^{\beta s}\right) \quad (9)$$

$$q_{xyl} = \left(\frac{q_{m,xyl}\, C_{xyl}}{K_{s,xyl} + C_{xyl}}\right)\left(\frac{K_{is,xyl}}{K_{is,xyl} + C_{xyl}}\right)\left(1 - \left(\frac{P}{P_{m,s,xyl}}\right)^{\beta s'}\right) \quad (10)$$

Substrate consumption:

$$r_s = q_s \times C_x \quad (11)$$

4.2.1.3. Ethanol production rate

The equations of specific ethanol production rate which were used for both microorganisms are represented by equations (12), (13), and (14). Used equation for ethanol production rate is shown in equation (15).

Specific ethanol production rate, *Z. mobilis* and *S. cerevisiae*:

$$v_{glu} = q_{glu} \times Y_{sp,glu} \quad (12)$$

$$v_{xyl} = q_{xyl} \times Y_{sp,xyl} \quad (13)$$

Specific ethanol production rate for mixed glucose and xylose:

$$v = v_{glu} + v_{xyl} \quad (14)$$

Ethanol production rate:

$$r_e = v \times C_x \quad (15)$$

4.2.2. Mass balance

The dynamic description of ethanol fermentation using unstructured models can be carried out with three differential equations for microorganism growth (r_x), substrate uptake (r_s), and ethanol formation (r_e) (Eqs. 16-18), which can be obtained from the mass balance in the reactor.

$$r_x = \frac{dC_x}{dt} \quad (16)$$

$$r_s = \frac{dC_s}{dt} \quad (17)$$

$$r_e = \frac{dP}{dt} \quad (18)$$

4.2.3. Parameter estimation

The kinetic parameters that are used in Eq. 1 to 18 were estimated from a non-linear regression fit to the literature experimental data for *S. cerevisiae* CEN.PK 113-7D and RWB 217 (Kuyper et al., 2005a, 2005b, 2004, 2003), and for *Z. mobilis* ZM4 (pZB5) (Leksawasdi et al., 2001) and through the minimization of the Residual Sum of Squares to achieve the correlation coefficient (statistical measure of how close the data in modelling are to the data in experiment) to be close to 1, defined by Eq. 19:

$$\text{Correlation coefficient (regression coefficient)} = 1 - \frac{\left(\text{Residual sum of squares } \sum_{p=1}^{np}(dp - xp)^2\right)}{\left(\text{Total sum of squares } \sum_{p=1}^{np}(dp - \overline{dp})^2\right)} \quad (19)$$

where xp and dp are the values predicted by the model and experimental data respectively, $\overline{dp}$ is the average of experimental values, and np is the number of experimental points. For the solutions of Eqs. 1 to 18, the MATLAB based Runge–Kutta method was used. Used yields (g of ethanol/g of consumed sugars) were found in literature: *S. cerevisiae* RWB 217 (Kuyper et al., 2005a, 2004, 2003), *S. cerevisiae* CEN.PK 113-7D (Kuyper et al., 2005a, 2005b), *Z. mobilis* ZM4 (Davis et al., 2006) and *Z. mobilis* ZM4 (pZB5) (Leksawasdi et al., 2001). The kinetic parameters of *Z. mobilis* ZM4 were estimated from obtained correction factors of *S. cerevisiae* (differences between kinetic parameters of CEN.PK 113-7D and RWB 217) and the estimated kinetic parameters of *Z. mobilis* ZM4 (pZB5).

4.2.4. Experimental work for validation

4.2.4.1. Strains

The strain of *Z. mobilis* ZM4 (ATCC 31821) was used.

4.2.4.2. Culture storage

For short-term storage, all cultures were stored in the solid rich medium (RM) (Goodman et al., 1982) containing per litre: 20 g glucose, 10 g yeast extract, 2 g KH_2PO_4, 15 g agar, 1000 ml DI water. Agar plates were stored at 4 °C. For long-term storage, the cultures were suspended in sterile 40% (v/v) glycerol and

stored in 1.5 mL volumes at -70 °C. For this, equal amounts of bacterial culture and 80% glycerol were mixed. The cell-glycerol mixture was kept at room temperature for half an hour prior to freezing.

4.2.4.3. Synthetic media

Inoculum medium consisted of 10 g L^{-1} yeast extract, 1 g L^{-1} $MgCl_2$, 1 g L^{-1} $(NH_4)_2SO4$, 1 g L^{-1} KH_2PO_4, with 44 g L^{-1} glucose. Yeast extract and inorganic salts (YEIS) solution were sterilized separately from glucose at 121 °C for 20 min.

4.2.4.4. Inoculum preparation and fermentation studies

Z. mobilis was subcultured in fresh inoculum media twice before inoculated into the fermentor. Inocula were incubated for 24 h at 30 °C in 250-ml conical flasks with 50 ml medium, in a shaker incubator at a speed of 120 rpm. A 10% (v_{inoc}/v_{ferm}) inoculum, i.e. volume of inoculum culture added to volume of fermentation medium that cells were inoculated into, was used to inoculate the fermentor to give an initial OD of 0.1. Experiments were conducted in a 7 L Brunswick reactor with a working volume of 5 litres, an agitation rate of 200 rpm, operated at 30 °C. The pH was controlled at 6.0 using 1 M NaOH. In this study, for *Z. mobilis* ZM4 (ATCC 31821), the initial biomass concentration (C_{x0}) was 0.05 g L^{-1}, initial ethanol concentration (P_0) was 0.01 g L^{-1} and initial glucose concentration (C_{glu0}) was 44 g L^{-1} as shown in Table 4.1.

4.2.4.5. Analytical procedures

Dry cell mass from 2 ml sample was determined by centrifuging for 10 minutes at 13000 rpm (Heraeus Biofuge Pico); washed with water and centrifuged again. The cell pellet was dried at 80 °C for 24 hours and stored in a desiccator before weighing. The optical density was determined at 660 nm by spectrophotometer (Gensys 10S UV-VIS) and kept at around 0.3 OD by dilution. Samples from the fermentation broth were analysed for glucose and ethanol concentrations by HPLC using an Aminex column HPX-87H (300*7.8mm) (Bio- Rad, Ion exclusion column) equipped with a refractive index detector. Separations were performed at 65 °C, eluted at 0.3 ml/min using 5 mM sulphuric acid. Standards containing analytical grade components were used periodically to confirm calibration accuracy. The samples and standards were filtered with a 0.22 µm syringe filter. The mobile phase was filtered (with a 0.45 µm membrane filter) and degassed in a sonic bath.

4.3. Results and Discussion

4.3.1. Modelling from literature experimental values using *S. cerevisiae*

The values of the kinetic parameters (Table 4.2) were derived and estimated from literature experimental data (Kuyper et al., 2005a, 2005b, 2004, 2003) as explained in the parameter estimation section. Majority of the estimated kinetic parameters of *S. cerevisiae* CEN.PK 113-7D in this study were similar to *S. cerevisiae* 1400 in the study of Krishnan and his group (Krishnan et al., 1995). As shown in Figure 4.2 and Figure 4.3, the glucose, and the mixed glucose/xylose model demonstrated good correlation - more than 0.9 - with the experimental data for glucose media (20 g L^{-1}) and mixed glucose/xylose media containing 20/20 g L^{-1} of each sugar. From the well-fitted models, the differences in the magnitude of the estimated kinetic parameters of the wild type and engineered strains of *S. cerevisiae* due to the introduction of the xylose utilization pathway were calculated. These are reported in Table 4.2 and were denoted as the correction factors. As shown in Table 4.2, the glucose limitation constants ($K_{s,glu}$, $K_{x,glu}$) in the engineered strain were three times higher than that of glucose limitation constants in wild type, indicating a higher affinity for glucose uptake in the wild type rather than in the engineered strain. In the engineered strain, simultaneous xylose and glucose conversion occurs, whereas in the wild type subsequent glucose and xylose conversion happens (Ho et al., 1998; Zhang et al., 1995). This change of sequence of uptake of the preferred carbon source may have led to the changes seen in the glucose kinetic parameters and resulted in faster glucose utilization in the wild type than in engineered strain (Lee and Rogers, 1983; Leksawasdi et al., 2001). It is postulated that higher affinity for glucose is required in the wild type strain as these cultures also lack supplementation from another carbon source (i.e. xylose). In this case, the wild type strains are required to scavenge for glucose to grow when glucose is near depletion.

The xylose limitation constants ($K_{s,xyl}$, $K_{x,xyl}$) in engineered strain are three times higher than that of glucose limitation constants ($K_{s,glu}$, $K_{x,glu}$) in the engineered strain, indicating a higher affinity for glucose rather than xylose in the engineered strain even though co-consumption occurs in the engineered strain (Kötter and Ciriacy, 1993). The change in xylose and glucose affinity may also have contributed to the expression profile of the 18 hexose transporters (Hxt1-17 and Gal2) (Moysés et al., 2016), specifically the high glucose affinity transporters - Hxt6, Hxt7, and Gal2 (with Km 1–2 mM) -, intermediate - Hxt2, Hxt4, and Hxt5 (with Km ~10 mM) -, or low - Hxt1 and Hxt3 (with Km 50–100 mM) (Diderich et al., 1999). Hxt1, Hxt4, Hxt5, Hxt7, and Gal2 have been implicated in xylose transport, taking up xylose with 200-fold lower affinity and slower rates than glucose (Hamacher et al., 2002; Sedlak and Ho, 2004). The presence of xylose may therefore influence the expression of these genes resulting in the lower glucose and xylose affinity profiles in the engineered strain compared to the wild type.

Table 4.2.Kinetic constants of *S. cerevisiae* (CEN.PK 113-7D and RWB 217) and the respective correction factors denoted in boxes. [Estimated] denotes estimated values from this study using a non-linear regression fit to literature experimental data (Kuyper et al., 2005a, 2005b, 2004, 2003)

Kinetics of wild type *S. cerevisiae* CEN.PK 113-7D		**Kinetics of engineered strain *S. cerevisiae*** RWB 217 **for glucose conversion**			**Kinetics of engineered strain *S. cerevisiae*** RWB 217 **for xylose conversion**		
	Value			Value			Value
$\mu_{m,glu}$	0.34 (Kuyper et al., 2005a, 2004)	$\mu_{m,glu}$		0.25 (Kuyper et al., 2005a, 2005b)	$\mu_{m,xyl}$		0.09 [Estimated]
$K_{x,glu}$	0.572 [Estimated], (Krishnan et al., 1995)	$K_{x,glu}$	×3	1.716 [Estimated]	$K_{x,xyl}$	×3	5.148 [Estimated]
$P_{m,x,glu}$	129.9 [Estimated], (Krishnan et al., 1995)	$P_{m,x,glu}$	÷1.5	86.6 [Estimated]	$P_{m,x,xyl}$	×1	86.6 [Estimated]
$K_{ix,glu}$	1127.8 [Estimated], (Krishnan et al., 1995)	$K_{ix,glu}$	×1	1127.8 [Estimated]	$K_{ix,xyl}$	×1	1127.8 [Estimated]
βx	0.25 [Estimated], (Krishnan et al., 1995)	βx	×3	0.75 [Estimated]	$\beta x'$	×3	2.25 [Estimated]
$q_{m,glu}$	3.06 [Estimated]	$q_{m,glu}$		3.04 (Kuyper et al., 2005a)	$q_{m,xyl}$		1.06 (Kuyper et al., 2005a)
$K_{s,glu}$	1.34 [Estimated], (Krishnan et al., 1995)	$K_{s,glu}$	×3	4.02 [Estimated]	$K_{s,xyl}$	×3	12.06 [Estimated]
$P_{m,s,glu}$	136.4 [Estimated], (Krishnan et al., 1995)	$P_{m,s,glu}$	÷1.5	90.93 [Estimated]	$P_{m,s,xyl}$	×1	90.93 [Estimated]
$K_{is,glu}$	4882.8 [Estimated], (Krishnan et al., 1995)	$K_{is,glu}$	×1	4882.8 [Estimated]	$K_{is,xyl}$	×1	4882.8 [Estimated]
βs	0.4 [Estimated]	βs	×3	1.2 [Estimated]	$\beta s'$	×3	3.6 [Estimated]
Y_{spglu}	0.4 (Kuyper et al., 2005a, 2004, 2003)	Y_{spglu}		0.43 (Kuyper et al., 2005a, 2005b)	Y_{spxyl}		0.43 (Kuyper et al., 2005a, 2005b)

The substrate inhibition constants ($K_{ix,glu}$, $K_{is,glu}$, $K_{ix,xyl}$, $K_{is,xyl}$) in the engineered and wild type strains were similar (Table 4.2), indicating that both glucose and xylose inhibit ethanol production, substrate consumption and biomass formation roughly at the same rate. High substrate concentrations are known to inhibit microbial growth and fermentation as a result of high osmotic pressure and low water activity (Casey and Ingledew, 1986), although it has been reported that substrate inhibition only becomes significant in the range of 15-25% (w/v) sugar (Krishnan et al., 1995). In this study, the substrate concentrations were well below the inhibitory threshold values, and therefore substrate inhibition effects had a small effect on ethanol production, substrate consumption and biomass formation and can, therefore, be neglected (Lee and Rogers, 1983).

The ethanol tolerance for growth in different microorganisms appears to be the result of adaptive and evolutionary changes in the cell composition (Ingram, 1989). Ethanol tolerance ($P_{m,x,glu}$, $P_{m,s,glu}$, $P_{m,x,xyl}$,

$P_{m,s,xyl}$) relates to the maximum concentration of ethanol above which ethanol is not produced, cells do not grow and substrate are not taken up. The maximum inhibitory ethanol concentrations in wild type ($P_{m,x,glu}$, $P_{m,s,glu}$, $P_{m,x,xyl}$, $P_{m,s,xyl}$) are 1.5 times that of the engineered strain ($P_{m,x,glu}$, $P_{m,s,glu}$, $P_{m,x,xyl}$, $P_{m,s,xyl}$), indicating growth is suppressed with a lower ethanol concentration in the engineered strain than wild type (Lee and Rogers, 1983; Leksawasdi et al., 2001). The inhibitory effects were also amplified by the ethanol inhibition constants (βs', βx', βs, βx). In the engineered strain, ethanol inhibition constants (βs', βx') when grown in xylose were three times higher than that of when grown in glucose (βs, βx) and nine times higher than that of wild type (βs, βx). The higher factor on the inhibitory term would therefore lead to a larger negative effect on the substrate uptake and biomass growth rates (Equations 3,4, 9 and 10). The reduction in ethanol tolerance in engineered strain may also be due to the changes in the membrane components to accommodate xylose utilization that eventually could lead to change in ethanol permeability issues.

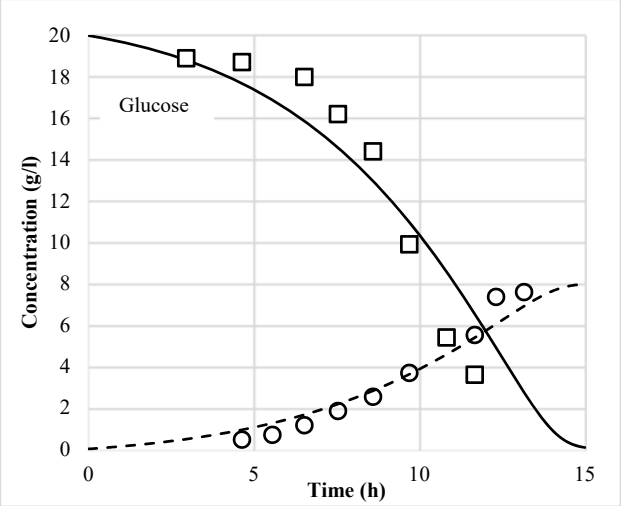

Figure 4.2.The concentration of ethanol and glucose over time (*S. cerevisiae* CEN.PK 113-7D). The solid line (—) denotes substrate concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote glucose concentration and open circles (○) denote ethanol concentration in the experiment (Kuyper et al., 2005a). The Correlation coefficient of glucose (0.9) and ethnaol (0.95)

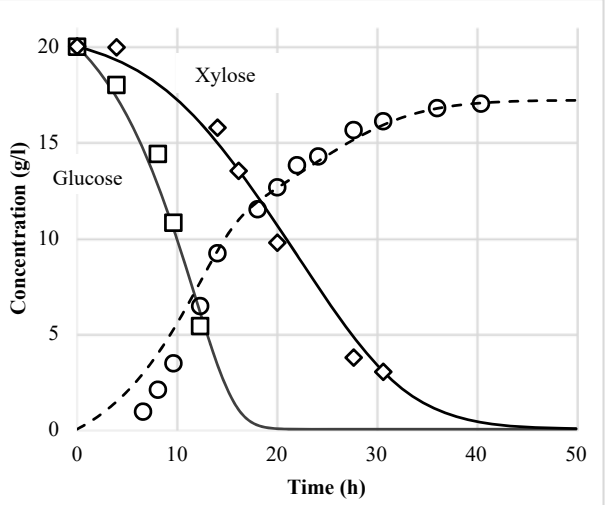

Figure 4.3.The concentration of ethanol, xylose and glucose over time (*S. cerevisiae* RWB217). The solid lines (—) denote substrate concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote glucose concentration, open diamonds (◊) denote xylose concentration and open circles (○) denote ethanol concentration in the experiments (Kuyper et al., 2005a, 2005b). The correlation of coefficient of glucose (0.94), xylose (0.94) and ethanol (0.97)

4.3.2. Modelling from literature experimental values using *Z. mobilis*

The kinetic parameters of engineered strain *Z. mobilis* ZM4 (pZB5) through linear and non-linear regression models from literature experimental results (Leksawasdi et al., 2001) were estimated and are listed in Table

4.3. Majority of these parameters showed similarities to those of Rogers and his co-workers (Leksawasdi et al., 2001). *Z. mobilis* ZM4 (pZB5) was selected because of the similar pentose metabolism pathway introduced in *S. cerevisiae* RWB 217. The average correlation coefficient of engineered strain ZM4 (pZB5) between modelling and experimental data (Leksawasdi et al., 2001) for substrate consumption was 0.98 and for bioethanol production was 0.98, showing acceptable fit (Figure 4.4a, Figure 4.4b) of the estimated parameters for *Z. mobilis* ZM4 (pZB5) (Table 4.3). The average correlation coefficient for biomass was however, at 0.75.

Table 4.3.Kinetic constants of *Zymomonas mobilis* (ZM4 and ZM4 (pZB5)), and the respective estimated correction factors denoted in box. [Estimated] denotes estimated values in this study from a non-linear regression fit to the literature experimental data (Leksawasdi et al., 2001)

Kinetics of wild type *Z. mobilis* ZM4		Kinetics of engineered strain *Z. mobilis* ZM4 (pZB5) for glucose conversion			Kinetics of engineered strain *Z. mobilis* ZM4 (pZB5) for xylose conversion		
	Value			Value			Value
$\mu_{m,glu}$	0.27 [Estimated]	$\mu_{m,glu}$		0.27 [Estimated]	$\mu_{m,xyl}$		0.09 [Estimated]
$K_{x,glu}$	0.48 [Estimated], (Lee and Rogers, 1983)	$K_{x,glu}$	×3	1.45 [Estimated], (Leksawasdi et al., 2001)	$K_{x,xyl}$	×3	4.32 [Estimated]
$P_{m,x,glu}$	85.8 [Estimated], (Lee and Rogers, 1983)	$P_{m,x,glu}$	÷1.5	57.2 [Estimated], (Leksawasdi et al., 2001)	$P_{m,x,xyl}$	×1	57.2 [Estimated], (Leksawasdi et al., 2001)
$K_{ix,glu}$	600 [Estimated]	$K_{ix,glu}$	×1	600 [Estimated]	$K_{ix,xyl}$	×1	600 [Estimated], (Leksawasdi et al., 2001)
$P_{ix,glu}$	28 [Estimated]	$P_{ix,glu}$	×1	28 [Estimated], (Leksawasdi et al., 2001)	$P_{ix,xyl}$	×1.5	42 [Estimated], (Leksawasdi et al., 2001)
$q_{m,glu}$	8 [Estimated]	$q_{m,glu}$		8 [Estimated]	$q_{m,xyl}$		1.77 [Estimated]
$K_{s,glu}$	2.1 [Estimated]	$K_{s,glu}$	×3	6.3 [Estimated], (Leksawasdi et al., 2001)	$K_{s,xyl}$	×3	18.9 [Estimated]
$P_{m,s,glu}$	127 [Estimated], (Lee and Rogers, 1983)	$P_{m,s,glu}$	÷1.5	84.66 [Estimated], (Leksawasdi et al., 2001)	$P_{m,s,xyl}$	×1	84.66 [Estimated], (Leksawasdi et al., 2001)
$K_{is,glu}$	600 [Estimated]	$K_{is,glu}$	×1	600 [Estimated]	$K_{is,xyl}$	×1	600 [Estimated], (Leksawasdi et al., 2001)
$P_{is,glu}$	35.5 [Estimated],	$P_{is,glu}$	×1	35.5 [Estimated], (Leksawasdi et al., 2001)	$P_{is,xyl}$	×1.5	56.25 [Estimated], (Leksawasdi et al., 2001)
Y_{spglu}	0.5 (Davis et al., 2006)	Y_{spglu}		0.48 (Leksawasdi et al., 2001)	Y_{spxyl}		0.48 (Leksawasdi et al., 2001)

The correction factors obtained from the *S. cerevisiae* RWB 217 and CEN.PK 113-7D comparison (Table 4.2) were used to estimate the kinetic parameters of *Z. mobilis* ZM4 (Table 4.3). Some of these parameters

showed similarities to those of Rogers and his co-workers (Lee and Rogers, 1983). The wild type parameters were eventually validated with literature experimental data (Davis et al., 2006; Lee and Rogers, 1983) in Figure 4.5. The average correlation coefficient of wild type *Z. mobilis* ZM4 between modelling and the literature experimental results (Lee and Rogers, 1983) for substrate consumption was 0.97, for bioethanol production was 0.977 and for biomass production was 0.82 when grown in 100, 150 and 200 g L^{-1} of glucose.

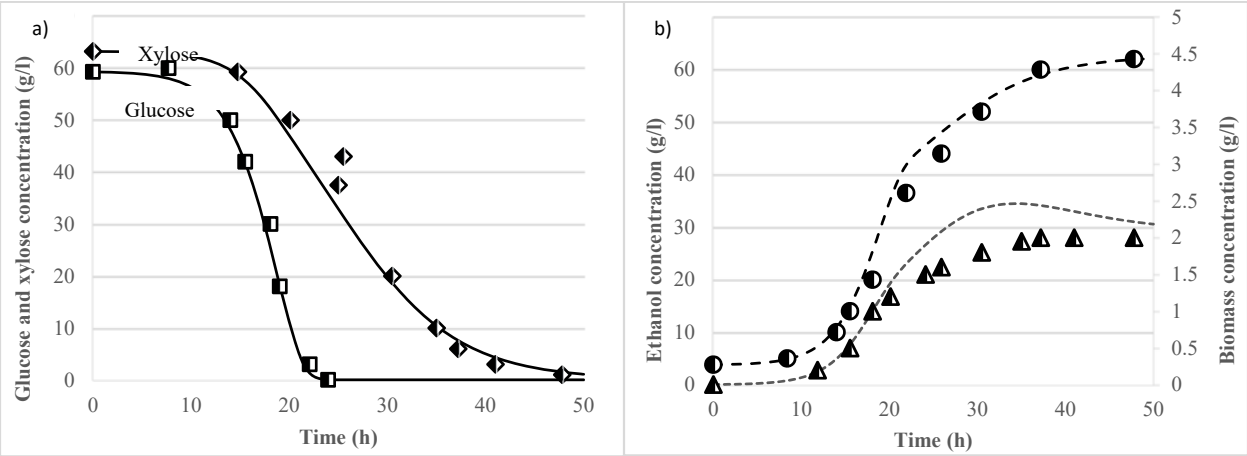

Figure 4.4. a) The concentration of glucose and xylose over time (*Z. mobilis* ZM4 (pZB5)). The solid lines (—) denote substrate concentration in modelling. Half-filled squares (◧) and half-filled diamonds (◆) denote 59.3 g L^{-1} glucose and 63.2 g L^{-1} xylose in the experiment (Leksawasdi et al., 2001). The average correlation coefficient for the substrate for 59.3/63.2 g L^{-1} glucose/xylose (0.98 ± 0.01). b) The concentration of ethanol and biomass over time (*Z. mobilis* ZM4 (pZB5)). The dotted line (…) denotes biomass concentration and dashed lines (--) denote ethanol concentration in modelling. Half-filled triangles (◭) using 59.3 g L^{-1} glucose and 63.2 g L^{-1} xylose denote biomass concentration in experiment. Half-filled circles (◐) using 65 g L^{-1} glucose and 65 g L^{-1} xylose denote ethanol concentration in the experiment (Leksawasdi et al., 2001). The correlation coefficient for ethanol at 59.3/63.2 g L^{-1} glucose/xylose (0.98). The correlation coefficient for biomass at 59.3/63.2 g L^{-1} glucose/xylose (0.75).

Ethanol concentration in the broth negatively affects substrate utilization, biomass and ethanol production when it reaches a certain point. The effect of ethanol is gradual and begins affecting the cells at a minimum concentration ($P_{ix,glu}$, $P_{is,glu}$, $P_{ix,xyl}$, $P_{is,xyl}$) and leading to complete inhibition at a maximum ethanol concentration ($P_{m,x,glu}$, $P_{m,s,glu}$, $P_{m,x,xyl}$, $P_{m,s,xyl}$). Table 4.2 and Table 4.3 show that either the maximum inhibitory ethanol concentration when grown in glucose ($P_{m,x,glu}$, $P_{m,s,glu}$) in wild type were 1.5 times that of engineered strain ($P_{m,x,glu}$, $P_{m,s,glu}$, $P_{m,x,xyl}$, $P_{m,s,xyl}$), reiterating the comparable higher ethanol tolerance of the wild type versus the engineered strains in both *Z. mobilis* and *S. cerevisiae*. On the other hand, the minimum concentration of ethanol affecting the cells when grown in glucose ($P_{ix,glu}$, $P_{is,glu}$) were similar in both engineered and wild type *Z. mobilis* strains. When grown in the presence of xylose, this minimum ethanol concentration ($P_{ix,xyl}$, $P_{is,xyl}$) in engineered strain were 1.5 times that of the minimum ethanol concentration

when grown in glucose ($P_{ix,glu}$, $P_{is,glu}$). This indicates an earlier onset of ethanol inhibition when grown on xylose as oppose to glucose as the carbon source.

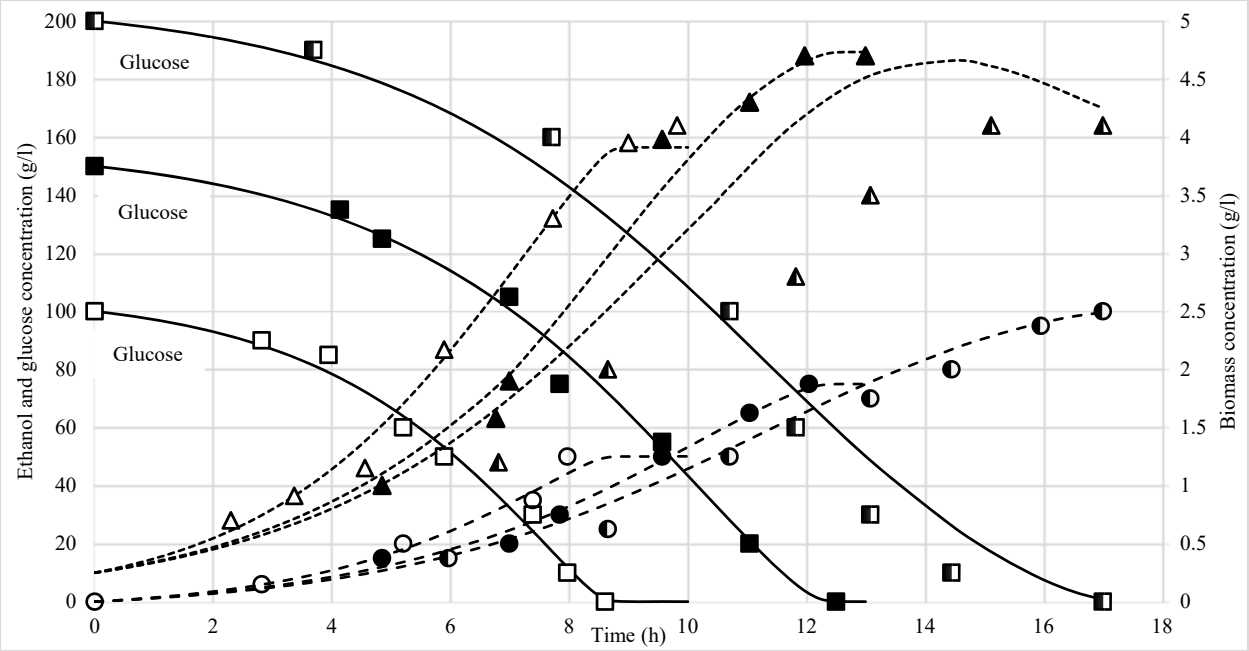

Figure 4.5.The concentration of ethanol, biomass and glucose over time (*Z. mobilis* ZM4). The solid lines (—) denote substrate concentration, dotted lines (…) denote biomass concentration and dashed lines (--) denote ethanol concentration in modelling. Open squares (□) denote 100 g L^{-1} glucose, full-filled squares (■) denote 150 g L^{-1} glucose and half-filled squares (◧) denote 200 g L^{-1} glucose in experiment. Open triangles (Δ) using 100 g L^{-1} glucose, full-filled triangles (▲) using 150 g L^{-1} glucose and half-filled triangles (◭) using 200 g L^{-1} glucose denote biomass concentration in experiment. Open circles (○) using 100 g L^{-1} glucose, full-filled circles (●) using 150 g L^{-1} glucose and half-filled circles (◐) using 200 g L^{-1} glucose denote ethanol concentration in the experiment (Lee and Rogers, 1983). The average correlation coefficient of glucose (0.97±0.048), biomass (0.82±0.34) and ethanol (0.977±0.011).

4.3.3. Validating modelling with experiments of this study using wild type of *Z. mobilis*

The fermentation time of this study was close to 10 hours for complete glucose consumption of 44 g L^{-1}, biomass growth of 1.74 g L^{-1} and ethanol production of 21.2 g L^{-1}; giving a yield, $Y_{ps,}$ of 0.48 g of ethanol/g of glucose. The experimental data of this study (Y_{xs}: 0.04 g of biomass/g of substrate, Y_{ps}: 0.48 g of ethanol/g of substrate) is close to the experimental results of Mokomele and his co-workers (Mokomele et al., 2013) (Y_{xs}: 0.035 g of biomass/g of substrate, Y_{ps}: 0.49 g of ethanol/g of substrate) and experimental result of Lee and his co-workers (Lee and Rogers, 1983) (Y_{xs}: 0.04 g of biomass/g of substrate, Y_{ps}: 0.48 g of ethanol/g of substrate) using same microorganism of *Z. mobilis* ATCC 31821 (ZM4) and the same growth conditions (30 °C, pH of 6).

The experimental results for glucose utilization, ethanol production, and biomass growth using *Z. mobilis* ATCC 31821 (ZM4) in batch for this study were compared to modelling results with estimated kinetics constants in Table 4.3 and kinetic models of Rogers and his collaborators (Lee and Rogers, 1983; Leksawasdi et al., 2001) (Equations 1,6,7,11,12 and 15). The model of this study fitted to experimental data with the correlation coefficient of 0.99 for bioethanol production, 0.98 for glucose consumption and 0.96 for biomass growth in comparison to the model by Mokomele and his co-workers (Mokomele et al., 2013) with the average correlation coefficient of 0.96 (data not shown). Although the fermentation time in the study of Mokomele and his co-workers (Mokomele et al., 2013) was shorter (8 h) when grown with initial glucose concentration of 50 g L^{-1}, the estimated value for the specific biomass production rate and the specific substrate consumption rate from this study gave a better fit to the experimental result. The correlation coefficients and comparison between the modelling and experiments of this study are shown in Figure 4.6, which indicates a good fit of the model to the experimental data.

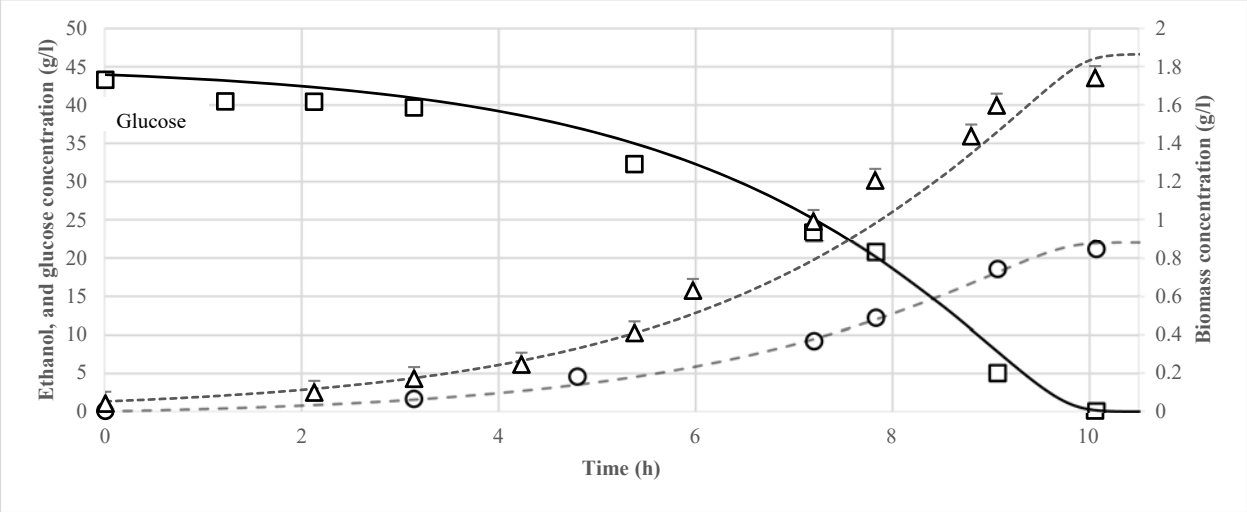

Figure 4.6.The concentration of glucose, biomass and ethanol over time (*Z. mobilis* ATCC 31821). The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote glucose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration in the experiment of this study. Correlation coefficient of glucose (0.98), biomass (0.96) and ethanol (0.99).

4.3.4. Sensitivity analysis

A sensitivity analysis was performed on the fermentation time by varying each model parameters by ±10% for both *S. cerevisiae* and *Z. mobilis* that is shown in Table 4.4 and Table 4.5. Based on this analysis, it was found that maximum specific growth rates ($\mu_{m,glu}$, $\mu_{m,xyl}$), maximum specific substrate utilization rates ($q_{m,glu}$, $q_{m,xyl}$) and ethanol inhibition parameters (βx, βs) had the most significant effects on the frmentation time. The fermentation time changed more than ±9% by changing ±10% of the specific growth rates ($\mu_{m,\,glu}$, $\mu_{m,}$

$_{xyl}$) / the specific substrate consumption rate ($q_{m,glu}$, $q_{m,xyl}$). As both these parameters are time dependent, it was assumed that any change in these parameters would lead to a significant change in the fermentation time.

Substrate inhibition ($K_{ix,glu}$, $K_{is,glu}$, $K_{ix,xly}$, $K_{is,xly}$) showed little effects on fermentation time for both *S. cerevisiae and Z. mobilis*. The fermentation time changed less than ±1.5% or less by implementing a ±10% substrate inhibition change in both microorganisms of either the wild type or engineered strains. It is very likely that the concentration of substrate supplied in the media in this study is not close to the inhibitory values for the microorganisms. Substrate limitation on the other hand was negligible in the wild types, whereas showed changes between ±3% to ± 1% in the engineered strains. The addition of a xylose utilization pathway and xylose as an additional carbon source may have led to morphological, physiological and metabolic changes in the cell, leading to changes in affinity for glucose and xylose utilization.

Table 4.4.Sensitivity analysis of fermentation time using *S. cerevisiae* by varying each model parameters by 10%

Wild type *S. cerevisiae* CEN.PK 113-7D		**Engineered strain *S. cerevisiae* RWB 217 for glucose conversion**		**Engineered strain *S. cerevisiae* RWB 217 for xylose conversion**	
	Fermentation time variation		Fermentation time variation		Fermentation time variation
$\mu_{m,glu}$, $q_{m,glu}$	±11.47%	$\mu_{m,glu}$, $q_{m,glu}$	±9.67%	$\mu_{m,xyl}$, $q_{m,xyl}$	±9.78%
$K_{x,glu}$, $K_{s,glu}$	±0.75%	$K_{x,glu}$, $K_{s,glu}$	±3.99%	$K_{x,xyl}$, $K_{s,xyl}$	±4.84%
$P_{m,x,glu}$, $P_{m,s,glu}$	±3.14%	$P_{m,x,glu}$, $P_{m,s,glu}$	±0.68%	$P_{m,x,xyl}$, $P_{m,s,xyl}$	±0.074%
$K_{ix,glu}$, $K_{is,glu}$	±0.53%	$K_{ix,glu}$, $K_{is,glu}$	0%	$K_{ix,xyl}$, $K_{is,xyl}$	0%
βx, βs	±7.13%	βx, βs	±2.31%	$\beta x'$, $\beta s'$	±4.44%

Table 4.5.Sensitivity analysis of fermentation time using *Zymomonas mobilis* by varying each model parameters by 10%

Wild type *Z. mobilis* ZM4		**Engineered strain *Z. mobilis* ZM4 (pZB5) for glucose conversion**		**Engineered strain *Z. mobilis* ZM4 (pZB5) for xylose conversion**	
	Fermentation time variation		Fermentation time variation		Fermentation time variation
$\mu_{m,glu}$, $q_{m,glu}$	±9.1%	$\mu_{m,glu}$, $q_{m,glu}$	±11.57%	$\mu_{m,xyl}$, $q_{m,xyl}$	±22.82%
$K_{x,glu}$, $K_{s,glu}$	±0.46%	$K_{x,glu}$, $K_{s,glu}$	±2.32%	$K_{x,xyl}$, $K_{s,xyl}$	±10.92%
$P_{m,x,glu}$, $P_{m,s,glu}$	±2.54%	$P_{m,x,glu}$, $P_{m,s,glu}$	±11.78%	$P_{m,x,xyl}$, $P_{m,s,xyl}$	±7.5%
$K_{ix,glu}$, $K_{is,glu}$	0	$K_{ix,glu}$, $K_{is,glu}$	±0.79%	$K_{ix,xyl}$, $K_{is,xyl}$	±1.54%
$P_{ix,glu}$, $P_{is,glu}$	±4.6%	$P_{ix,glu}$, $P_{is,glu}$	±18.9%	$P_{ix,xyl}$, $P_{is,xyl}$	±36.67%

The largest effects on fermentation time resulted from the changes in product inhibition in engineered *Zymomonas mobilis* ZM4 (pZB5), resulting in a ±19% to ±37% higher fermentation time. However, this

effect was not replicated in *S. cerevisiae*, which only resulted in a ±2% to ±4%. The higher resistance of *S. cerevisiae* to ethanol may be a key factor in this finding. Similarly, the maximum ethanol concentration above which the process would be stopped ($P_{m,x,glu}$, $P_{m,s,glu}$, $P_{m,x,xyl}$, $P_{m,s,xyl}$) in engineered *Saccharomyces cerevisiae* RWB 217 showed no significant change, but in engineered *Zymomonas mobilis* ZM4 (pZB5), changes in fermentation time was around ±7% to ±12%.

4.4. Conclusion

The kinetic parameter relationship of glucose and xylose utilization between the different constructs of the same species, for example, a wild type and engineered strain were examined and showed a consistent correction factor between the strains. The two microorganisms with an engineered pentose metabolism pathway were selected: 1) *Saccharomyces cerevisiae* CEN.PK 113-7D vs RWB 217 (xylose isomerase gene from the fungus *Piromyces sp.* E2 expressed), 2) *Zymomonas mobilis* ZM4 vs ZM4 (pZB5) (xylose isomerase gene from the bacteria *Escherichia coli* expressed). Even though the origin of the xylose isomerase genes was different, differences between estimated kinetic parameters of wild type and engineered strains from linear and non-linear regression adhered to a conserved pattern. Literature experimental results validated these results in *S. cerevisiae* for both wild type (CEN.PK 113-7D) and engineered strains (RWB 217) (Kuyper et al., 2005a, 2005b, 2004, 2003) and in *Z. mobilis* for both wild type (ZM4) and engineered strains (ZM4(pZB5)) (Lee and Rogers, 1983; Leksawasdi et al., 2001). Final validation of the proposed method of estimating kinetics using correction factors was confirmed with experimental data generated in this study for *Z. mobilis* ZM4.

This study showed that there was conserved regulation of xylose and glucose utilization when a recombinant xylose pathway was introduced to its parent strain. This conservation was also noticed across genus when a similar pathway is introduced (*Saccharomyces* vs *Zymomonas*). Although *S. cerevisiae* and *Z. mobilis* utilizes a different glycolytic pathway, it was found that the kinetic parameters altered with equal consistency when changes were introduced to the cell's metabolism for xylose utilization to ethanol production. This may be the result of the tight regulation of the glycolysis pathway, regulated by various mechanisms such as feedback inhibition, isoenzymes and post-translational modifications to the glycolytic enzymes. The multitude of control schemes on the pathway ensures that the metabolic flux on the main carbon trunk for the synthesis of major intermediates, products and biomass is maintained and uninterrupted.

The set of correction factors obtained in this study is a key indication that modelling remains an essential tool for process prediction and optimization. However, available kinetic data for the development of these models remain a stumbling block when proper experimental data are not available. A suggestion here is to approach the model from a black box concept and by simplifying the understanding of metabolic pathways

could lead to a generic *in silico* model for process development and optimization. The information gained from this approach could eventually contribute to the development of an *in silico* cell when simulating complex metabolic pathways which remains challenging, tedious and time consuming.

4.5. Declaration of interest

The authors declare no competing financial interest.

4.6. Acknowledgments

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4.7. Nomenclature

C_x: Cell concentration in medium (mass/unit volume)

C_{xyl}: Xylose concentration (mass/unit volume)

C_{glu}: Glucose concentration (mass/unit volume)

C_{x0}: Initial cell concentration in medium (mass/unit volume)

C_{xyl0}: Initial xylose concentration (mass/unit volume)

C_{glu0}: Initial glucose concentration (mass/unit volume)

$K_{s,glu}$: Glucose limitation constant for substrate uptake rate (mass/unit volume)

$K_{s,xyl:}$ Xylose limitation constant for substrate uptake rate (mass/unit volume)

$K_{x,glu:}$ Glucose limitation constant for biomass production rate (mass/unit volume)

$K_{x,xyl:}$ Xylose limitation constant for biomass production rate (mass/unit volume)

$K_{is,glu:}$ Glucose inhibition constant for substrate uptake rate (mass/unit volume)

$K_{is,xyl}$: Xylose inhibition constant for substrate uptake rate (mass/unit volume)

$K_{ix,glu:}$ Glucose inhibition constant for biomass production rate (mass/unit volume)

$K_{ix,xyl}$: Xylose inhibition constant for biomass production rate (mass/unit volume)

P: Ethanol concentration (mass/unit volume)

P_0: Initial ethanol concentration (mass/unit volume)

$P_{m,x,\ glu}$: Maximum ethanol concentration above which cells do not grow in glucose (mass/unit volume)

$P_{m,x,xyl}$: Maximum ethanol concentration above which cells do not grow in xylose (mass/unit volume)

$P_{m,s,\ glu}$: Maximum ethanol concentration above which glucose do not uptake (mass/unit volume)

$P_{m,s,xyl}$: Maximum ethanol concentration above which xylose do not uptake (mass/unit volume)

$P_{ix,\ glu}$: Minimum ethanol concentration above which cells production is affected negatively when grown in glucose (mass/unit volume)

$P_{ix,xyl}$: Minimum ethanol concentration above which cells production is affected negatively when grown in xylose (mass/unit volume)

$P_{is,\ glu}$: Minimum ethanol concentration above which glucose consumption is affected negatively (mass/unit volume)

$P_{is,xyl}$: Minimum ethanol concentration above which xylose consumption is affected negatively (mass/unit volume)

$q_{m,glu:}$ Maximum specific glucose utilization rate (1/unit time)

$q_{m,xyl}$: Maximum specific xylose utilization rate (1/unit time)

$q_{glu:}$ Specific glucose utilization rate (1/unit time)

q_{xyl}: Specific xylose utilization rate (1/unit time)

r_s: Substrate consumption rate (mass/unit volume/unit time)

r_x: Biomass production rate (mass/unit volume/unit time)

r_e: Ethanol production rate (mass/unit volume/unit time)

$Y_{SP,\ glu}$: Product yield constant (g-product/g-glucose)

$Y_{SP,\ xyl}$: Product yield constant (g-product/g-xylose)

$\mu_{m,glu}$: Maximum specific growth rate in glucose (1/unit time)

$\mu_{m,xyl}$: Maximum specific growth rate in glucose (1/unit time)

$\mu_{xyl:}$ Specific growth rate in xylose (1/unit time),

μ_{glu}: Specific growth rate in glucose (1/unit time)

ν_{xyl}: Specific rate of product formation in xylose (1/unit time)

ν_{glu}: Specific rate of product formation in glucose (1/unit time)

βs: Product inhibition constant in glucose for uptake substrate (dimensionless)

$\beta s'$: Product inhibition constant in xylose for uptake substrate (dimensionless)

βx: Product inhibition constant in glucose for growth of biomass (dimensionless)

$\beta x'$: Product inhibition constant in xylose for growth of biomass (dimensionless)

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5. Kinetic model and kinetic parameters of *P. stipitis* for bioethanol fermentation from xylose

After selecting the suitable kinetic model and kinetic parameters for glucose conversion using *Z. mobilis*, the kinetic parameters for xylose conversion using *P. stipitis* should be estimated for the best fit between experiment and modelling. This study involves the development of an available kinetic model in Chapter 2B that includes oxygen inhibition and oxygen limitation constants for bioethanol production from xylose using MATLAB from observing experimental data. Validation of the model through experimental observations were done. The optimum aeration level and optimum initial biomass concentration were investigated for maximum ethanol yields and productivities.

The effective factors of substrate limitation, substrate inhibition, ethanol inhibition, oxygen limitation and oxygen inhibition on growth rate have yet to be modelled simultaneously to each other for bioethanol production using *P. stipitis* from xylose. The main goal of the work presented in this paper was to develop a mathematical model capable of describing cell, substrate, oxygen and ethanol concentrations through experimental observations validating anaerobic and microaerobic batch fermentations for ethanol production from xylose using *P. stipitis* CBS 5773 (ATCC 58376). The model and kinetic parameters that were suggested in this research can be used for bioethanol fermentations from xylose using *P. stipitis* ATCC 58376 at different aeration levels, initial xylose concentrations, initial biomass concentrations and initial ethanol concentrations to establish xylose, oxygen, biomass and ethanol concentrations over time.

This paper addresses objective (1), (2), (3) and (4). Different aeration levels and different inoculum sizes were tested to determine the optimum processing condition.

Author contribution:

The manuscript was written by Nosaibeh Nosrati Ghods (NNG) and edited by Prof. Susan T.L. Harrison (STLH), Associate Prof. Adeniyi J. Isafiade (AJI) and Dr Siew L. Tai (SLT). STLH and SLT guided in design of research approach and in interpretation of findings. STLH funded the entire cost of the experimental work. Technical assistance during the commissioning stage was provided by SLT and STLH. The primary author designed the study, performed the experiments, developed the model and statistically analysed the data used to validate the model. NNG encoded the model in Matlab and provided the model output. STLH approved the final design. The financial support of the National Research Foundation of South Africa Competitive Programme for Rated Researchers (CPRR: 87744), kindly prepared by Prof. Duncan M. Fraser and Associate Prof. Adeniyi J. Isafiade; the financial support of the research development

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Paper title:

Analysis of ethanol production from xylose using *Pichia stipitis* in microaerobic condition through experimental observations and kinetic modelling

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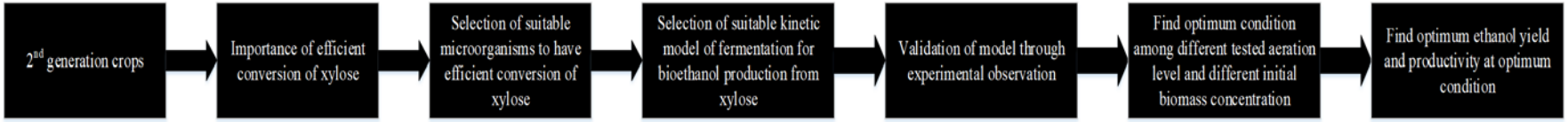
Biochemical Engineering Journal (intended)

Abstract

Secondary, non-food sources of sugars are essential for the production of biopolymers, bio-based platform chemicals, and biofuels. Hydrolysis of biomass (e.g. bagasse) yields xylose as one of the primary sugars for fermentation to bioethanol, along with glucose. The selection of a suitable microorganism and consequently selection of suitable aeration levels for the selected microorganism plays a significant role in xylose conversion. This work focuses on the kinetics of bioethanol production from xylose using *Pichia stipitis* as the microorganism with the development of a MATLAB mathematical model (combined Andrews and Levenspiel's models), considering the effect of substrate, oxygen, cell and product concentrations on growth rate. Experiments were carried out to validate the kinetic model in an anaerobic and microaerobic growth conditions in a batch process. The results showed good correlation with an average correlation coefficient of 0.865 for bioethanol production, 0.91 for xylose consumption and 0.86 for biomass growth. Different aeration levels of 0, 0.1 and 0.2 vvm and different initial biomass concentration of 0.3, 1.5 and 3 g L^{-1} were tested to find the optimum condition (0.1 vvm and 3 g L^{-1}). The optimum ethanol yield and productivity were found to be 0.459 g g^{-1} and 0.95 g L^{-1} h^{-1} respectively.

Keywords: bioethanol, fermentation, kinetic model, microaerobic, *Pichia stipitis*, xylose

CHAPTER 5: KINETIC MODEL AND KINETIC PARAMETERS OF *P. stipitis* FOR BIOETHANOL FERMENTATION FROM XYLOSE



5.1. Introduction

Biomass can be used as a sustainable energy source to meet the current challenges of energy production (Srirangan et al., 2012). Any type of fuel made from biomass is labelled biofuel (Srirangan et al., 2012). Liquid biofuels such as bioethanol are intended as substituting or as an addition to fossil fuels (Srirangan et al., 2012). Bioethanol can be made from sugar/starch-based feedstocks and from lignocellulosic feedstocks (Cáceres, 2010). Sugar/starch-based feedstocks are readily available and have high product yields, but are food sources. Hence, lignocellulosic biomass has been investigated as an alternative feedstock in order not to temper with food security (Mohr and Raman, 2013). If the cellulose and hemicellulose of lignocellulose are converted to sugars using pretreatment steps and hydrolysis, hexose and pentose sugars are released for fermentation (Menon and Rao, 2012; Pereira et al., 2011). Xylose a pentose sugar is the second most abundant sugar in nature for the production of bioethanol from biomass (Lachke, 2002).

A high conversion of xylose from bagasse to ethanol is a precondition for a cost-effective process for bioethanol production (Canilha et al., 2012). The efficient conversion of xylose is one of the major issues in the use of lignocellulosic raw material for ethanol production when efficiently converted, this could lead to an estimated increase of 25% in overall ethanol production (Nigam, 2001). Thus, suitable microorganisms for xylose conversion must, therefore, be considered. *Saccharomyces cerevisiae* and *Zymomonas mobilis* are the benchmark microorganisms for bioethanol production (Banerjee, 2010) demonstrating high ethanol tolerance and yield, but are unable to ferment xylose (Banerjee, 2010). Among xylose-fermenting microorganisms, *Pichia stipitis* is a well-known pentose-fermenting yeast and the highest ethanol yield (0.47 g g^{-1}) and good productivity (0.27 g $L^{-1}h^{-1}$) was achieved by *P. stipitis* CBS 5773 (ATCC 58376) in 88 h compared to *Candida shehatae* ATCC 22984 with an ethanol yield of 0.4 g g^{-1} and productivity of 0.28 g $L^{-1}h^{-1}$ in 72 h from 50 g L^{-1} of xylose under microaerobic conditions (Hamidimotlagh et al., 2007; Laplace et al., 1991a, 1991b). Best taken ethanol yield (0.46 g g^{-1}) (Nakanishi et al., 2017) and productivity (1.04 g $L^{-1}h^{-1}$) (Hou, 2012) of *Spathaspora passalidarum* which can grow under both anaerobic and microaerobic condition was compared with best taken ethanol yield (0.47 g g^{-1}) and productivity (1.5 g $L^{-1}h^{-1}$) of *P. stipitis* as mentioned in Chapter 2, part 1 of Ph.D. thesis, *Spathaspora passalidarum* and *P. stipitis* have similar glucose tolerance of 35% (w/v) (Rodrussamee et al., 2018) and have a little bit better ethanol tolerance (40 g L^{-1}) (Su et al., 2015) than *P. stipitis* (35 g L^{-1}) (Hamidimotlagh et al., 2007), even though it is not good enough to have a batch or fed-batch using high substrate concentration.

For *P. stipitis*, oxygen availability is considered one of the major factors that affect xylose conversion efficiency into ethanol. *P. stipitis* cells grown aerobically took up xylose 27 times faster than anaerobically (Balat et al., 2008; Fu et al., 2009; Gírio et al., 2010; Skoog et al., 1990), indicating possible high energy

requirements for the conversion or transport of xylose (Skoog et al., 1990). Typically, the preference for bioethanol production is anaerobic for glucose conversion and microaerobic for xylose conversion. Skoog et al. (1990) showed maximum specific ethanol yield and productivity for xylose fermentation by *P. stipitis* when the oxygen transfer rate (OTR) was below 1 mmol/lh (Skoog et al., 1990). The other important factor for high bioethanol yields and productivity is the selection of a suitable inoculum size. The efficient fermentation of xylose by Fu et al. (2009) (0.5 g g^{-1} and 1.126 g L^{-1} h^{-1}) (Fu et al., 2009) and Moniruzzaman et al. (1997) (0.44 g g^{-1} and 3.44 g L^{-1} h^{-1}) (Moniruzzaman et al., 1997) is directly attributed to the large initial biomass concentration of around 3.5 g L^{-1}.

The usage of suitable mathematical models, for example, kinetic models, to define the performance of bioethanol in fermentation is necessary. It can lessen the number of experiments required and make available mathematical demonstrations that can quantitatively define the structure of the activity as necessitated for optimization, design, and control (Dunn et al., 2003). There are two steps in judging how good a model is with respect to the system. The model implemented should ascertain the assumptions correctly (model verification) and whether the assumptions that have been made are reasonable with respect to the real system (model validation). Previous study (Hannoun and Stephanopoulos, 1990) tested different aeration levels for bioethanol production from glucose using *S. cerevisiae*. Farias et al. (2014), who modelled the kinetics of ethanol production by *Pichia stipitis* on xylose, only considered the effect of sugar and product concentrations on growth rate, but not dissolved oxygen concentration. Experiments were validated in oxygen-limited conditions, but the optimum aeration level was not confirmed in modelling nor in the experiments. This study involves the development of a novel kinetic model that includes oxygen inhibition and oxygen limitation constants for the of bioethanol production from xylose using MATLAB. Validation of the model through experimental observation was done. The optimum aeration level and optimum initial biomass concentration were investigated for maximum ethanol yield and productivities in experiment of this study.

The effective factors of substrate limitation, substrate inhibition, ethanol inhibition, oxygen inhibition and oxygen limitation constants for bioethanol production from xylose using Matlab. The main goal of this work was to develop a mathematical model capable of describing cell, substrate, oxygen and ethanol concentrations through experimental observation validating anaerobic and microaerobic batch fermentations for ethanol production from xylose using *P. stipitis* CBS 5773 (ATCC 58376). The model and kinetic parameters that were suggested in this research can be used for fermentation of bioethanol production from xylose using *P. stipitis* ATCC 58376 at different aeration levels, initial xylose concentrations, initial biomass concentrations and initial ethanol concentrations to establish xylose, oxygen, biomass and ethanol concentrations over time.

5.2. Material and Methods

5.2.1. Strains

The strain of *P. stipitis* ATCC 58376 was used.

5.2.2. Culture storage

For short-term storage, all cultures were stored in solid Yeast extract peptone dextrose (YEPD) (Goodman et al., 1982) containing per litre: 20 g glucose, 10 g yeast extract, 20 g peptone, 20 g agar, 1000 ml de-ionized water. Agar plates were stored at 4 °C. For long-term storage, the cultures were suspended in sterile 40% (v/v) glycerol and stored in 1.5 mL volumes at -60 °C. For this, equal volumes of bacterial culture and 80% glycerol were mixed. The cell-glycerol mixture was kept at room temperature for half an hour prior to freezing.

5.2.3. Synthetic media

Inoculum medium consisted of 10 g L^{-1} yeast extract, 0.5 g L^{-1} $MgCl_2$ (1.065 g L^{-1} $MgCl_2.6H_2O$), 0.5 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} KH_2PO_4, with 24 g L^{-1} xylose. Xylose solution was sterilized separately. Media were sterilized at 121 °C for 20 min. 0.01 v/v of antifoam 204 were added to synthetic media when grown under microaerobic condition. The synthetic media was made by combining the various components.

5.2.4. Inoculum preparation and fermentation studies

P. stipitis was subcultured in fresh inoculum media twice before being inoculated into the fermentor. Inocula were incubated for 48-72 h (depending on the requested initial biomass concentration) at 30 °C in 250 ml conical flasks with 50 ml medium, in a shaker incubator at a speed of 120 rpm. Experiments were conducted in a Brunswick reactor with a working volume of 5 litres with an agitation rate of 200 rpm, at 30 °C. The pH was controlled at 5 using NaOH 1 M. Experiments were done in duplicate.

5.2.5. Analytical procedures

Dry cell mass from 2 ml sample was determined by centrifuging for 10 minutes at 13000 rpm (Heraeus Biofuge Pico); washed with water and centrifuged again. The cell pellet was dried at 80 °C oven for 24 hours and stored in a desiccator before weighing. The optical density was determined at 600 nm by spectrophotometer (Gensys 10S UV-VIS) and kept at around 0.3 OD by dilution. Samples from the fermentation broth were analysed for xylose and ethanol concentrations by HPLC using an Aminex column HPX-87H (300*7.8mm) (Bio- Rad, Ion exclusion column) equipped with a refractive index detector. Separations were performed at 65 °C, eluted at 0.3 ml/min using 5 mM sulphuric acid. Standards containing

analytical grade components were used periodically to confirm calibration accuracy. The samples and standards were filtered with a 0.22 µm syringe filter. The mobile phase was filtered (with a 0.45 µm membrane filter) and degassed in a sonic bath.

5.2.6. Kinetic model

The combined kinetic models of Levenspiel (Levenspiel, 1972) and Andrews (Andrews, 1968) described by Farias et al. (2014) for *P. stipitis* (Lee and Rogers, 1983; Leksawasdi et al., 2001) were further developed in this study by considering the oxygen term as it was not considered in previous studies along with the substrate and product terms for biomass growth rate. The kinetic models developed were biomass production rate, oxygen uptake rate, substrate uptake rate and ethanol production rate.

5.2.6.1. Biomass production rate

The specific biomass growth rate using *P. stipitis* for xylose is represented by Equation (1). Biomass growth rate was then calculated from Equation (2).

Specific biomass growth rate:

$$\mu_{xyl} = \left(\frac{\mu_{m,xyl}\, C_{xyl}}{K_{x,xyl} + C_{xyl} + \frac{C_{xyl}^{2}}{K_{ix,xyl}}} \right) \left(\frac{\alpha_{ix\ oxy} - C_o}{\alpha_{x,oxy}} \right) \left(1 - \frac{P}{P_{m,x,xyl}} \right)^{\beta x} \quad (1)$$

Biomass growth rate:

$$r_x = \mu_{xyl} \times C_x \quad (2)$$

5.2.6.2. Substrate and oxygen uptake rate

The specific glucose uptake rate for *P. stipitis* is represented by Equation (3). Equations (4) and (5) respectively illustrate substrate and oxygen consumption rate which were used in this paper.

Specific substrate consumption, *P. stipitis*:

$$q_{xyl} = \frac{\mu_{xyl}}{Y_{sx}^{max}} + m_x \quad (3)$$

Substrate consumption rate:

$$r_s = q_{xyl} \times C_x \quad (4)$$

Oxygen consumption rate:

$$r_o = \left(\frac{\mu_{xyl}}{Y_{ox}^{max}} + m_o \right) \times C_x \quad (5)$$

5.2.6.3. Ethanol production rate

The equations of specific ethanol production rate that were used for *P. stipitis* are represented by Equation (6). The equation used for ethanol production rate is shown in Equation (7).

Specific ethanol production, *P. stipitis*:

$$v_{xyl} = Y_{sp} \times q_{xyl} \quad (6)$$

Ethanol production rate:

$$r_e = v_{xyl} \times C_x \, (7)$$

5.2.7. Mass balance

The dynamic description of ethanol fermentation using unstructured models can be carried out with four differential equations for microorganism growth, substrate uptake, ethanol formation, and oxygen uptake (Eqs. 8-11), which can be obtained from the mass balance in the reactor.

$$\frac{dC_x}{dt} = r_x \quad (8), \quad 0\frac{g}{l} < C_{x0} < 4.5\frac{g}{l}$$

$$\frac{dC_s}{dt} = -r_s \quad (9)$$

$$\frac{dP}{dt} = r_e \quad (10)$$

$$\frac{dC_o}{dt} = K_L a \times (C_o{}^* - C_o) - r_o \, (11)$$

5.2.8. Parameter estimation

The kinetic parameters that are used in Eq. 1 to 7 were found from a literature data (Farias et al., 2014; Grootjen et al., 1991; Rizzi et al., 1989) and the rest estimated from a non-linear regression fit to the experimental data of this study (substrate, biomass and product concentrations versus time) and literature experimental data of Fu (Fu, 2008) through the minimization of the Residual Sum of Squares to achieve the correlation coefficient (statistical measure of how close the data in modelling are to the data in experiment) to be close to 1, defined by Eq. 12:

$$\text{Correlation coefficient (regression coefficient)} = 1 - \frac{\left(\text{Residual sum of squares } \sum_{p=1}^{np}(dp - xp)^2\right)}{\left(\text{Total sum of squares } \sum_{p=1}^{np}(dp - \overline{dp})^2\right)} \quad (12)$$

where xp and dp are the values predicted by the model and experimental data respectively, $\overline{dp}$ is the average of experimental values, and np is the number of experimental points. For the solutions of Eqs. 1 to 11, the MATLAB based Runge–Kutta method was used.

5.2.9. Statistical analysis

The data of ethanol yield and productivity with changes of initial biomass concentration and aeration level were analysed using one way ANOVA. For the initial biomass yield, Cxo = 1.5 and 3 g/l were compared, while for aeration level, vvm of 0.1 and 0.2 were compared. In each comparison, 3 time points were taken. In this case, this resulted in two (n = 2) groups per comparison with six (m = 6) data time points per comparison. This resuts in a degree of freedom of between groups (n-1) of 1 and a degee of freedom within groups (m - n) of 4. Significant changes were measured when P-value < 0.1.

5.3. Results and Discussion

5.3.1. Kinetic constants of *Pichia stipitis*

The values of the kinetic parameters (Table 5.1a and 5.1b) were obtained from literature modelling data (Table 5.1a) (Farias et al., 2014; Grootjen et al., 1991; Rizzi et al., 1989) and experimental results of this study (Table 5.1b) as explained in parameter estimation. The aeration levels and initial biomass concentrations were varied in the experiments of this study to improve kinetic parameters obtained from Farias et al. (Farias et al., 2014).

Table 5.1.Kinetic constants obtained from literature and experiment of this study

a) Kinetic constants obtained from literature			**b) Kinetic constants obtained from experiment of this study**				
		Reference		**Anaerobic at any Cx0**	**C_{x0}=0.3 at VVM of 0.1/ VVM of 0.2**	**C_{x0}=1.5 at VVM of 0.1/ VVM of 0.2**	**C_{x0}=3.0 at VVM of 0.1/ VVM of 0.2**
Y_{sx}^{max} (g g^{-1})	0.12	(Farias et al., 2014)	K_La (h^{-1})	-	2.6/5.6	2.6	2.6
βx(dimensionless)	4.5	(Farias et al., 2014)	μ_{m_xyl} (h^{-1})	0.0257	0.035	0.06/0.035	0.15/0.08
$K_{x,xyl}$ (g L^{-1})	1.67	(Farias et al., 2014)	Y_{sp} (g g^{-1})	0.45	0.45/0.4	0.45/0.4	0.45/0.4
$P_{m,x,xyl}$ (g L^{-1})	56	(Farias et al., 2014)	$\alpha_{ix,oxy}$ (200% oxygen saturation)[1] (g L^{-1})	-	0.015	0.015	0.015
$K_{ix,xyl}$ (g L^{-1})	24.4	(Farias et al., 2014)	$\alpha_{x,oxy}$ (100% oxygen saturation)[1] (g L^{-1})	-	0.0075	0.0075	0.0075
m_x (g $g^{-1}h^{-1}$)	0.027	(Farias et al., 2014)					
m_o (g $g^{-1}h^{-1}$)	0.0072	(Grootjen et al., 1991)					
Y_{ox}^{max} (g g^{-1})	1.95	(Rizzi et al., 1989)					

[1](American Public Health Association, 1999; Hannoun and Stephanopoulos, 1990)

5.3.2. Validating modelling with experimental data of this study

Oxygen and initial biomass concentration are important factors in the conversion of xylose to ethanol using *P. stipitis* (Farias et al., 2017, 2014; Fu et al., 2009).

Therefore, different aeration levels were tested on three different initial biomass concentration of 0.3, 1.5, 3 g L^{-1}. The tested aeration levels were 0, 0.1 and 0.2 vvm to find the best aeration level and best initial biomass concentration for bioethanol production from xylose using *P. stipitis*. Initial values of biomass, xylose and ethanol that were used in the experiment of this study for *P. stipitis* are shown in Table 5.2.

Table 5.2.The initial concentrations, ethanol yield and productivity, a correlation coefficient

	***P. Stipitis*, anaer.* (initial biomass: 0.3 g L^{-1})**	***P. Stipitis*, microaer.* (initial biomass: 0.3 g L^{-1}, 0.1 vvm)**	***P. Stipitis*, microaer.* (initial biomass: 0.3 g L^{-1}, 0.2 vvm)**	***P. Stipitis*, anaer.* (initial biomass: 1.5 g L^{-1})**	***P. Stipitis*, microaer.* (initial biomass: 1.5 g L^{-1}, 0.1 vvm)**	***P. Stipitis*, microaer.* (initial biomass: 1.5 g L^{-1}, 0.2 vvm)**	***P. Stipitis*, anaer.* (initial biomass: 3 g L^{-1})**	***P. Stipitis*, microaer.* (initial biomass: 3 g L^{-1}, 0.1 vvm)**
C_{x0} (g L^{-1})	0.3	0.31	0.31	1.51	1.49	1.55	3.15	3.003
P_0 (g L^{-1})	0.5	0.5	0.83	0.00	0.00	0.00	0.15	0.55
C_{xyl0} (g L^{-1})	20.3	23.22	24	14.8	16.25	16	14.75	26.6
Correlation coefficient (Xylose)	0.97	0.96	0.97	0.94	0.95	0.96	0.87	0.65
Correlation coefficient (Ethanol)	0.88	0.9	0.86	0.92	0.96	0.9	0.98	0.52
Correlation coefficient (Biomass)	0.92	0.97	0.91	0.91	0.95	0.83	0.61	0.80
Xylose conversion time (h)	180	95	125	60	19	39	42	17
Y_{sp} (g g^{-1})	0.34	0.406	0.37	0.443	0.445	0.401	0.45	0.459
Ethanol productivity (g L^{-1} h^{-1})	0.039	0.099	0.072	0.115	0.38	0.256	0.158	0.95
Final biomass concentration (g L^{-1})	1.85	3.06	3.2	2.9	3.37	3.8	5.04	7.1
Y_{xs} (g g^{-1})	13.097	8.41	8.27	10.5	8.69	6.96	7.29	6.49
Y_{xp} (g g^{-1})	4.46	3.5	3.1	4.66	3.86	2.79	2.99	3.00

*Microaer: microaerobic, Anaer: anaerobic

Results of modelling and experimental work for *P. stipitis* in terms of biomass, ethanol, and substrate concentration were compared and shown in Figures 5.1 to 5.6. The correlation coefficients and comparison

between the modelling and experiments of this study are given in Table 5.2. The model of this study indicated a good fit to the experimental data with an average correlation coefficient of 0.865 for bioethanol production, 0.91 for xylose consumption and 0.86 for biomass growth.

5.3.2.1. Initial biomass concentration of 0.3 g L^{-1}

Anaerobic and microaerobic conditions were tested with initial biomass concentration of 0.3 g L^{-1}. Under anaerobic conditions, 20 g L^{-1} of xylose was exhausted in 180 h, whereas under microaerobic conditions of 0.1 vvm and 0.2 vvm was 98 h and 125 h respectively for complete conversion of 24 g L^{-1} of xylose. The obtained ethanol yield and productivity were 0.34 g g^{-1} and 0.0386 g L^{-1} h^{-1} (Figure 5.1) under anaerobic condition; 0.4057 g g^{-1} and 0.099 g L^{-1} h^{-1} with 0.1 vvm; and 0.37 g g^{-1} and 0.37 g L^{-1} h^{-1} with 0.3 vvm (Figure 5.2 and Figure 5.3). The maximum biomass concentration under anaerobic condition was 1.85 g L^{-1} (Figure 5.1) and from microaerobic condition with 0.1 vvm was 3.06 g L^{-1} and with 0.2 vvm was 3.2 g L^{-1}. Lower cell growth was observed during anaerobic condition that shows the dependence of *P. stipitis* on oxygen. Results showed that air flow of 0.1 vvm gave a better fermentation performance, although the ethanol productivity remained low using initial biomass of 0.3 g L^{-1} (Table 5.2). Optimum aeration level was 0.1 vvm when 10% initial biomass concentration was used. It was noted that ethanol re-assimilation or ethanol evaporation could occur towards the end of xylose consumption as shown in Figure 5.1 (7.2 g L^{-1} ethanol and 1.4 g L^{-1} xylose compared to 6.9 g L^{-1} ethanol and 0.47 g L^{-1} xylose in an anaerobic condition). The calculated dissolved oxygen (C_o) from modelling changes from 0.0053 g L^{-1} to zero during the 45 h fermentation time.

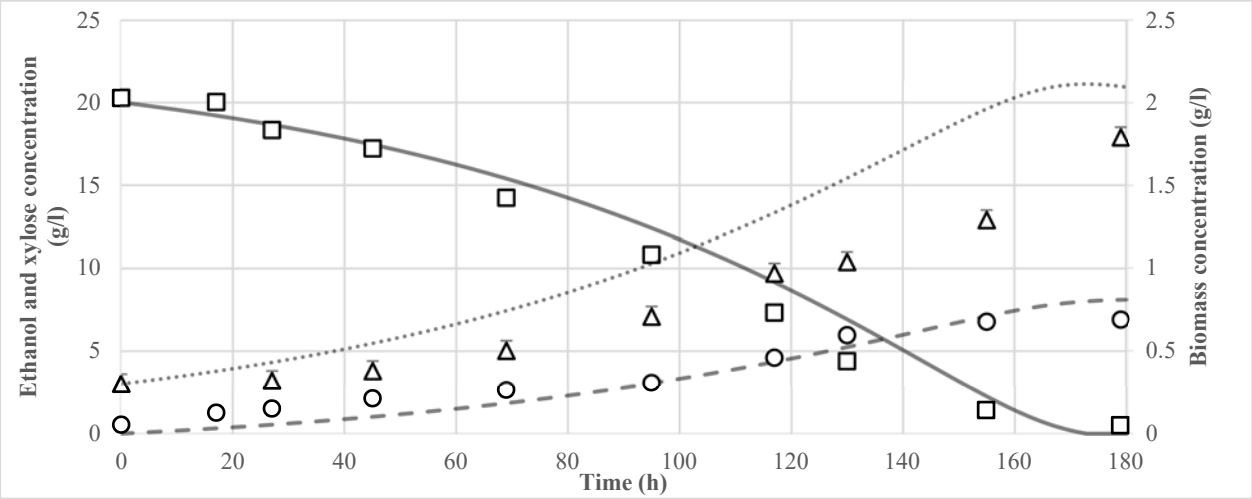


Figure 5.1.Xylose, ethanol and biomass concentration versus time under the anaerobic condition, the initial biomass concentration of 0.3 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study.

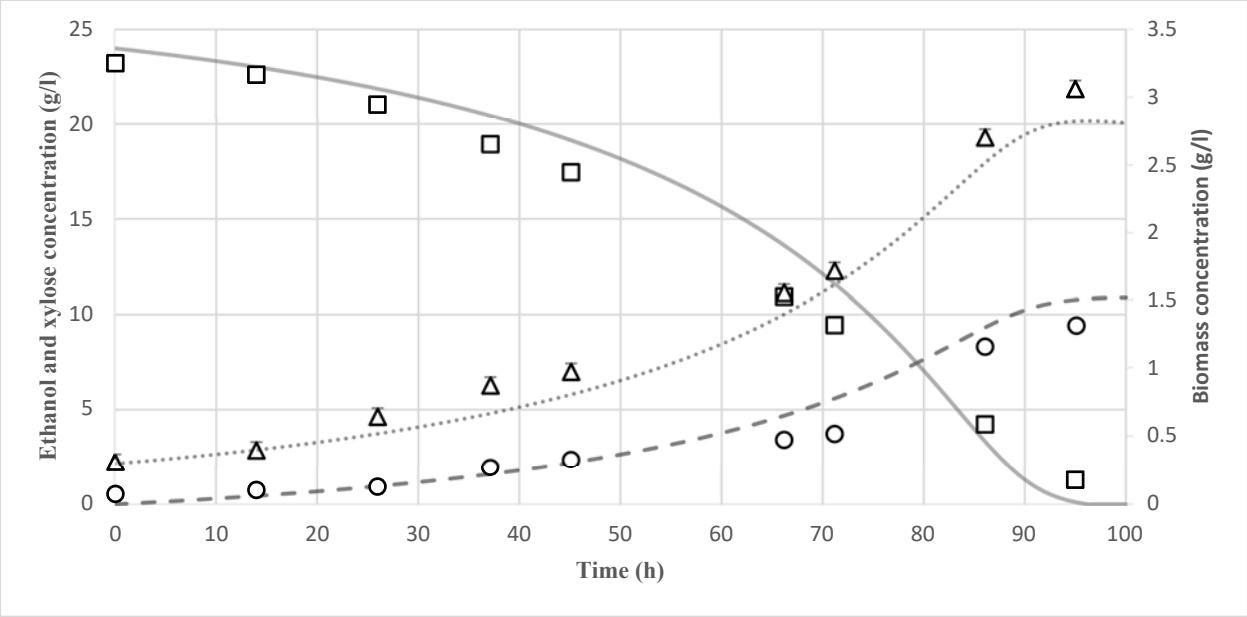


Figure 5.2.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.1 vvm), the initial biomass concentration of 0.3 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study.

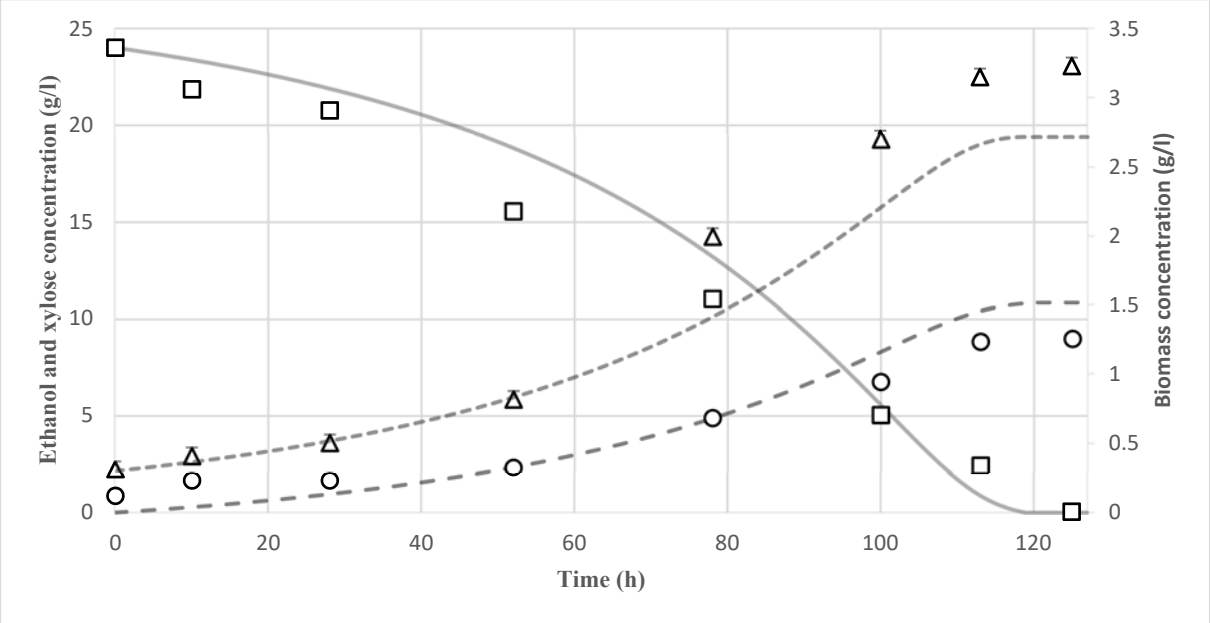


Figure 5.3.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.2 vvm), the initial biomass concentration of 0.3 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study.

5.3.2.2. Initial biomass concentration of 1.5 g L^{-1}

To find the effects of aeration level when there is a larger amount of initial biomass concentration, fermentations were initiated with the other initial biomass concentration of 1.5 g L^{-1} using 0, 0.1 and 0.2 vvm. The fermentation time decreased from 60 h to 19 h and then increased to 39 h when the air flow was increased from 0 to 0.1 and then to 0.2 vvm. The ethanol productivity showed the similar increase from 0.115 g L^{-1} h^{-1} to 0.38 and then decreased to 0.256 g L^{-1} h^{-1} accordingly. The ethanol yield was varied from 0.443 g g^{-1} under the anaerobic conditions to 0.401 in 0.2 vvm and 0.445 g g^{-1} in 0.1 vvm. The highest biomass concentration under the anaerobic conditions is 2.9 g L^{-1} (Figure 5.4) while the microaerobic conditions yielded 3.37 g L^{-1} at 0.1 vvm and 3.8 g L^{-1} at 0.2 vvm (Figure 5.5 and Figure 5.6). Increase of air flowrate from 0.1 to 0.2 vvm resulted in increasing of biomass concentration from 3.37 g L^{-1} to 3.8 g L^{-1} and decreasing ethanol yield from 0.445 to 0.401 g g^{-1}, consequently, decreasing ethanol concentration from 7.225 g L^{-1} to 6.41 g L^{-1}, showing 0.1 vvm is an optimum aeration level with initial biomass concentration of 1.5 g L^{-1}. Highest ethanol concentration of 6.41 g L^{-1} with 0.2 vvm occurred in 25 h, however xylose was consumed completely in 39 h with 6.25 g L^{-1}. Results of the modelling of this study and experimental data of previous study (Silva et al., 2011) showed that the level of oxygen dissolved in the medium, Co was zero under the microaerobic conditions tested in this experiment when using high initial biomass concentration of 1.5 g L^{-1}, possibly indicating that all the oxygen supplied was quickly and immediately consumed by the high initial *P. stipitis* concentration.

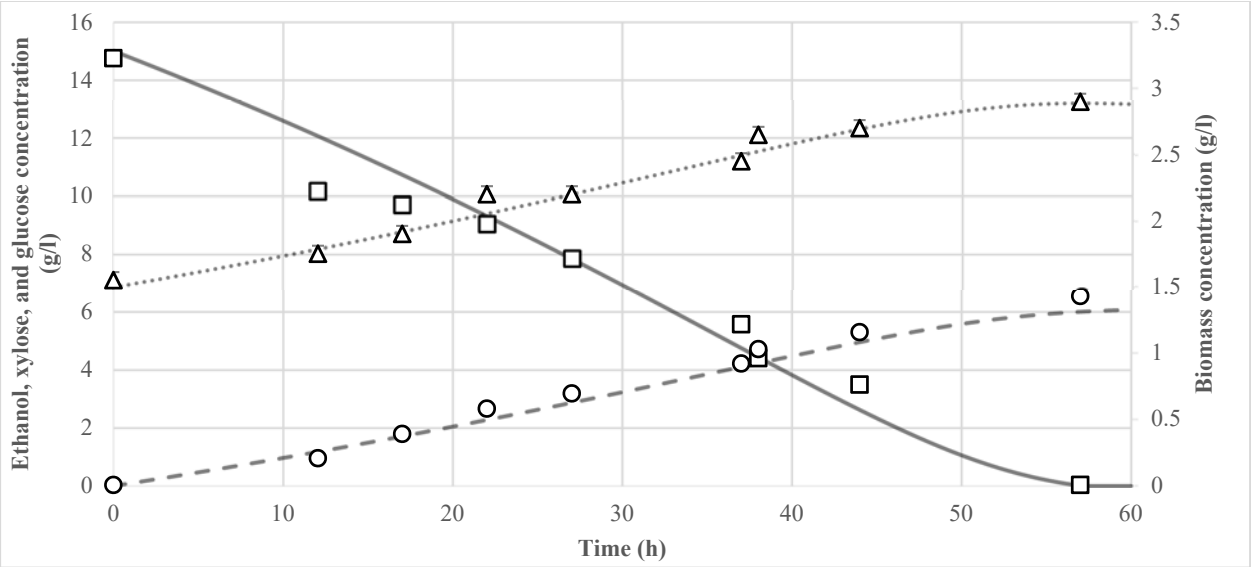


Figure 5.4.Xylose, ethanol and biomass concentration versus time under the anaerobic condition, the initial biomass concentration of 1.5 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study.

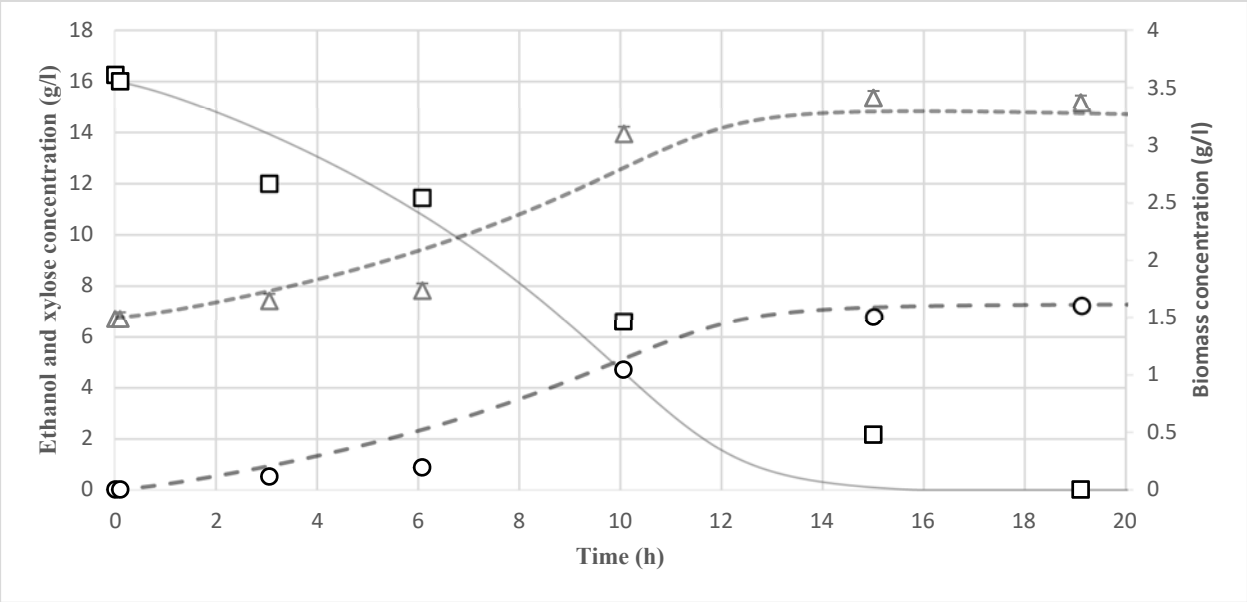


Figure 5.5.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.1 vvm), the initial biomass concentration of 1.5 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study.

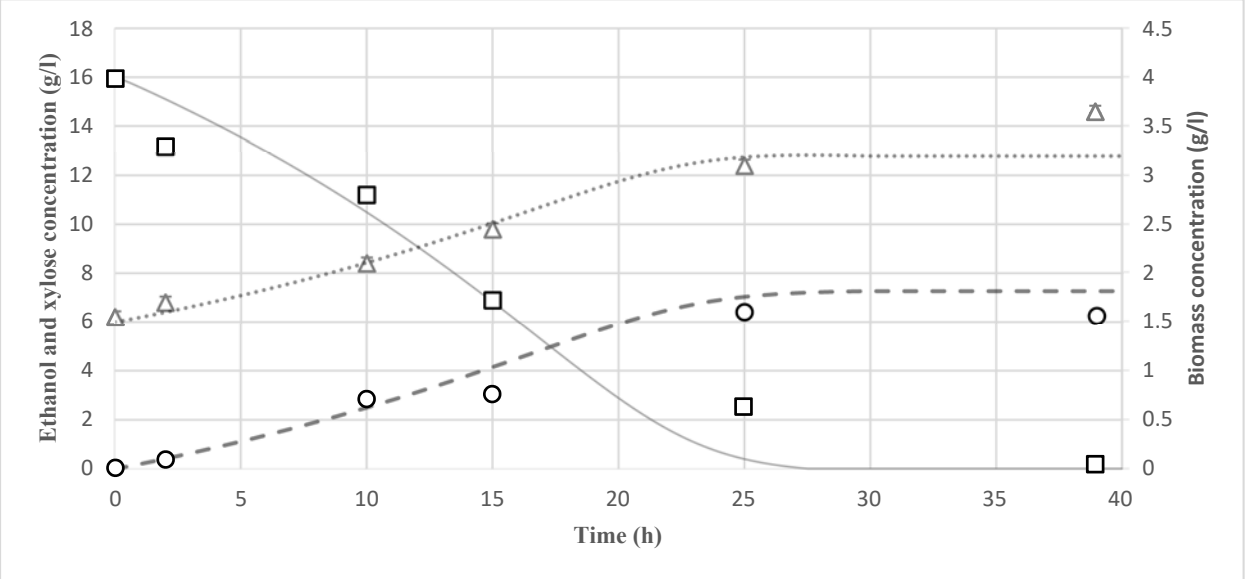


Figure 5.6.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.2 vvm), the initial biomass concentration of 1.5 g L^{-1}. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study.

5.3.2.3. The initial biomass concentration of 3 g L^{-1}

Fermentations were initiated with an initial biomass concentration of 3 g L^{-1} for the anaerobic and the microaerobic condition of 0.1 vvm. All xylose was converted to ethanol in 42 h under the anaerobic conditions, whereas, it took 17 h under microaerobic condition of 0.1 vvm of air. The highest ethanol yield was obtained in 13 h using the microaerobic condition and 96% of xylose conversion that shows the possibility of ethanol re-assimilation or ethanol evaporation. The ethanol yield was 0.45 g g^{-1} under the anaerobic conditions and increased to 0.46 in the microaerobic condition. Ethanol productivity was 0.158 g L^{-1} h^{-1} in the anaerobic and 0.95 g L^{-1} h^{-1} in the microaerobic condition which was the highest among all different tested conditions. The final biomass concentration was 5.04 g L^{-1} in the anaerobic condition and was 7.1 g L^{-1} in the microaerobic condition shown in Figure 5.7 and Figure 5.8. As with the study of initial biomass concentration of 1.5 g l-1, the level of Co in the medium was also reported as zero using high initial biomass concentration of 3 g L^{-1}.

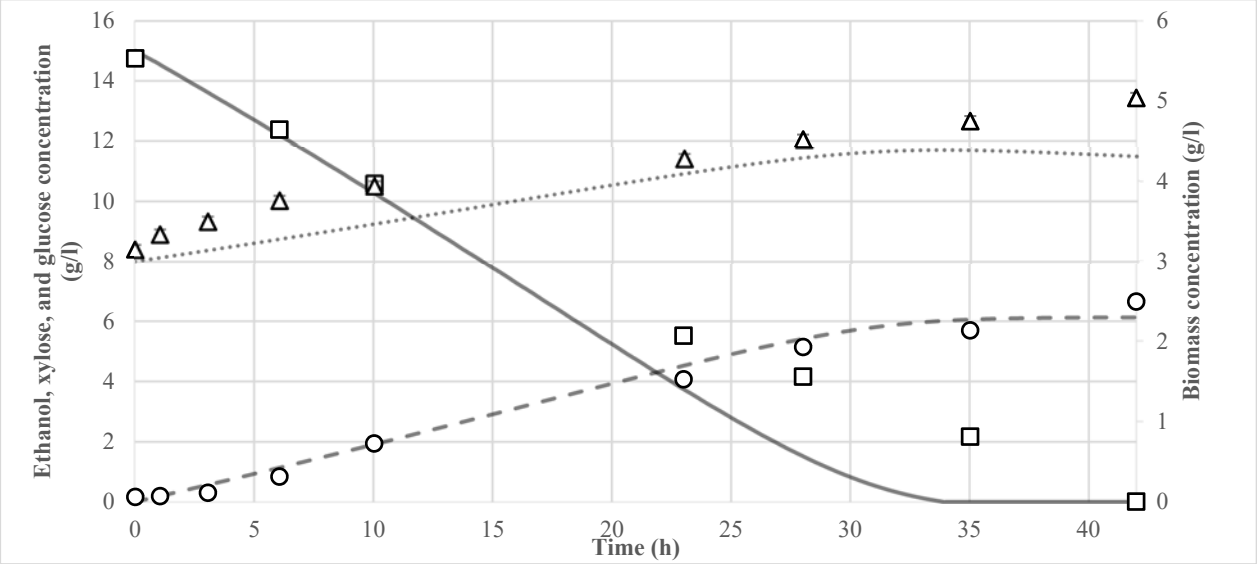


Figure 5.7.Xylose, ethanol and biomass concentration versus time under the anaerobic condition, the initial biomass of 3 g L^{-1}, free cell. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study.

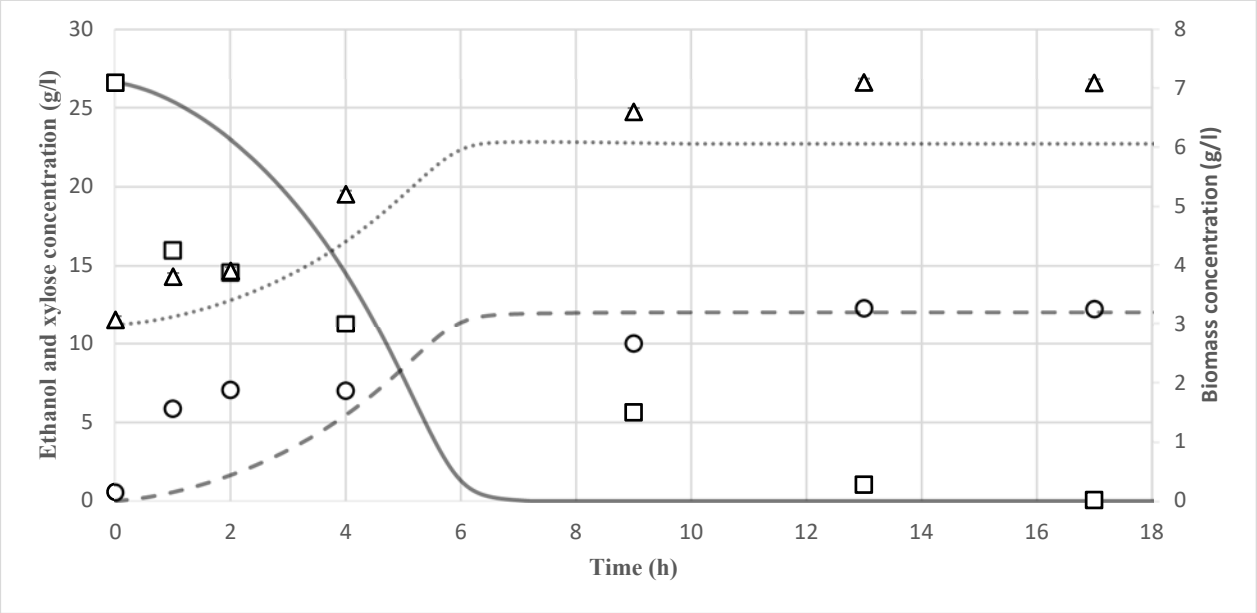


Figure 5.8.Xylose, ethanol and biomass concentration versus time under the microaerobic condition (0.1 vvm), the initial biomass of 3 g L^{-1}, free cell. The solid line (—) denotes substrate concentration, dotted line (…) denotes biomass concentration and dashed line (--) denotes ethanol concentration in modelling. Open squares (□) denote xylose concentration, open triangles (Δ) denote biomass concentration and open circles (○) denote ethanol concentration for experimental data of this study.

5.3.3. Comparison of performance

Comparison among different initial biomass concentration and different aeration level show that increasing initial biomass concentration increase productivity and finding optimum microaerobic condition has effect on both yield and productivity. The three selected time courses are: first initial biomass of 0.3 g L^{-1} with air flow level of 0.1 vvm, first initial biomass of 1.5 g L^{-1} with air flow level of 0.1 vvm, and first initial biomass of 3 g L^{-1} with air flow level of 0.1 vvm. The fermentation time, ethanol yield and productivity are represented in Table 5.2. Air has more effect than inoculum size on growth of microorganisms and consequently in ethanol yield and productivity for xylose conversion using *P. stipitis*. As shown in Table 5.2, there was an increase in ethanol yield (0.4057 to 0.445 and then to 0.459) and productivity (0.099 to 0.38 and then to 0.95 g L^{-1} h^{-1}) with increasing first initial biomass concentration from 0.3 to 1.5 and then to 3 g L^{-1} using 0.1 vvm which was the best aeration level. The highest reported value for free cells of *P. stipitis* in batch culture is in 0.1 vvm and initial biomass concentration of 3 g L^{-1}.

5.3.4. Statistical analysis

One-way Analysis of Variance (ANOVA) was used in the determination of the significance of changes to the initial biomass concentration and aeration levels to the outputs of ethanol productivity and yield during the batch processes carried out in Table 5. 3.

ANOVA analysis showed that aeration level has a stronger effect compared to initial biomass concentrations on ethanol yield and productivity. With a P-value of < 0.1, both criteria of aeration levels and initial biomass concentrations significantly affected ethanol productivities. However, when P-values were dropped to 0.05, only aeration levels gave significant changes. In all cases, the ethanol yields were not significantly affected by changes in vvm and Cxo.

Table 5. 3. Summary of Anova result.

Different aeration level		
Parameter	F	P-value*
Ethanol yield	3.33	0.142
Ethanol productivity	33.96	0.004
Different initial biomass concentration		
Parameter	F	P-value*
Ethanol yield	0.65	0.467
Ethanol productivity	6.98	0.057

* for significant P-values less than 0.1, F value > 4.54. for significant P-value less than 0.05, F value > 7.71

5.4. Conclusion

P. stipitis consumed xylose efficiently and reaching high conversion rates. Ethanol can be produced in the anaerobic condition. However, the xylose conversion without aeration failed to achieve high ethanol productivity (0.158 g L^{-1} h^{-1} using the anaerobic and 0.95 g L^{-1} h^{-1} using the microaerobic condition). It was thus confirmed that oxygen limited supply was essential for an efficient xylose fermentation. A non-structured model of Andrews–Levenspiel was used and the model predictions were in good agreement with experimental observations, thus allowing us to systematically investigate the kinetics characteristics and describe xylose consumption and ethanol yield of this yeast under the microaerobic conditions in different aeration level and different initial biomass concentration. The biomass concentration of *P. stipitis* on xylose increased with the increase of inoculum size and aeration level. Higher initial biomass concentration showed faster sugar utilization (0.95 g L^{-1} h^{-1} using initial biomass concentration of 3 g L^{-1}). The approach used in this work can be useful for process prediction and control, as well as for simulation and optimization of the fermentative process. The results showed that increase in both initial biomass concentration and operating under optimum oxygenation levels benefitted the ethanol production and yield by *P. stipitis* on xylose.

5.5. Declaration of interest

The authors declare no competing financial interest.

5.6. Acknowledgment

The financial support of the National Research Foundation of South Africa Competitive Programme for Rated Researchers (CPRR: 87744), kindly prepared by Prof. Duncan M. Fraser and Dr. Adeniyi J. Isafiade; the financial support of the research development grant from the University Research Council (URC) of the University of Cape Town, the financial support of Centre for Bioprocess Engineering Research (CeBER) through the SARChI Chair in Bioprocess Engineering (GUN 64788) of Prof. Susan T. Harrison are gratefully acknowledged.

5.7. Nomenclature:

C_x: Cell concentration in medium (mass/unit volume)

C_{xyl}: Xylose concentration (mass/unit volume)

C_{xyl0}: Input substrate concentration (mass/unit volume)

C_{x0}: Input biomass concentration (mass/unit volume)

C_o: Dissolved oxygen concentration in medium during the fermentation (mass/unit volume)

C_o^*: Dissolved oxygen concentration at saturation (mass/unit volume) (C_o^* at 0.21 % v/v O_2 and 30 ºC: 7.5 mg L^{-1})

$K_{x,xyl:}$ Xylose limitation constant for biomass production rate (mass/unit volume)

$K_{ix,xyl}$: Xylose inhibition constant for biomass production rate (mass/unit volume)

$K_L a$: Volumetric oxygen transfer coefficient (1/unit time)

m_x: Maintenance energy coefficient of biomass (g-substrate/ g-biomass/ unit time)

m_o: Maintenance energy coefficient of oxygen (g-substrate/ g-oxygen/ unit time)

VVM: Volume of air/volume of medium/time (unit volume/ unit volume/ unit time)

$\alpha_{ix,oxy}$: Oxygen inhibition constant in xylose for growth of biomass (mass/unit volume)

$\alpha_{x,oxy}$: Product limitation constant in xylose for growth of biomass (mass/unit volume)

P: Ethanol concentration (mass/unit volume)

P_0: Input product concentration (mass/unit volume)

$P_{m,x,xyl}$: Maximum ethanol concentration in xylose above which cells do not grow (mass/unit volume)

q_{xyl}: Specific xylose utilization rate (1/unit time)

r_s: Substrate consumption rate (mass/unit volume/unit time)

r_x: Biomass production rate (mass/unit volume/unit time)

r_e: Ethanol production rate (mass/unit volume/unit time)

r_o: Oxygen consumption rate (mass/unit volume/unit time)

Y_{ox}^{max}: Maximum cell yield constant from oxygen in xylose (g-bimass/g-oxygen)

Y_{sp}: Product yield constant from xylose (g-product/g-substrate)

Y_{xp}: Ethanol yield constant from biomass (g-product/ g-biomass)

Y_{xs}: Substrate yield constant from biomass (g-substrate/g-biomass)

Y_{sx}^{max}: Maximum cell yield constant from xylose (g-biomass/g-substrate)

$\mu_{m,xyl}$: Maximum specific growth rate in xylose (1/unit time)

$\mu_{xyl:}$ Specific growth rate in xylose (1/unit time),

ν_{xyl}: Specific rate of product formation in xylose (1/unit time)

βx: Product inhibition constant in xylose for growth of biomass (dimensionless)

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6. Kinetic model of bioethanol fermentation using Aspen Custom Modeller (ACM)

In this paper, novel custom kinetic models for bioethanol fermentation, developed in Aspen Custom Modeller (ACM), within an Aspen Plus flow sheet instead of a stoichiometry/kinetic model in Aspen Plus was presented. The following paper addresses objective (4). Stoichiometry models can be implemented with less information and effort. However, stoichiometry models do not represent the continuous experimental data sufficiently because of uncertainty in the estimation of reaction conversion and the non-dependency on dilution rates in CSTR.

Kinetic models represent experimental and industrial data more accurately, but since it is not possible to develop complex kinetic models of microbial systems in Aspen Plus alone, these models were developed using an equation-oriented approach that was then integrated into Aspen Plus. This paper is under preparation to be published in the Computers and Chemical Engineering Journal.

Author contribution:

The manuscript was written by Nosaibeh Nosrati Ghods (NNG) and edited by Prof. Susan T.L. Harrison (STLH), Associate Prof. Adeniyi J. Isafiade(AJI) and Dr. Siew L. Tai (SLT). The modelling through ACM was done by the primary author. Mr. Muven Naidoo helped in some part of modelling as a computational support. Required information of industrial input and output for bioethanol production in Brazil were prepared from CTBE by Prof. Antonio Bonomi and Dr. Edvaldo Morais. The financial support of the National Research Foundation of South Africa Competitive Programme for Rated Researchers (CPRR: 87744), kindly prepared by Prof. Duncan M. Fraser and Associate Prof. Adeniyi J. Isafiade; the financial support of the research development grant from the University Research Council (URC) of the University of Cape Town, the financial support of Centre for Bioprocess Engineering Research (CeBER) through the SARChI Chair in Bioprocess Engineering (GUN 64788) of Prof. Susan T.L. Harrison are gratefully acknowledged.

Paper title:

Embedding the equation-oriented kinetic model for bioethanol fermentation in a sequential modular flowsheet simulator

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Abstract:

Commercial processes used to produce first generation biofuels (e.g. oil seeds, starch-rich grains, sugar-rich plants) are mature. In this study, a kinetic model using equation-oriented process modelling tools (Aspen Custom Modeller) for a biological industrial reactor with cell recycles was embedded in a process simulation environment (Aspen Plus) through a C++ compiler. To embed a native module in a process simulation environment, the legacy codes written in the procedural language for Aspen Custom Modeller (ACM) is treated as a black box in order to integrate the system into the component based framework. The compliance of the custom modeller to Aspen Plus allows for easy integration of the developed reactor simulator for the fermentation process of bioethanol production using kinetic models. The aim of this research is to improve the ability to use custom kinetic models of fermentation processes (e.g. bioethanol production), developed in equation-oriented modelling tools, within a sequential modular flowsheet simulator. The obtained result showed the accuracy of the selected kinetic model in ACM with less than 1% difference to the industrial data, while a stoichiometry model in ACM and Aspen Plus showed approximately 9% difference to the industrial data. Furthermore, when a continuous process was carried out, the results between the model and experimental results showed good agreement. The simulation shows ACM integrated into Aspen Plus allows for complex biological processes to be accurately predicted for biomass growth, ethanol production and sugar consumption.

Keywords: bioethanol, oriented process modelling tools, sequential modular flowsheet simulator, fermentation

6.1. Introduction

Simulation has become a powerful tool to support the design of industrial processes and is now a critical step in the design of process (Rhodes, 1996). Simulation tools for chemical design processes, which are relevant to this study, can be divided into two categories, namely sequential modular (or block oriented) flowsheet simulators and equation-oriented process modelling tools (Hensen, 2005). Sequential modular flowsheet simulator (SMFS) environments (e.g. Aspen Plus, Aspen HYSYS, and PRO/II) are widely used by process engineers because of their ease of use and robustness in handling large-scale process simulation problems (e.g. large number of unit operations, process streams and chemical components) (Gani et al., 2012; Hensen, 2005). However, most SMFSs are limited in type and number of process unit operations models, and also limited to mainly continuous steady-state processes (Dimian et al., 2014). The equation-oriented environments (e.g. gPROMS and Aspen Custom Modeller) can be used for steady-state and dynamic process simulation and optimization, as well as for parameter estimation and experimental design (Hensen, 2005; Nawaz, 2015) . Equation-oriented process modelling tools offer the opportunity to develop custom process unit operation models without the need to develop numerical solution methods for the model equations (Gani et al., 2012). Therefore, it would be beneficial to combine these two simulation environments. The use of a custom process unit operation model, developed using equation-oriented modelling tools, and combined in a SMFS, requires interfacing between the different software programmes. In the early nineties, the idea of an open interface for integration data between process simulation software of various origins was put forward by academic institutions and industry. Several CAPE-OPEN (Computer Aided Process Engineering) projects were initiated to develop standards and to explore the possibilities for open interfaces for integration of process unit operations, thermodynamic and physical property packages, and numerical solvers between the various process simulation tools (COLaN, 2016).

Aspen Custom Modeller (ACM) can be used to develop custom process unit operation models which are not available in the Aspen Plus model library (Brinkmann et al., 2003). After development, the custom model can be used as a user model block inside a sequential modular flowsheet simulator (Dimian et al., 2014). For consistent flowsheet simulation and optimization, the use of a custom model in Aspen Plus requires interfacing with equation-oriented modelling tools (Hensen, 2005; Nikolić, 2016). Testing the status and performance of the software interoperability, as well as examining the custom model performance in Aspen Plus with experimental and specifically industrial results would be essential.

Hence, this paper describes the development of the custom kinetic model and its interfacing with Aspen Plus and the validation with experimental and industrial data. The current practice for simulating a unit operation present in a process flowsheet, in which the required kinetic model does not exist in the model library, is a shortcut method to obtain the mass and energy balances. To allow a deeper understanding of the impacts of inserting kinetic model for fermentation process on a small scale and an industrial plant,

simulations of bioethanol fermentation process were carried. A schematic picture of the unit model interface in Aspen Tech (ACM and Aspen Plus) is shown in Figure 6.1.

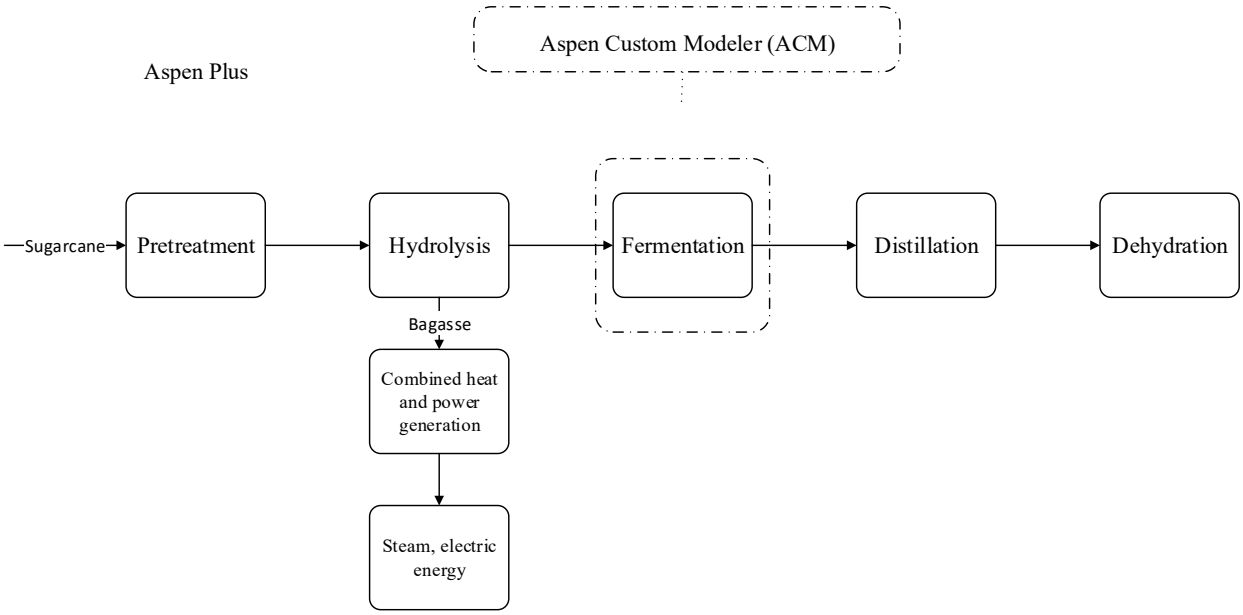

Figure 6.1.The custom model block of a process unit operation in a sequential modular flowsheet simulator for bioethanol production process from sugarcane (Dias et al., 2010)

6.2. Materials and Methods

6.2.1. Experimental work for validation

6.2.1.1. Strains

The strain of *Z. mobilis* ZM4 (ATCC 31821) was used.

6.2.1.2. Culture storage

For short-term storage, all cultures were stored in the solid rich medium (RM) (Goodman et al., 1982) containing per litre: 20 g glucose, 10 g yeast extract, 2 g KH_2PO_4, 15 g agar, 1000 ml DI water. Agar plates were stored at 4 °C. For long-term storage, the cultures were suspended in sterile 40% (v/v) glycerol and stored in 1.5 mL volumes at -60 °C. For this, equal amounts of bacterial culture and 80% glycerol were mixed. The cell-glycerol mixture was kept at room temperature for half an hour prior to freezing.

6.2.1.3. Synthetic media

Inoculum medium consisted of 10 g L^{-1} yeast extract, 1 g L^{-1} $MgCl_2$, 1 g L^{-1} $(NH_4)_2SO4$, 1 g L^{-1} KH_2PO_4, with 44 g L^{-1} glucose. Yeast extract and inorganic salts (YEIS) solution were sterilized separately from glucose at 121 °C for 20 min.

6.2.1.4. Inoculum preparation and fermentation studies

Z. mobilis was subcultured in fresh inoculum media twice before inoculated into the fermentor. Inocula were incubated for 24 h at 30 °C in 250-ml conical flasks with 50 ml medium, in a shaker incubator at a speed of 120 rpm. Initial biomass of 2.1 g/l was used to inoculate the fermentor. Experiments were conducted in a 7 L Brunswick reactor with a working volume of 5 litres, an agitation rate of 200 rpm, and at 30 °C. The pH was controlled at 6.0 using 1 M NaOH. In this study for *Z. mobilis* ZM4 (ATCC 31821), the initial biomass concentration (C_{x0}) was 2.1 g L^{-1}, initial ethanol concentration (P_0) was 0.001 g L^{-1}, initial glucose concentration (C_{glu0}) was 48.78 g L^{-1} as shown in Table 6.1.

Table 6.1.Initial concentrations of *Z. mobilis* strains which was used for modelling and experiment of this study

	Wild type *Z. mobilis* ZM4 (g L^{-1})
C_{glu0}	48.78
P_0	0.001
C_{x0}	2.1

6.2.1.5. Analytical procedures

Dry cell mass from 2 ml sample was determined by centrifuging for 10 minutes at 13000 rpm (Heraeus Biofuge Pico); washed with water and centrifuged again. The cell pellet was dried at 80 °C for 24 hours and stored in a desiccator before weighing. The optical density was determined at 660 nm by spectrophotometer (Gensys 10S UV-VIS) and kept at around 0.3 OD by dilution. Samples from the fermentation broth were analysed for glucose and ethanol concentrations by HPLC using an Aminex column HPX-87H (300*7.8mm) (Bio- Rad, Ion exclusion column) equipped with a refractive index detector. Separations were performed at 65 °C, eluted at 0.3 ml/min using 5 mM sulphuric acid. Standards containing analytical grade components were used periodically to confirm calibration accuracy. The samples and standards were filtered with a 0.22 µm syringe filter. The mobile phase was filtered (with a 0.45 µm membrane filter) and degassed in a sonic bath.

6.2.2. Computational methods

Modelling was carried out using ACM version 9.0 and Aspen Plus version 9.0. The custom kinetic model for fermentation process of bioethanol production was developed in ACM and embedded in Aspen Plus. The model includes equations of vapour-liquid equilibrium (VLE) (Eq.1), mass (Eq.2) and energy balance (Eq.3) (e.g. molecular weight, thermodynamic phase equilibria, kinetic equation).

VLE equation (modified Raoult's law):

$$y \times P_{tot} = L_{mole\text{-}fraction} \times \gamma \times P_{vap} \qquad (1)$$

Mass balance:

$$Z \times F + (r_{rate} \times R_{vol}) = y \times V + L_{mole\text{-}fraction} \times L_{molar\text{-}flow} \quad (2)$$

Energy balance:

$$H_{in} \times F + Q = L_{molar\text{-}flow} \times H_L + V \times H_v \quad (3)$$

6.2.2.1. Kinetic models

The kinetic model in this paper incorporates substrate limitation, substrate inhibition, and product inhibition functions, which are based on the modified Monod form expressions for glucose conversion using *Zymomonas mobilis* (Lee and Rogers, 1983; Leksawasdi et al., 2001) and glucose conversion using *Saccharomyces cerevisiae* (Krishnan et al., 1999, 1995).

6.2.2.1.1. Biomass production rate

The specific biomass growth rate for all microorganisms of *S. cerevisiae* 1400 and *Z. mobilis* ZM4 for glucose are represented by Equations (4), (5a) and (5b). Biomass growth rate was calculated from Equation (6).

S. cerevisiae:

$$\mu_{glu} = \left(\frac{\mu_{m,glu} C_{glu}}{K_{x,glu} + C_{glu}}\right)\left(\frac{K_{ix,glu}}{K_{ix,glu} + C_{glu}}\right)\left(1 - \left(\frac{P}{P_{ix,glu}}\right)^{\beta x}\right) \quad (4)$$

Z. mobilis:

$C_{glu} \leq 100$ g L^{-1}

$$\mu_{glu} = \left(\frac{\mu_{m,glu} C_{glu}}{K_{x,glu} + C_{glu}}\right)\left(1 - \frac{P - P_{ix,glu}}{P_{m,x,glu} - P_{ix,glu}}\right) \quad (5a)$$

$C_{glu} > 100$ g L^{-1}

$$\mu_{glu} = \left(\frac{\mu_{m,glu} C_{glu}}{K_{x,glu} + C_{glu}}\right)\left(1 - \frac{P - P_{ix,glu}}{P_{m,x,glu} - P_{ix,glu}}\right)\left(\frac{K_{ix,glu}}{K_{ix,glu} + C_{glu}}\right) \quad (5b)$$

Biomass growth rate:

$$r_x = \mu_{glu} \times C_x \quad (6)$$

6.2.2.1.2. Substrate uptake rate

The specific glucose uptake rate for *S. cerevisiae* and *Z. mobilis* are represented by Equations (7) and (8) respectively. Equation (9) illustrates substrate consumption rate that was used in this paper.

S. cerevisiae:

$$q_{glu} = \left(\frac{q_{m,glu} C_{glu}}{K_{s,glu} + C_{glu}}\right)\left(\frac{K_{is,glu}}{K_{is,glu} + C_{glu}}\right)\left(1 - \left(\frac{P}{P_{is,glu}}\right)^{\beta s}\right) \quad (7)$$

Z. mobilis

$$q_{glu} = \left(\frac{q_{m,glu} C_{glu}}{K_{s,glu} + C_{glu}}\right)\left(1 - \frac{P - P_{is,glu}}{P_{m,s,glu} - P_{ix,glu}}\right)\left(\frac{K_{is,glu}}{K_{is,glu} + C_{glu}}\right) \quad (8)$$

Substrate conversion rate:

$$r_{glu} = q_{glu} \times C_x \quad (9)$$

6.2.2.1.3. Ethanol production rate

The specific ethanol production rate for *S. cerevisiae* and *Z. mobilis* are represented by Equations (10). Ethanol production rate is shown in Equation (11).

S. cerevisiae and *Z. mobilis*

$$v_{glu} = q_{glu} \times Y_{sp,glu} \quad (10)$$

Ethanol production rate:

$$r_e = v_{glu} \times C_x \quad (11)$$

6.2.2.2. Kinetic constants

The values of the kinetic parameters are given in Table 6.2.

Table 6.2.Kinetics of *Z. mobilis* and *S. cerevisiae*

Kinetics of *Z. mobilis*			**Kinetics of *S. cerevisiae***		
	Value	References		Value	References
$\mu_{max,glu}$	0.27	Chapter 4 of Ph.D. thesis	$\mu_{max,glu}$	0.34	Chapter 4 of Ph.D. thesis
$K_{x,glu}$	0.48		$K_{x,glu}$	0.572	
$P_{max,x,glu}$	85.8		$P_{max,x,glu}$	129.9	
$K_{ix,glu}$	600		$K_{ix,glu}$	1127.8	
$P_{ix,glu}$	28		βx	0.25	
$q_{max,glu}$	8		$q_{max,glu}$	3.06	
$K_{s,glu}$	2.1		$K_{s,glu}$	1.34	
$P_{max,s,glu}$	127		$P_{max,s,glu}$	136.4	
$K_{is,glu}$	600		$K_{is,glu}$	4882.8	
$P_{is,glu}$	35.5		βs	0.4	
Y_{spglu}	0.5		Y_{spglu}	0.4	

6.2.2.3. Stoichiometry

The ethanol production, biomass growth, sugar consumption, water and carbon dioxide production rates were calculated by Equations 12-15 and the by-products (glycerol, and acetic acid) were calculated by Equations 14-15. The conversions stated in Equations 12-15 are based on data provided by Brazilian Bioethanol Science and Technology Laboratory (CTBE's Aspen simulation) from a typical industrial unit (Bonomi et al., 2011). The use of these conversions to represent by-product formation simulates the

real process because the stoichiometric coefficients were calculated in order for the process reactions to meet the number of by-products obtained in the industry (Bonomi et al., 2011). The fractional conversion is based on data provided by CTBE from industry (Bonomi et al., 2011).

Glucose conversion:

$C_6H_{12}O_6 \rightarrow 2\ C_2H_6O + 2\ CO_2$, fractional conversion: 90% of glucose (mole) (12)

$3.3\ C_6H_{12}O_6 + 2.8\ NH_4OH + 2.1\ CO_2 \rightarrow CH_3COOH + 6.9\ H_2O + 19.6\ CH_{1.8}O_{0.9}N_{0.145}$, fractional conversion: 100% of NH_4OH (mole) (13)

$0.6\ C_6H_{12}O_6 + 0.5\ H_2O \rightarrow 0.5\ CO_2 + C_3H_8O_3$, fractional conversion: 52.09% of glucose (mole) (14)

$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$, fractional conversion: 1.315% of glucose (mole) (15)

6.2.2.4. Mass balance

Mass balance of CSTR, including, microorganism growth (r_x), substrate uptake (r_{glu}), and ethanol formation (r_e)

are given in Equations 16 to 18.

$$r_x = D \times (C_{x0} - C_x) \quad (16)$$

$$r_{glu} = D \times (C_{glu0} - C_{glu}) \quad (17)$$

$$r_e = D \times (P_0 - \mathrm{P}) \quad (18)$$

6.2.2.5. Aspen Tech interfaces (ACM – Aspen Plus)

Two functionalities were available in ACM to export a custom model for implementation in Aspen Plus, namely flowsheet export or model export. Changing the list of components, the physical property package and parameters is not possible in flowsheet export and is less flexible than an model export (Hensen, 2005). Hence, the functionality of a model export to create an ACM user model in Aspen Plus was used.

6.2.2.6. Process of interfacing

The same versions of process simulation software for the development of the work processes were used in order to prevent issues of backward compatibility and to retain consistency in the property database version. The procedures and issues are divided into cases that have to do with physical property interfacing and with unit model interfacing.

Biomass properties

Biomass properties was obtained from the National Renewable Energy Laboratory (NREL) document in Aspen Tech (NREL, 2004) and by entering additional information manually in the database manager.

Activity coefficient

It is suggested to use the conditional statement in the thermodynamic activity equation to avoid the calculated limiting activity coefficient to be too large or out of bounds in Aspen Plus property capabilities. The limiting activity coefficient becomes a problem when the mole fraction of the solute approaches zero, which is often caused by the initial value used in Aspen Plus.

Information exchange

The custom model was designed to give information on the conditions (e.g. temperature, pressure and composition) for which the property was calculated. Together with this information, the state of the phase (gas, liquid or solid) property or procedure was specified. In ACM the property was calculated on molar - or mass basis, the component fraction was in kmol/kmol, the temperature was in Celsius and pressure was in bar. In ACM, the order of the components was the order of the components defined in the component list (normally alphabetical order), which is not necessarily the same as in Aspen Plus. The name of the components in the component list of ACM was equal to the name entered for the components in Aspen Plus when creating the property definition file.

Port connection

Model blocks were connected by streams representing the flow of mass or energy in Aspen Plus. Aspen Plus streams were connected to the ports of the custom model. The variables representing an Aspen Plus material stream were set to SI units to allow for automatic conversion as the ACM user block in Aspen Plus calculates in metric units instead of in SI units. The input port and two output ports (vapours and liquids) were defined as condition statements in ACM containing the mole fraction, molar flow rate, molar volume, temperature and pressure.

Link with physical property package

ACM have its user block to define the property method. The physical property package from Aspen Plus was used, as there was a physical property interface between Aspen Plus and ACM.

Initialisation set

The set of model equations created for the custom kinetic model of fermentation process cannot be solved simultaneous, but several intermediate modelling steps are required to approach the final results. To ensure that the exported model converges in Aspen Plus, an initialization set was exported from ACM with the custom model. This initialization set contained the values of all variables near the values of the final result for a specific application.

A visual basic script (PreSolve) was created for ACM. This PreSolve script in Aspen Plus runs just before the actual run solving the equations, and sets the values of all variables to the values defined in the script.

Design specification

Variables and parameters defined in the ACM user block was connected to other parameters or variables of other models of process unit operations in a SMFS.

6.3. Results and discussion

6.3.1. Comparing kinetic and stoichiometry model with literature experimental data and experiment of this study

The results of the kinetic model developed in ACM and from stoichiometry in Aspen Plus in comparison with the experimental result of this study under anaerobic continuous stirred tank reactor using *Z. mobilis* (0.07 h^{-1}) for bioethanol fermentation from 48.78 g L^{-1} of glucose are shown in Figure 6.2.

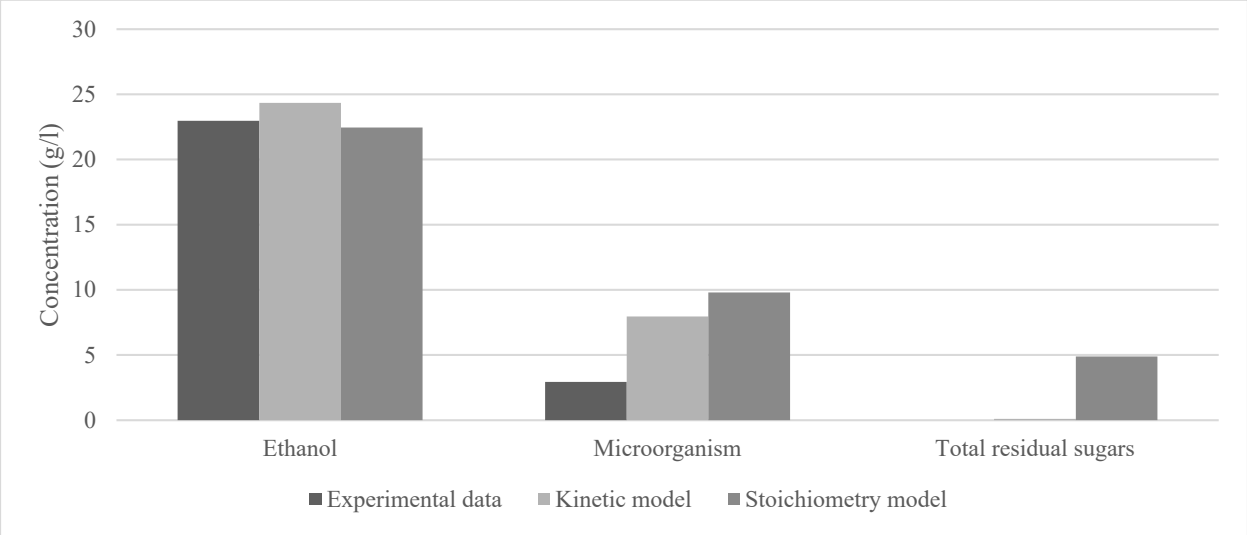

Figure 6.2.Comparison of the kinetic and stoichiometry model of fermentation data on ethanol, microorganisms and residual sugars with experiment of this study for bioethanol production from glucose

The kinetic model had a better agreement with experimental data than stoichiometry model as shown in Figure 6.2. The ethanol, cell and residual glucose concentrations in the experiment of this study were 22.97, 2.94 and 0.0 g/l respectively compared to 24.34, 7.95, 0.1 g/l from the kinetic model developed in ACM and embedded in Aspen Plus, and 22.45, 9.79, 4.88 g/l from stoichiometric model in Aspen Plus.

In the other example, the results of the kinetic model in ACM and stoichiometry in Aspen Plus in comparison with literature experimental results using *Zymomonas mobilis* (Lee et al., 1980) for the fermentation process of bioethanol production from initial concentration of 170 g L^{-1} of glucose is shown in Figure 6.3.

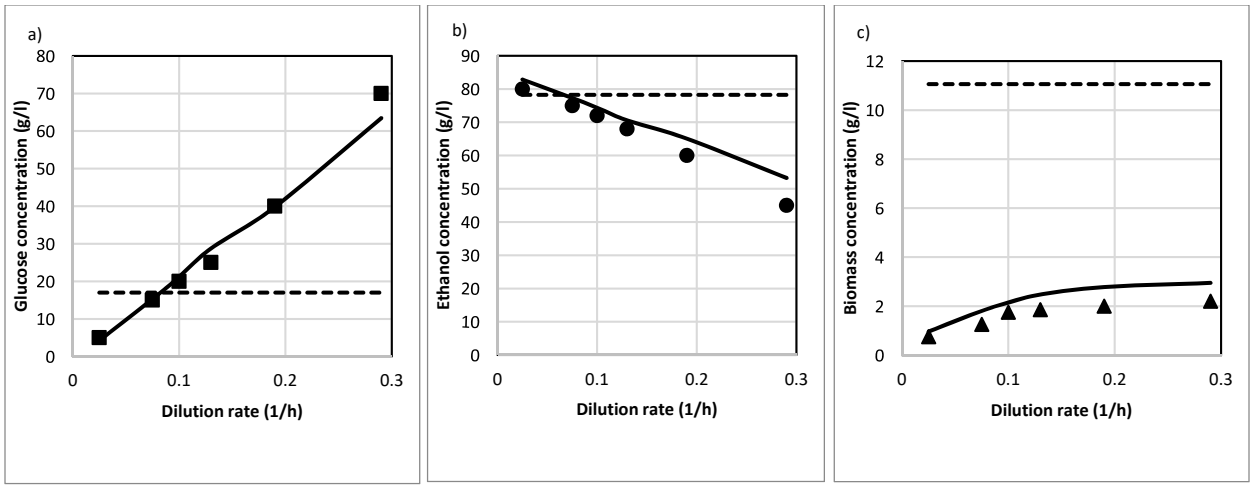

Figure 6.3.Ethanol production from 170 g L^{-1} glucose using *Z. mobilis* (Lee et al., 1980). a) square (■) is for glucose concentration, dashed line (---) is for glucose concentration using stoichiometry model, solid line (—) is for glucose concentration using kinetic model; b) circle (●) is for ethanol concentration, dashed line (---) is for ethanol concentration using stoichiometry model, solid line (—) is for ethanol concentration using kinetic model; c) triangle (▲) is for biomass concentration in experimental result, dashed line (---) is for biomass concentration using stoichiometry model, solid line (—) is for biomass concentration using kinetic model.

The results show that kinetic models have better agreement with the experimental results for concentrations of ethanol and biomass production, and substrate consumption at different dilution rates from same substrate concentration than stoichiometric model.

The dilution rate that is an important factor in CSTR play no role when stoichiometry model is used and resulted in a constant ethanol (78.25 g L^{-1}), biomass (11.06 g L^{-1}) and substrate concentrations (17 g L^{-1}), Figure 6.3. Stoichiometry models only relates to initial concentration and not dilution rate as shown in Equations 16-17. In addition, finding reliable and suitable reaction conversion in stoichiometry models is difficult and estimation from these models tends to predict less than accurate results.

As an example, X (glucose coefficient) =1, Y (ethanol coefficient) =2 and Z (CO_2 coefficient) =2 are the coefficient of Equation 12. Reaction rate of stoichiometry model for ethanol is given in Equation 18. The reaction rate of Equation 18 is then substituted in Equation 19. As it is given in Equation 20, that the dilution rate is cancelled that shows that stoichiometric models in CSTR are independent of the dilution rate.

$$r_e = D \times C_{glu0} \times \text{conversion rate} \times [Y/X]/MW_{glu} \quad (19)$$

$$D \times (P_0 - P)/MW_e = D \times C_{glu0;} \times \text{conversion rate} \times [Y/X]/MW_{glu} \quad (20)$$

6.3.2. Comparing kinetic and stoichiometry model with industrial data

Ethanol production, substrate consumption, by-product production, and microbial growth from glucose in fermentation using ACM for kinetic model and using Aspen Plus for stoichiometry were compared with the industrial output provided by CTBE in Figure 6.4. The conversions for the stoichiometric model were

calculated based on industrial data from a typical industrial unit. The mass fraction of acetic acid over ethanol ratio in industry in Brazil is 3. 6 % (wt) and with modelling in ACM embedded in Aspen Plus resulted in 3.2% (wt) while a stoichiometric model in Aspen Plus resulted in 3.1%. The glycerol amount over ethanol in the industry was reported to be (6.33% wt) is lower than using kinetic model (6.9% wt) and stoichiometry (6.6% wt). Residual sugars over ethanol in industry was (0.25% wt) and showed comparable result to the ACM kinetic model (0.3% wt) while the stoichiometric model under determined this value as 0.13% (wt). Biomass over ethanol production in industry was 5.85% (wt) and was modelled to 4.64% (wt), when kinetic model was used, whereas, it was over predicted with 14.5% (wt) when stoichiometry was used. Overall, the differences in results between industrial data and kinetic model were less than 1% (wt) that makes ACM an acceptable tool as a simulator when considering industrial data. When industrial data were represented by stoichiometric conversions, the components could not fit well. In particular, the biomass over ethanol fraction was out of range, as the stoichiometric equations were interdependent and the sensitivity of the conversions affected each component more readily. A small variation in the conversion led to large misinterpretation of the final outputs.

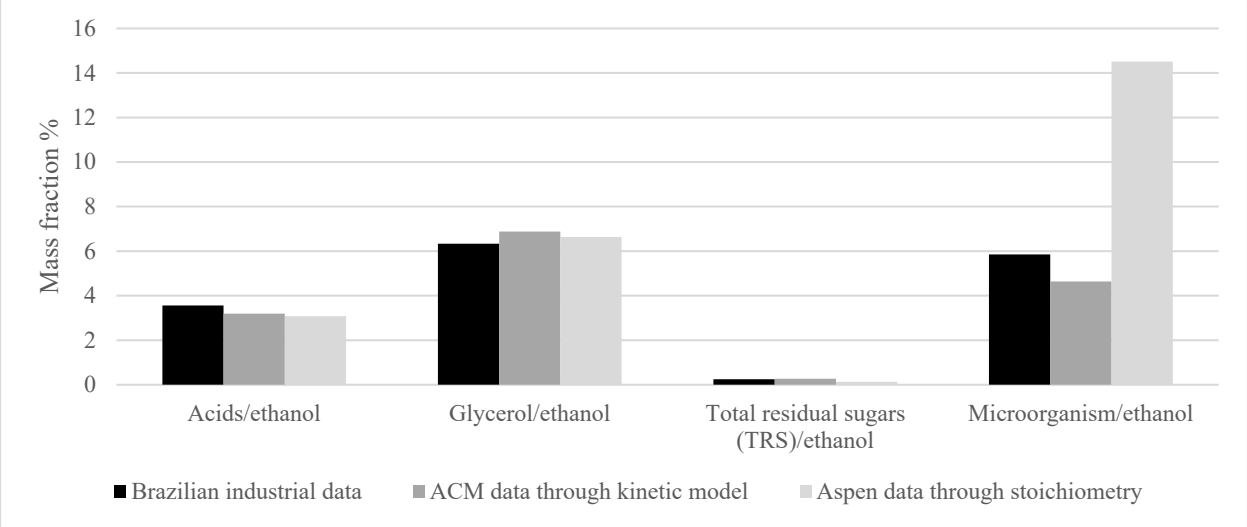

Figure 6.4.Comparison of the kinetic and stoichiometry model of fermentation data on glycerol, acids, residual sugars and microorganisms over ethanol production with industrial data for bioethanol production from glucose (Bonomi et al., 2011)

6.4. Conclusion

Stoichiometry models can be implemented with less information and effort. However, stoichiometry models do not represent continuous experimental data sufficiently because of uncertainty in the estimation of reaction conversions and the absence of dilution rates for CSTRs. In this study, it was shown that kinetic models developed in ACM and embedded in Aspen Plus represented experimental and industrial data accurately. Since it is not possible to develop complex kinetic models of microbial systems in Aspen Plus,

these models were developed using an equation- oriented approach. The custom kinetic model which was developed in ACM, served as a case study to test the current status of software interfaces and custom model performance in Aspen Plus Although the interfacing between ACM and Aspen Plus was challenging, implementations of proper estimations, additional scripts and assumptions were required to maximise proper integration between the two platforms. The estimations for products and substrate utilization were as accurate as if using other kinetic model platforms (such as Scilab or MATLAB), however modelling of biomass formation still requires further optimization. The complexity of estimating biomass formation remains a key issue in modelling in general. Consideration of including additional parameters such as by-product inhibition and limitation, and maximum biomass concentrations in the kinetic model could be considered. However, predicting the behaviour of biological factors such as cell-to-cell interaction, cell sizes and the cell's intracellular metabolic state are less accessible in kinetic models as these remain relatively unknown in mechanism for growth regulation.

6.5. Nomenclature:

C_x: Cell concentration in medium (mass/unit volume)

C_{xo}: Initial biomass concentration (mass/unit volume)

C_{glu}: Glucose concentration (mass/unit volume)

C_{glu0}: Initial glucose concentration (mass/unit volume)

D: Dilution rate (1/unit time)

F: Feed molar flow (mole/unit time)

H_{in}: Feed enthalpy (energy unit/mole/unit time)

H_L: Liquid enthalpy (energy unit/mole/unit time)

H_v: Vapour enthalpy (energy unit/mole/unit time)

$K_{s,glu}$: Glucose limitation constant for substrate uptake rate (mass/unit volume)

$K_{x,glu:}$ Glucose limitation constant for biomass production rate (mass/unit volume)

$K_{is,glu:}$ Glucose inhibition constant for substrate uptake rate (mass/unit volume)

$K_{ix,glu:}$ Glucose inhibition constant for biomass production rate (mass/unit volume)

$L_{mole\text{-}fraction}$: Liquid mole fraction (dimensionless)

$L_{molar\text{-}flow}$: liquid molar flow (mole/unit time)

MW_e: Molecular weight of ethanol (mass/mole)

MW_{glu}: Molecular weight of glucose (mass/mole)

P: Ethanol concentration (mass/unit volume)

P_0: Initial ethanol concentration (mass/unit volume)

$P_{m,\,x,\,glu}$: Maximum ethanol concentration in glucose above which cells do not grow (mass/unit volume)

$P_{m,\,s,\,glu}$: Maximum ethanol concentration in glucose above which substrate do not uptake (mass/unit volume)

$P_{ix,\,glu}$: Minimum ethanol concentration above which cells production is affected negatively when grown in glucose (mass/unit volume)

$P_{is,\,glu}$: Minimum ethanol concentration above which glucose consumption is affected negatively (mass/unit volume)

P_{tot}: Total vapour pressure (unit pressure)

P_{vap}: Vapour pressure of the pure component (unit pressure)

$q_{m,glu:}$ Maximum specific glucose utilization rate (1/unit time)

$q_{glu:}$ Specific glucose utilization rate (1/unit time)

Q: Heat (unit energy/unit time)

r_{glu}: Glucose consumption rate (mass/unit volume/unit time)

r_x: Biomass production rate (mass/unit volume/unit time)

r_e: Ethanol production rate (mass/unit volume/unit time)

r_{rate}: Reaction rate (mass /unit volume/unit time)

R_{vol}: Reactor volume (unit volume)

V: Vapour molar flow (mole/unit time)

y: Vapor mole fraction (dimensionless)

$Y_{SP,glu}$: Product yield constant from glucose (g-product/g-glucose)

Z: Inlet mole fraction (dimensionless)

$\mu_{m,glu}$: Maximum specific growth rate in glucose (1/unit time)

μ_{glu}: Specific growth rate in glucose (1/unit time)

ν_{glu}: Specific rate of product formation in glucose (1/unit time)

βs: Product inhibition constant in glucose for uptake substrate (dimensionless)

βx: Product inhibition constant in glucose for growth of biomass (dimensionless)

γ: Activity coefficient (dimensionless)

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7. Co-fermentation for bioethanol production from mixed glucose and xylose using *Zymomonas mobilis* and *Pichia stipitis*

In this research, process conditions were manipulated to increase ethanol yield and productivity. This included using immobilization techniques, using high inoculum sizes, using two reactors in series in a continuous fashion (first reactor for glucose conversion and the second reactor for xylose conversion), using low agitation rates of 50 rpm and finally carrying out cultivation in a fluidized bed with nitrogen sparging. This chapter addresses objective (5) of the Ph.D. project.

7.1. Introduction

In order to increase the ethanol yield and productivity for bioethanol fermentation from glucose and xylose, optimization of process conditions presented in the literature or explored in earlier aspects of this thesis and additional manipulation of process conditions were considered. Additional approaches included cell immobilization, high cell inoculum sizes and use of a fluidized bed reactor, as stated in Table 7.1 a, Table 7.1 b, Table 7.1 c and Table 7. 1 d. Xylose and glucose are the two most abundant sugars in bagasse and can be converted to ethanol in fermentation using suitable microorganisms. A major problem associated with the efficient fermentation of glucose and xylose to bioethanol is the lack of industrially suitable microorganisms. Some species like *Z. mobilis* ferment glucose to ethanol efficiently but are unable to ferment the pentose sugars (Dien et al., 2003). Other species like *P. stipitis* can convert both glucose and xylose, but have low ethanol tolerance (Hamidimotlagh et al., 2007; Laplace et al., 1991a, 1991b). For co-fermentation of glucose and xylose, co-culturing two microorganisms such as *Z. mobilis* and *P. stipitis* for efficient fermentation of the glucose and xylose respectively is an option as *Z. mobilis* and *P. stipitis* showed good synergy (Fu et al., 2009). However, in co-culturing, xylose conversion only occurs when the glucose concentration is less than 2.3 g L^{-1} due to catabolite repression. Furthermore, ethanol tolerance of the microorganisms differs, with some lower than others (e.g. *P. stipitis*), resulting in inhibition of microbial growth. Moreover, oxygen requirements differ across microorganisms creating sub-optimum growth conditions when co-cultured. For instance, *Z. mobilis* prefers anaerobic conditions while xylose conversion to ethanol by *P. stipitis* is optimal under microaerobic conditions. Hence, the separation of *Z. mobilis* cells from *P. stipitis* cells is a prerequisite for successful co-utilization of sugars by these two strains (Fu et al., 2009). Two stage sequential batch fermentation in one reactor, growing microorganisms in series rather than simultaneously, has been shown to resolve issues of catabolite repression and differing oxygen

requirements, providing efficient conversion of pentoses and hexoses to ethanol (Grootjen et al., 1991b) by allowing glucose to be utilized first followed by xylose.

Table 7.1 a. Physicochem conditions and immobilization technique for bioethanol fermentation by *Zymomonas mobilis* for glucose conversion and by *Pichia stipitis* for xylose conversion

Process condition	Microorganism	Optimum value/Advantages			References
pH	*P. stipitis*	pH 3.5-4.5: increase specific ethanol production and growth rate.	Growth rate (h^{-1})	0.15	(du Preez et al., 1986; McMillan, 1993)
			Specific ethanol productivity ($g\ g^{-1}\ h^{-1}$)	0.30	
		pH 4.5-5.5: increase ethanol yield and volumetric productivity.	Ethanol yield ($g\ g^{-1}$)	0.43	
			Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	0.86	
		pH 5.0 (high inoculum size)	Ethanol yield ($g\ g^{-1}$)	0.459	Chapter 5 of the Ph.D. thesis
			Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	0.95	
	Z. mobilis	pH 5.5-7.0	Ethanol yield ($g\ g^{-1}$)	0.5	(Panesar et al., 2006)
			Ethanol productivity ($g\ g^{-1}\ h^{-1}$)	5.67	
		pH 6.0	Ethanol yield ($g\ g^{-1}$)	0.495	Chapter 4 of the Ph.D. thesis
			Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	2.18	
Temperature	*P. stipitis*	30 °C	Ethanol yield ($g\ g^{-1}$)	0.43	(du Preez et al., 1986; McMillan, 1993)
			Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	0.86	
		30 °C (high inoculum size)	Ethanol yield ($g\ g^{-1}$)	0.459	Chapter 5 of the Ph.D. thesis
			Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	0.95	
	Z. mobilis	25-30 °C	Ethanol yield ($g\ g^{-1}$)	0.5	(Panesar et al., 2006)
			Ethanol productivity ($g\ g^{-1}\ h^{-1}$)	5.67	
		30 °C	Ethanol yield ($g\ g^{-1}$)	0.495	Chapter 4 of the Ph.D. thesis
			Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	2.18	
Immobilization technique	Entrapment in calcium alginate is one of the most common methods for whole cell immobilization because of ease and non-toxicity.				(Rosevear, 2008)
	Advantages: reduces the risk of contamination in continuous processes, enhances xylose and glucose conversion by reducing catabolite repression owing to diffusion limitation restricting glucose concentration inside the beads, increases ethanol yield and productivity because of reducing ethanol inhibition and concentrating the cells into a small volume, relative ease of product separation, reuse of biocatalyst.				(Abbi et al., 1996; Ghorbani et al., 2011)

Table 7.1 b. Aeration conditions of bioethanol fermentation by *Zymomonas mobilis* for glucose conversion and by *Pichia stipitis* for xylose conversion

Process condition	Microorganism	Optimum value/Advantages			References
Aeration level	*P. stipitis*	Microaerobic condition (below 1 mmol/lh): shows higher ethanol productivity and yield for xylose fermentation than anaerobic or aerobic.			(Balat et al., 2008; Fu et al., 2009; Gírio et al., 2010; Skoog et al., 1990)
			Anaerobic	Oxygen limited (microaerobic)	(McMillan, 1993)
		Ethanol yield (g g^{-1})	0.25-0.42	0.40-0.48	
		Ethanol productivity (g L^{-1} h^{-1})	0.10-0.20	0.30-0.90	
		1 vvm	Ethanol yield (g g^{-1})	0.459	Chapter 5 of the Ph.D. thesis
			Ethanol productivity (g L^{-1} h^{-1})	0.95	
	Z. mobilis	Anaerobic conditions: consume glucose more quickly, grew *Z. mobilis* more rapidly and show higher ethanol productivity and yield over aerobic or microaerobic.			Chapter 4 of the Ph.D. thesis, (Balat et al., 2008; Fu et al., 2009; Gírio et al., 2010; Yang et al., 2009)

Table 7.1 c. Reactor configuration of bioethanol fermentation by *Zymomonas mobilis* for glucose conversion and by *Pichia stipitis* for xylose conversion

Process condition	Microorganism	Optimum value/Advantages				References
Reactor configuration	Fluidized-bed bioreactors provide a much lower physical disruption (e.g. attrition) of the immobilized cells than conventional mechanically stirred bioreactors. Fluidized-bed bioreactors can be operated with smaller size particles, without the drawbacks of clogging, high liquid pressure drop, a creation of preferential flow paths, or particle compression due to bed weight.					(Godia and Sola, 1995)
	Fluidized-bed reactor results in higher overall ethanol productivity than in packed-bed and stirred tank reactors.					(Klein and Kressdorf, 1983)
	Engineered *Z mobilis*	Continuous fluidized bed reactor, CP4 (pZB5) (D:0.5)	Continuous fluidized bed reactor, CP4 (pZB5) (D:0.25)	Continuous membrane reactor with cell recycling, ZM4 (pZB5)	Continuous stirred tank reactor, AX101	(Joachimsthal and Rogers, 2000; Krishnan et al., 2000; Lawford and Rousseau, 2002)
	Yield	0.42	0.45	0.5	0.44	
	Productivity	15.3	8.6	5	3.54	

Table 7. 1 d. Substrate and product conditions of bioethanol fermentation by *Zymomonas mobilis* for glucose conversion and by *Pichia stipitis* for xylose conversion

Process condition	Microorganism	Optimum value/Advantages					References
Initial total sugar concentration	*P. stipitis*	Initial sugar concentration (g L^{-1})	35	85	114		(Laplace et al., 1991a)
		Ethanol yield (g g^{-1})	0.44	0.44	0.45		
		Ethanol productivity (g g^{-1} h^{-1})	0.18	0.19	0.14		
	Z. mobilis	Initial sugar concentration (g L^{-1})	20	50	80	110	170
		Ethanol yield (g g^{-1})	0.49	0.48	0.48	0.5	0.3
		Ethanol productivity (g g^{-1} h^{-1})	3.05	3.6	1.35	1.2	1.3
Maximal product concentration when cell growth ceases	*P. stipitis*	56 g L^{-1}					Chapter 4 of the Ph.D. thesis, (Farias et al., 2014)
	Z. mobilis	86 g L^{-1}					Chapter 4 of the Ph.D. thesis, (Lee and Rogers, 1983)
Minimum ethanol concentration above which cells production is affected negatively	*P. stipitis*	35 g L^{-1}					(Hamidimotlagh et al., 2007)
	Z. mobilis	28 g L^{-1}					Chapter 4 of the Ph.D. thesis

However, the time required to inactivate the glucose fermenting bacterial cells prior to the introduction of the xylose fermenting cells prolongs the fermentation time and decreases ethanol productivity. Also, the issue of low ethanol tolerance of xylose-fermenting microorganisms is still prevalent in the two-stage sequential batch fermentation in one bioreactor used to avoid catabolite repression. Hence, two-stage fermentation in two reactors in series along with optimized process conditions (e.g. process configuration, immobilization technique, cell type) has potential to enable improved ethanol yield and productivity. In particular, the preferred operating conditions for each stage can be selected. In Table 7.1 a, Table 7.1 b, Table 7.1 c and Table 7. 1 d, these conditions are explored. As it is justified in Table 7.1 c, fluidized bed

reactors are examples of a process configuration that result in good ethanol yields and productivities when immobilized cells are used.

This study examines the co-culture of *Z. mobilis* and *P. stipitis*in a two-stage, reactors-in-series configuration, employing four novel fermentation schemes: i) initial biomass of 2.1 g L^{-1} of suspended *Z. mobilis* using anaerobic batch stirred tank reactor with 200 rpm for glucose conversion in the first fermentation stage and initial biomass of 2.1 g L^{-1} of suspended *P. stipitis* microaerobic batch culture for xylose conversion in the second reactor, ii) initial biomass of 2.1 g L^{-1} of immobilized *Z. mobilis* using anaerobic batch stirred tank reactor with 200 rpm in the first fermentation stage for glucose conversion, and an immobilized *P. stipitis* using microaerobic batch stirred tank reactor with 200 rpm in a second reactor for xylose conversion, iii) immobilized *Z. mobilis* using 0.05 h^{-1} dilution rate in anaerobic continuous stirred tank reactor with 50 rpm in the first reactor for glucose conversion, and suspended *P. stipitis* using 0.025 1/h dilution rate in microaerobic continuous stirred tank reactor with 200 rpm in a second reactor for xylose conversion, iv) immobilized *Z. mobilis* using 0.07 l/h dilution rate in anaerobic continuous fluidized bed in the first reactor for glucose conversion, and suspended *P. stipitis* using 0.035 1/h dilution rate in microaerobic continuous stirred tank reactor with 200 rpm in a second reactor for xylose conversion.

7.2. Material and Methods

7.2.1. Microorganisms and culture storage

The strains of *Z. mobilis* ZM4 (ATCC 31821) and *P. stipitis* ATCC 58376 were used.

For short-term storage, *Z. mobilis* were stored in solid rich medium (RM) (Goodman et al., 1982) containing per litre: 20 g glucose, 10 g yeast extract, 2 g KH_2PO_4, 15 g agar, 1000 ml DI water, and *P. stipitis* were stored in solid YEPD (Goodman et al., 1982) containing per litre: 20 g glucose, 10 g yeast extract, 20 g peptone, 20 g agar, 1000 ml DI water. Agar plates were stored at 4 °C. For long-term storage, the cultures were stored in 40% (v/v) glycerol in 1.5 mL volumes at -60 °C. For this, equal amounts of bacterial or yeast culture and 80% sterile glycerol solution were mixed.

7.2.2. Synthetic media

Inoculum medium for *Z. mobilis* consisted of 10 g L^{-1} yeast extract, 1 g L^{-1} $MgCl_2$, 1 g L^{-1} $(NH_4)_2SO4$, 1 g L^{-1} KH_2PO_4, with 44 g L^{-1} glucose. Inoculum medium for *P. stipitis* consisted of 10 g L^{-1} yeast extract, 0.5 g L^{-1} $MgCl_2$ (1.065 g L^{-1} $MgCl_2.6H_2O$), 0.5 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} KH_2PO_4, with 24 g L^{-1} xylose (inorganic salts were varied with changing sugar quantity). In all cases, yeast extract and inorganic salts (YEIS) solution were sterilized separately from glucose.

7.2.3. Inoculum preparation and fermentation studies

A two-stage inoculum train was used for *Z. mobilis* and *P. stipitis*. Inocula of *Z. mobilis* were incubated for 24 h at 30 °C in 250-ml conical flasks containing 50 ml medium, in a shaker incubator at 120 rpm. The inoculum addition of suspended *Z. mobilis* was calculated to provide a starting concentration of 2.1 g L^{-1}. Experiments were conducted in a Brunswick reactor at 30 °C with working volume of 2.5 and 5 litres. The pH was controlled at 6 using 1 M NaOH and 1 M HCl. Inocula of *P. stipitis* were incubated for 48 h at 30 °C in 250 ml conical flasks containing 50 ml medium, in a shaker incubator at 120 rpm. The inoculum addition of suspended *P. stipitis* was calculated to provide a starting concentration of 3 g L^{-1}. Experiments were conducted in a reactor at 30 °C. The pH was controlled at 5 using NaOH 1 M and 1 M HCl.

7.2.4. Immobilization

Z. mobilis and *P. stipitis* were immobilized in calcium alginate beads based on the mentioned process in studies of Fu et al.(2009) and Becerra et al. (2001). The process of immobilization is shown in Figure 7. 1. *Z. mobilis* storage medium was 10.0 g L^{-1} glucose, 5.0 g L^{-1} yeast extract and 10 g L^{-1} CaCl2 and for *P. stipitis* storage medium was 10.0 g L^{-1} xylose, 5.0 g L^{-1} yeast extract and 10 g L^{-1} $CaCl_2$. The distance between needle and CaCl2 solution used was 20 cm. Beads were activated with 2 g L^{-1} CaSO4 and 10 g L^{-1} glucose for 10 h. 2 g L^{-1} $CaSO_4.2H_2O$ were added to medium to prevent bead damage in continuous process. A muslin cloth was used on the outlet port to retain and prevent of passing of immobilized glucose-fermenting microorganism into the second stage fermentation.

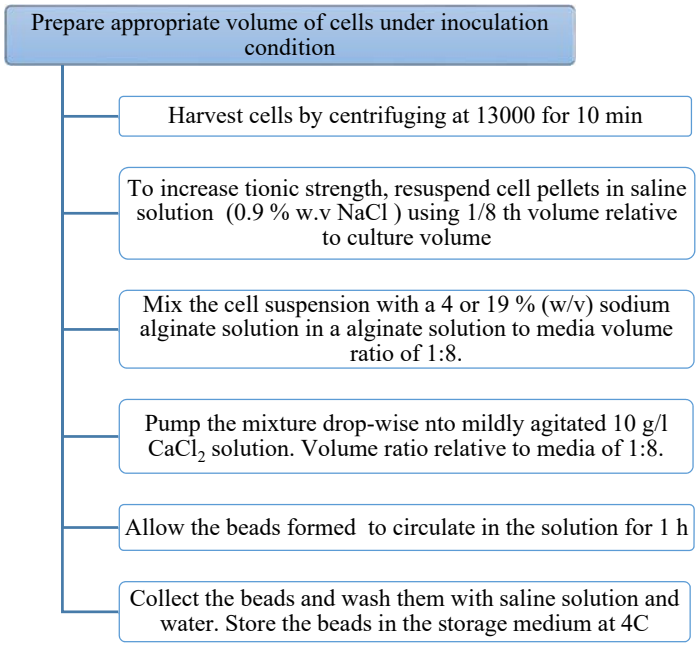


Figure 7. 1. The process of immobilization in this study (adapted from Becerra et al., 2001; Fu et al., 2009)

7.2.5. Reactor configuration

Different reactor configuration processes were tested in this study:

A. Sequential suspended high inoculum sizes of *Z. mobilis* and *P. stipitis* in one batch stirred tank reactor,
B. Sequential immobilized *Z. mobilis* and *P. stipitis* in one batch stirred tank reactor,
C. Immobilized *Z. mobilis* in the first continuous stirred tank reactor and high inoculum size of suspended *P. stipitis* in the second microaerobic continuous stirred tank reactor,
D. Immobilized *Z. mobilis* in the first continuous fluidized bed and high inoculum size of suspended *P. stipitis* in the second microaerobic continuous stirred tank reactor.

7.2.6. Analytical procedures

Dry cell mass was determined by centrifuging a 2 ml sample for 10 minutes at 13000 rpm (Heraeus Biofuge Pico); washed with water and centrifuged again. The cell pellet was dried at 80 °C for 24 hours and stored in a desiccator before weighing. The optical density was determined at 660 nm for *Z. mobilis* and 600 nm for *P. stipitis* by spectrophotometer (Gensys 10S UV-VIS) and kept at around 0.3 OD by dilution. Samples from the fermentation broth were analysed for glucose and ethanol concentrations by HPLC using an Aminex column HPX-87H (300*7.8mm) (Bio- Rad, Ion exclusion column) equipped with a refractive index detector. Separations were performed at 65 °C, eluted at 0.3 ml/min using 5 mM sulphuric acid. Standards containing analytical grade components were used periodically to confirm calibration accuracy. The samples and standards were filtered with a syringe filter (0.22 μm). The mobile phase was filtered (with a 0.45 μm membrane filter) and degassed in a sonic bath.

7.3. Results and Discussion

All approaches tested in this study for bioethanol fermentation from glucose and xylose provided a separate glucose and xylose conversion, thereby addressing the problem of differing oxygen requirements of *Z. mobilis* and *P. stipitis*, and of glucose catabolite repression inhibiting xylose-fermenting microorganisms. Due to the time required to inactivate the bacterial cells and prolonged fermentation time due to separate batch fermentation stages, the sequential batch culture in one reactor was considered to be less desirable than the sequential continuous culture of the two microorganisms in two reactors in series, for example, immobilized *Z. mobilis* in continuous-fluidized-bed-reactor/CSTR for glucose conversion in first reactor and suspended *P. stipitis* in CSTR for xylose conversion in second reactor.

7.3.1. Sequential batch culture of the high inoculum sizes of *Z. mobilis* and *P. stipitis* in one batch stirred tank reactor

The use of high inoculum concentration is known to reduce the lag period and batch fermentation time, raising productivity as stated in Table 7. 2. However, the relationship between product yield and inoculum

size has not been clearly reported (Erten et al., 2006; Wanderley et al., 2014), although a general increase in yields are expected with increasing inoculum sizes from mass balance. In addition, the efficient fermentation of xylose by Fu et al. (2009) (0.5 g g^{-1} and 1.126 g L^{-1} h^{-1}) and Moniruzzaman et al. (1997) (0.44 g g^{-1} and 3.44 g L^{-1} h^{-1}) is directly attributed to the large inoculum size (50% v_{cells}/v_{ferm}) and type of microorganisms selected.

Table 7. 2. Ethanol yield (Ysp) and ethanol productivity (Qp) on glucose across three inoculum sizes of Saccharomyces cerevisiae UFPEDA 1238 (Wanderley et al., 2014)

Inoculum (g L^{-1})	Y_{sp} (g g^{-1})	Q_p (g L^{-1} h^{-1})
0.4	0.28	1.04
4.0	0.35	1.84
8.0	0.43	3.10

The fermentation profile of the two stage sequential batch with suspended culture using high inoculum *Z. mobilis* ATCC 31821 (initial biomass of 2.1 g L^{-1}) for glucose conversion and high inoculum *P. stipitis* ATCC 58376 (initial biomass of 3 g L^{-1}) for xylose conversion are illustrated in Figure 7. 2 and Figure 7. 3.

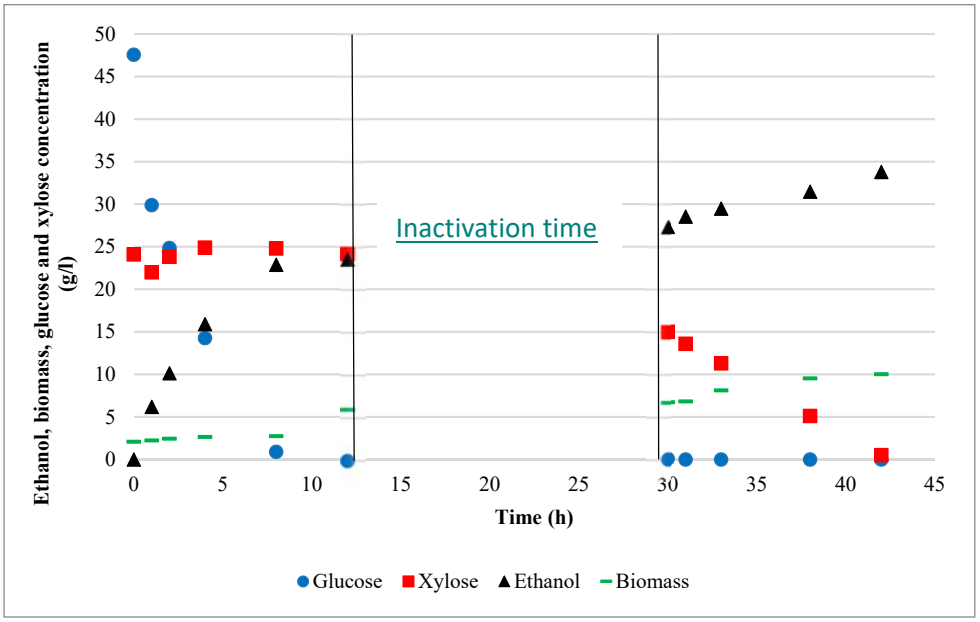


Figure 7. 2. Fermentation profile of the sequential batch suspended cultures (xylose, glucose, ethanol and biomass concentration versus time). Circle (●) is for glucose concentration, square (■) is for xylose concentration, triangle (▲) is for ethanol concentration, line (—) is for biomass concentration.

The obtained ethanol yield was 0.47 g g^{-1} and productivity was 1.35 g L^{-1} h^{-1} from 100% glucose conversion and 98% xylose conversion in 25 h of fermentation time (12 h for glucose conversion and 13 h for xylose conversion) that excludes the time required for inactivating the glucose-fermenting microorganism. Ethanol

productivity dropped to 0.8 g L^{-1} h^{-1} when the 17 h of inactivation time was taken into account (5 h autoclaving and 12 h for DO probe polarization).

The fermentation profile of the two stage sequential batch culture in one reactor using immobilized *Z. mobilis* ATCC for glucose conversion and immobilized *P. stipitis* ATCC 58376 for xylose conversion are illustrated in Figure 7. 3 and Table 7. 3.

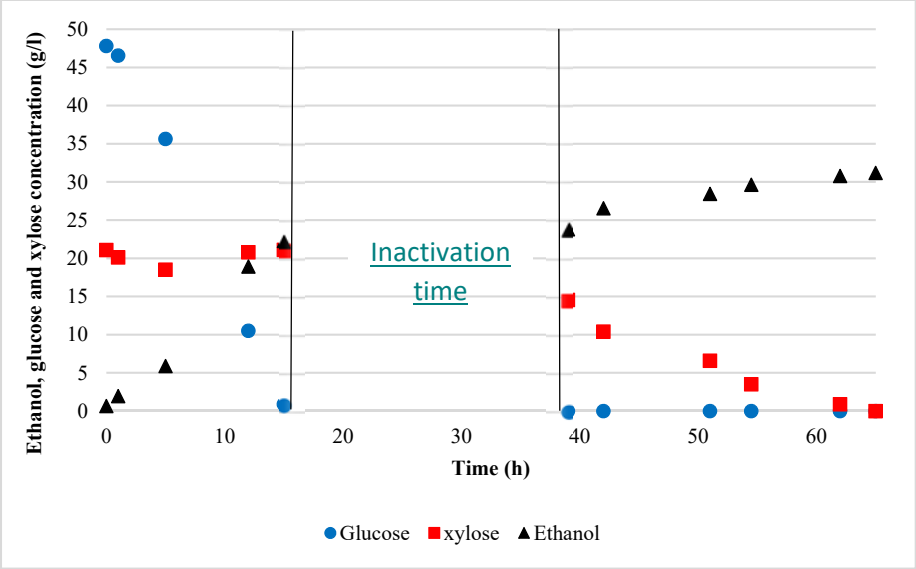


Figure 7. 3. Fermentation profile of the sequential batch immobilized cultures (xylose, glucose and ethanol concentration versus time). Circle (●) is for glucose concentration, square (■) is for xylose concentration, triangle (▲) is for ethanol concentration.

The obtained ethanol yield was 0.47 g g^{-1} and productivity was 0.67 g L^{-1} h^{-1} in 48 h of fermentation time (15 h for glucose conversion and 33 h for xylose conversion), but without considering the inactivation time required for the glucose-fermenting microorganism inactivation. Ethanol productivity dropped from 0.48 g L^{-1} h^{-1} to 0.45 g L^{-1} h^{-1} when the 17 h of inactivation time was considered (5 h for autoclaving and cooling, 12 h for DO probe polarization). The ethanol productivity of both glucose (from 1.96 to 1.48 g L^{-1} h^{-1}) and xylose (from 0.79 to 0.25 g L^{-1} h^{-1}) considerably decreased when immobilized cultures were used over suspended cultures. The ethanol yield for xylose conversion using *P. stipitis* decreased from 0.43 g g^{-1} using suspended culture to 0.38 g g^{-1} using immobilized culture. The decrease in yields and productivities with immobilized cells may be due to mass transfer limitations of sugars from media across the beads or vice versa. Also, another mass transfer issue on oxygen transfer for xylose conversion using *P. stipitis* could occur inside the beads, leading to sub-optimum micro-aeration conditions. The risk of a diauxic shift from growth on glucose to growth on ethanol is minimized in a sequential process in which the cells are removed immediately after glucose exhaustion, avoiding ethanol consumption (Guan et al., 2013). Fu and Peiris

(2007) achieved a reasonable yield (0.33 g g^{-1}) using suspended *Z. mobilis* and *P. tannophilus* in a sequential manner.

Table 7. 3. Comparison of different fermentation parameters for bioethanol production at different conditions in batch process using pure sugars

	Glucose used (g L^{-1}) /conversion rate	Xylose used (g L^{-1}) /conversion rate	Initial /final biomass concentration (g L^{-1})	Ethanol concentration (g L^{-1})	Fermentation time (h)	Ethanol yield (g g^{-1})	Ethanol productivity (g L^{-1} h^{-1})
Sequential batch, one reactor, high inoculum, suspended culture							
1st stage	47.5/100%		2.10/2.75	23.56	12	0.496	1.96
2nd stage		23.7/98%	3.25/7.28	10.22	13	0.43	0.79
Overall	47.5	23.7/98%	6.00/10.03	33.78	25	0.47	1.35
Considering inactivation time	47.5	23.7/98%	6.00/10.03	33.78	42	0.47	0.80
Sequential batch, one reactor, both cultures immobilised							
1st stage	46.9/98%		2.08	22.28	15	0.475	1.48
2nd stage	0.9/100%	21.1/100%	3.17	8.30	33	0.38	0.25
Overall	47.8	21.1		31.20	48	0.47	0.67
Considering inactivation time	47.8	21.1		31.20	65	0.45	0.48

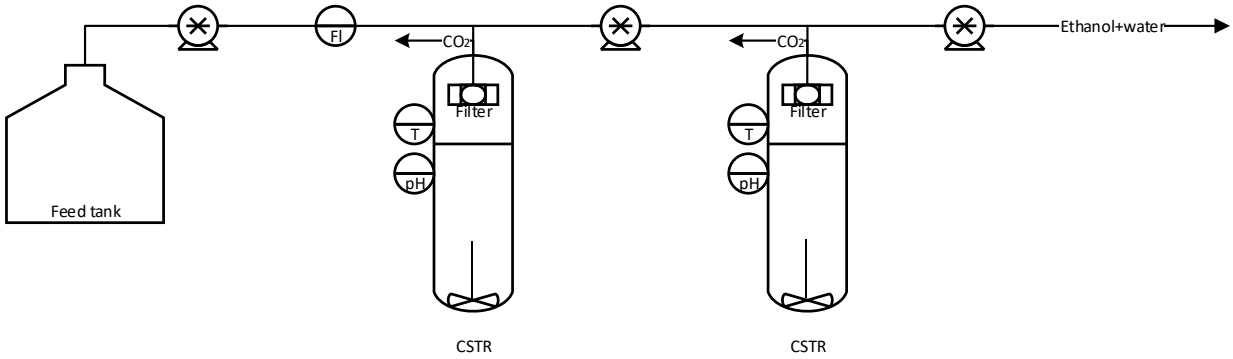
The initial fermentation was carried out with *Z. mobilis* with no aeration, followed by the inactivation of the bacterium after depletion of glucose. The second fermentation with *P. tannophilus* was carried out under limited aeration. Similarly, Fu et al. (2009) performed fermentation with sequential cultivation of *Z. mobilis* and *P. stipitis* obtaining yield of 0.47 g g^{-1} and a productivity of 0.83 g L^{-1} h^{-1}, with complete glucose and xylose fermentation in 2.5 hours and 26 hours respectively (inactivation time of glucose-fermenting microorganism is not considered). The separation of *Z. mobilis* cells from *P. stipitis* cells is a prerequisite for successful co-utilization of sugars by these two strains (Fu et al., 2009). In this study, using a high inoculum concentration of *Z. mobilis* and *P. stipitis* subsequently maintained the ethanol yield to 0.47 g g^{-1} and improved ethanol productivity to 0.8 g L^{-1} h^{-1} (inactivation time of glucose-fermenting microorganism is not considered). Immobilization of these cells however did not show any improvement in ethanol yield (0.45 g g^{-1}) and productivity (0.48 g L^{-1} h^{-1}). The encapsulation of these cells provided the cells protection from shear stress but the barrier introduced may have limited mass transfer for the diffusion of substrate and products. In this setup, the productivities were found to be lower than most studies.

7.3.2. Two stage fermentation in two bioreactors in a continuous process

The effect of immobilised cultures in sequential batch process in one reactor tested and from shown data, mass transfer limitation appeared to have restricted *P. stipitis* performance. Hence, the fermentation

parameters of the two-reactor system using immobilized *Z. mobilis* for glucose conversion in the first reactor and free *P. stipitis* for xylose conversion in the second reactor are presented in Table 7. 4. Two continuous reactors in series were tested under two different conditions:

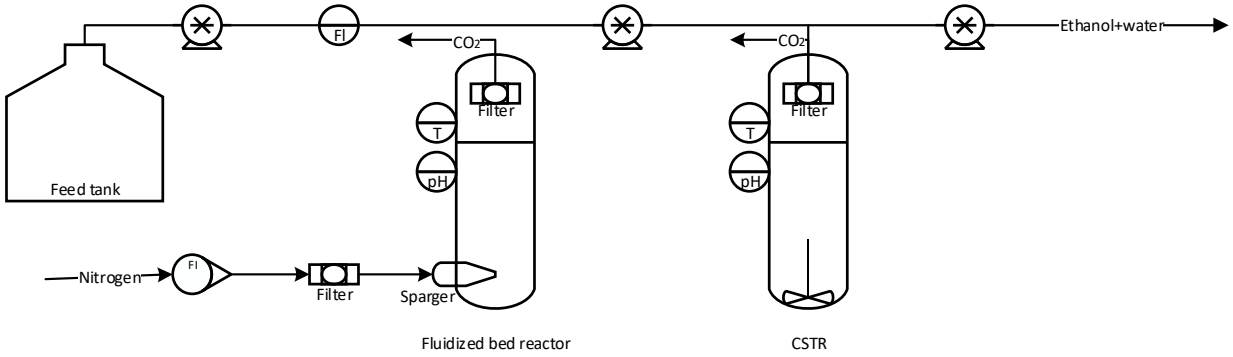
i) Immobilized *Z. mobilis* was inoculated into the first continuous stirred tank reactor which was operated anaerobically at 50 rpm and a dilution rate of 0.05 1/h, working volume of 5 litres. A high inoculum size of suspended *P. stipitis* (initial biomass of 3 g L^{-1}) was inoculated into the second microaerobic continuous stirred tank reactor operated at 200 rpm and a dilution rate of 0.025 h^{-1}, working volume of 2.5 litres (Figure 7. 4).



T: Temperature, Fl: Flowmeter

Figure 7. 4. Schematic diagram of two CSTR in series

ii) Immobilized *Z. mobilis* was inoculated in anaerobic continuous fluidized bed, dilution rate of 0.07 1/h, working volume of 5 litres. A high inoculum size of suspended *P. stipitis* (initial biomass of 3 g L^{-1}) was inoculated into the second microaerobic continuous stirred tank reactor agitated at 200 rpm and dilution rate at 0.035 1/h, working volume of 2.5 litre (Figure 7. 5).



T: Temperature, Fl: Flowmeter

Figure 7. 5. Schematic diagram of CSTR and fluidized bed reactor in series

Among the different methods tested in this study, the two stage continuous fermentation in two reactors in series using immobilized *Z. mobilis* in fluidized bed reactor for glucose conversion and suspended *P. stipitis*

in CSTR for xylose conversion proceeded efficiently, giving a high ethanol yield and productivity (0.47 g g^{-1}, 0.97 g L^{-1} h^{-1}). In contrast, two stage fermentation in two reactors in series using immobilized *Z. mobilis* in CSTR for glucose conversion and suspended *P. stipitis* in CSTR for xylose conversion resulted in a poor fermentation performance (0.43 g g^{-1}, 0.67 g L^{-1} h^{-1}). Glucose concentrations measured in the first reactor at steady state with immobilized *Z. mobilis* using CSTR at a dilution rate of 0.05 h^{-1}, showed ineffective glucose conversion because of mass transfer limitation in the first reactor and resulted in incomplete conversion of glucose when CSTR was used. This glucose was carried into the second reactor and consumed by *P. stipitis* prior to xylose fermentation. Fluidized-bed bioreactors provided better mass transfer leading to complete glucose and xylose conversion, as well as impoved ethanol yields and ethanol productivities even with a higher dilution rate.

Table 7. 4. Comparison of different fermentation parameters for bioethanol production at different conditions during continuous culture using pure sugars

	Glucose used (g L^{-1}) /conversion rate	Xylose used (g L^{-1}) /conversion rate	Initial /final biomass concentration (g L^{-1})	Ethanol concentration (g L^{-1})	Dilution rate (h^{-1})	Ethanol yield (g g^{-1})	Ethanol productivity (g L^{-1} h^{-1})
Immobilized *Z. mobilis* in CSTR (1st reactor) and suspended *P. stipitis* in CSTR (2nd reactor)							
1st reactor	30.63/77%		1.98	12.30	0.050	0.40	0.61
2nd reactor	10.84/100%	21.97/100%	3.11/6.90	14.55	0.025	0.44	0.36
Overall	41.47	21.97		26.85		0.43	0.67
Immobilized *Z. mobilis* in continuous fluidized bed reactor (1st reactor) and suspended *P. stipitis* in CSTR (2nd reactor)							
1st reactor	37.82/100%		2.05	17.77	0.070	0.47	1.24
2nd reactor	0	20.39/100%	3.07/7.01	9.37	0.035	0.46	0.33
Overall	37.82	20.39		21.14		0.47	0.95

Hence, in the fluidized bed reactor setup, all glucose was converted at the selected dilution rate (0.07 1/h) and allowed immediate xylose utilization by *P. stipitis* in the second reactor (0.035 1/h). Compared to the two- reactor system used by Grootjen et al. (Grootjen et al., 1991a) with *S. cerevisiae* and *P. stipitis*, and the two-reactor system used by Chaudhary et al. (Chaudhary and Ghosh, 2014) with suspended and low innoculum sizes of *Z. mobilis* and *P. stipitis*, the two-reactor system in this present study with immobilized *Z. mobilis* and high inoculum size of *P. stipitis* was more efficient and economical. The overall ethanol productivity of this system (using immobilized *Z. mobilis* in fluidized bed reactor for first reactor and high inoculum of suspended *P. stipitis* in CSTR for the second reactor) was found to be 1.14 g L^{-1} h^{-1}, which is higher compared to that achieved by Grootjen et al. (Grootjen et al., 1991a) i.e. 0.43 g L^{-1} h^{-1} in the case of

the two-reactor system using *S. cerevisiae* and *P. stipitis*, and 0.51 g L^{-1} h^{-1} in the three-reactor system with *P. stipitis* alone. Also, the overall ethanol yield of this system was found to be 0.48 g g^{-1}, which is higher as compared to that achieved by Chaudhary et al. (Chaudhary and Ghosh, 2014) i.e. 0.46 g g^{-1} in the case of the two-reactor system using suspended *Z. mobilis* and suspended *P. stipitis*. Productivity was higher in continuous processes than batch processes as the additional time required for inactivating the glucose-fermenting microorganism before adding xylose-fermenting microorganism were long (17 hours) and contributed significantly in the overall batch time (Fu et al., 2009).

7.4. Conclusion

To overcome the lack of suitable microorganisms for co-fermentation of bioethanol production from the mixture of glucose and xylose, two microorganisms in co-culture have been suggested. However, issues such as catabolite repression and differences of aeration requirement of the microorganisms used in co-culturing results in poor performance when using two microorganisms in the same reactor at the same time (Grootjen et al., 1991b; Hamidimotlagh et al., 2007; Kordowska-Wiater and Targoński, 2002; Laplace et al., 1993; Lebeau et al., 1997). To circumvent this, co-fermentation can be achieved by two stage fermentation, either sequentially in a single reactor or in two reactor(s) in series. Two stage batch fermentation in one reactor (sequential batch culture) gave low ethanol productivities because of the additional time required for inactivating the glucose-fermenting microorganism before the addition of the xylose-fermenting microorganism (Fu et al., 2009). Also, evaporation of ethanol occurred during the heat inactivation of the first microorganism. It was noted that using a high inoculum size culture did improve the ethanol yields and productivities in a batch system (Wanderley et al., 2014), as, from a flux point of view, more available carbon would be dedicated in making products rather than biomass. Two stage batch fermentation in one reactor using two immobilised cultures were also tested, however mass transfer limitation on *P. stipitis* occured.

The combination of *Z. mobilis* and *Pichia stipitis* showed good performance. Thus, a sequential immobilized culture of *Z. mobilis* and suspended *P. stipitis* using two continuous reactors in series with *Z. mobilis* in the first (glucose consumption); and *P. stipitis* in the second (xylose fermentation) reactor was suggested. Immobilized *Z. mobilis* in a continuous fluidized bed reactor (0.47 g g^{-1}, 1.249 g L^{-1} h^{-1}) outperformed immobilized *Z. mobilis* in CSTR in the first reactor (0.40 g g^{-1}, 0.61 g L^{-1} h^{-1}), with ethanol yield and productivity. Fluidized-bed bioreactors provide a much lower physical disruption (e.g. attrition) of the immobilized cells than conventional mechanically stirred bioreactors (Godia and Sola, 1995). Fluidized-bed bioreactors can be operated with smaller size particles, without the drawbacks of clogging, high liquid pressure drop, creation of preferential flow paths, or particle compression due to bed weight (Godia and Sola, 1995). It was found that the combination of running the fermentation in a continuous

fashion, with immobilized *Z. mobilis* in a fluidized bed resulted in the highest ethanol yield and productivity for the co-fermentation of the glucose and xylose feed mixture. Fluidizing the immobilized cells allowed for sufficient mass transfer and is not burdened by the harsh shear forces from the stirrer of a CSTR. Subsequently, coupling this glucose conversion with a continuous suspended *P. stipitis* culture under microaerobic condition allowed for the efficient conversion of xylose to ethanol (0.95 g L^{-1} h^{-1}, 0.47 g g^{-1}).

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8. Conclusions and Recommendations

This concluding chapter presents an overview of the contribution of each of the preceding chapters to the overall aims of the Ph.D. study. In addition, concluding remarks and recommendations for future studies are presented. This study focused on improving ethanol yields and productivities for bioethanol fermentation from glucose and xylose through integration of modelling and experimental observation. To provide a fundamental basis, the thesis focuses on the efficient fermentation of the mixed sugars and does not consider the other potential inhibitors present in hydrolysates. Following review of fermentation of mixed sugars to ethanol in Chapter 2A, it was found that naturally occurring microorganisms do not ferment both sugars efficiently simultaneously. Options to achieve this included engineering the strains, using co-fermentation or using sequential fermentation. Following an analysis of strengths and weaknesses, the sequential fermentation using two microorganisms was selected as most promising route and studied further.

In Chapter 2B, the suitable bio-kinetic models, considering required kinetic parameters of sugar utilization, oxygen utilization (where applicable), ethanol production, and biomass growth for the microbial cultures of interest were determined, addressing the first objective. The second objective of modelling the batch and continuous fermentation system for fermentation of glucose only, xylose only, and a mixture of glucose and xylose is addressed in Chapters 4, 5 and 7 respectively. Experimental studies to address the third objective are carried out using planktonic cultures for glucose only in Chapter 4, xylose only in Chapter 5, and mixture of glucose and xylose as well as planktonic and immobilised cultures in Chapter 7. Optimal values for key process parameters that result in the closest fit with the experimental observations were determined in Chapters 4 to 6. The fifth and final objective of manipulation of process conditions using immobilization, high inoculum sizes, differing aeration rates and stirrer speed was carried out experimentally and comparison of ethanol yields and productivities under these conditions reported in Chapter 7.

At high substrate and product concentrations, substrate or product or both are inhibitors for substrate consumption, biomass growth and bioethanol production. Hence, along with substrate limitation, substrate and product inhibition were considered when developing a kinetic model, building on the consideration of only one of these in most literature studies to date. To improve the sensitivity and accuracy of the kinetic models for better agreement with experimental results, effective factors of substrate limitation (e.g. sugars and/or oxygen), product inhibition (e.g. ethanol), and substrate inhibition (e.g. sugars and/or oxygen) were considered in this thesis. Rogers model was used for *Z. mobilis* and Andrews and Levenspiel model was

selected for *P. stipitis*, after which, was improved by experimental observation and the consideration of an oxygen term.

After selecting suitable kinetic models, finding the precise kinetic parameters plays an important role in supporting the accuracy and usability of the model. To find the accurate kinetic parameters for glucose conversion using *Z. mobilis*, the kinetic parameter relationship of glucose and xylose utilization between the different constructs of the same culture, for example, a wild type and engineered strain were examined. Here, novelty was found in intra (wild type vs engineered) and inter species (*S. cerevisiae* vs *Z. mobilis*) kinetic parameter dependency for bioethanol production when glucose or xylose or both were used as carbon source, and when the same xylose utilization pathway was engineered in these strains. As an example, the substrate limitation constants in the engineered strain of xylose ($K_{s,xyl}$, $K_{p,xyl}$, $K_{x,xyl}$) and of glucose ($K_{s,glu}$, $K_{p,glu}$, $K_{x,glu}$) were nine and three times higher in the engineered strain compared with that of the wild type ($K_{s,glu}$, $K_{p,glu}$, $K_{x,glu}$) respectively. *Saccharomyces cerevisiae* CEN.PK 113-7D vs RWB 217 using the xylose isomerase gene and *Z. mobilis* ZM4 vs ZM4 (pZB5) using the xylose isomerase gene were selected. Correction factors were found from estimated kinetic parameters from literature experimental data (Kuyper et al., 2005a, 2005b, 2004, 2003) using linear and non-linear regression when a xylose fermentation route was inserted recombinantly (*S. cerevisiae* RWB 217) into the native culture (*S. cerevisiae* CEN.PK 113-7D). This set of correction factors together with the estimated kinetic parameters of engineered strain *Z. mobilis* ZM4 (pZB5) from literature experimental data, were used to estimate the kinetic parameters of the wild-type *Z. mobilis* ZM4. The predicted rates were then validated with experimental data generated in this study and showed good agreement between them. A sensitivity analysis was performed on the retention time by varying each model parameters by ±10% for both *S. cerevisiae* and *Z. mobilis*. The largest effects on retention time resulted from the changes in product inhibition in engineered *Zymomonas mobilis* ZM4 (pZB5), resulting in a ±19% to ±37% higher retention time. However, this effect was not replicated in *S. cerevisiae*, which only resulted in a ±2% to ±4%. The higher resistance of *S. cerevisiae* to ethanol may be a key factor in this finding. Similarly, the maximum ethanol concentration above which the process would be stopped ($P_{m,x,glu}$, $P_{m,s,glu}$, $P_{m,x,xyl}$, $P_{m,s,xyl}$) in engineered *Saccharomyces cerevisiae* RWB 217 showed no significant change, but in engineered *Zymomonas mobilis* ZM4 (pZB5), changes in retention time was around ±7% to ±12%. The conserved correction factors in both *S. cerevisiae* and *Z. mobilis* when genetically modified using the same enzyme of xylose isomerase (XI) in converting D-xylose to D-xylulose showed a strong regulation in the central carbon pathway. This conservation suggests that organisms react to this mutation in a similar manner to ensure optimal growth by regulating carbon fluxes for the production of biomass and bioethanol.

To build on the modelling aspect of this study, the oxygen term in the kinetic models for bioethanol fermentation from xylose using *P. stipitis* was considered, as optimum performance was found under

microaerobic conditions yet literature models have not included this term. The addition of the oxygen term improved agreement between modelling and experimental results. With this knowledge, this PhD project used an integrated modelling and experimental approach to investigate the optimal oxygenation level for xylose conversion using *P. stipitis*. To obtain accurate kinetic parameters through non-linear regression model, experiments were done with varying aeration levels and different initial biomass concentrations. This approach was useful for process prediction and control, as well as for simulation and optimization of the fermentative process. Different aeration levels of 0, 0.1 and 0.2 vvm and different initial biomass concentration of 0.3, 1.5 and 3 g L^{-1} were tested to find the optimum condition (0.1 vvm and 3 g L^{-1}). The optimum ethanol yield and productivity were found to be 0.459 g g^{-1} and 0.95 g L^{-1} h^{-1} respectively. The results showed that increase in both initial biomass concentration and operation under micro-oxygenation levels benefitted the ethanol production and yield by *P. stipitis* on xylose. It also concluded that the addition of the oxygen effectiveness factors in the model allowed for optimization of aeration in the fermentation system.

Stoichiometric models can be implemented with less information and effort. However, stoichiometric models do not represent experimental data sufficiently because of uncertainty in the estimation of reaction conversion and absence of dilution rates in the CSTR model. Kinetic models therefore would represent processes more accurately. Since it is not possible to develop complex kinetic models for microbial systems in Aspen Plus alone, these models were developed using an equation-oriented approach. The custom kinetic model for bioethanol fermentation was developed in Aspen Custom Modeller (ACM), which serves as a case study to test the current status of software interfaces and custom model performance in Aspen Plus. This custom model was exported and interfaced with the Aspen Plus flowsheet simulator and was designed to function just like any library models available in Aspen Plus. The novel custom kinetic model developed in Aspen Custom Modeller, within an Aspen Plus instead of a standard stoichiometry model in Aspen Plus resulted in a functional kinetic model that predicted experimental and industrial data well.

The use of lignocellulosic substrates as feedstocks for biocommodities, particularly the liquid biofuel ethanol, provides an opportunity for ample carbon source, but presents the challenge of hydrolysates containing both glucose and xylose. To date, no suitable wild type organisms concomitantly ferment both sugars of glucose and xylose efficiently to ethanol, owing to catabolite repression by glucose and product toxicity in co-culturing which causes suboptimal performance when growing two microorganisms together (Grootjen et al., 1991b; Hamidimotlagh et al., 2007; Kordowska-Wiater and Targoński, 2002; Laplace et al., 1993; Lebeau et al., 1997). To solve this issue, co-fermentation was achieved by two stage fermentation either in one or two reactors in series. Fu and Peiris (2007) achieved a reasonable ethanol yield (0.33 g g^{-1}) using sequential *Z. mobilis* and *P. tannophilus* in a batch process using one reactor. Similarly, Fu et al. (2009) performed fermentations with different xylose-fermenting microorganisms in sequential cultivation

of *Z. mobilis* and *P. stipitis* obtaining a yield of 0.47 g g^{-1}, productivity of 0.83 g L^{-1} h^{-1}, and with complete glucose and xylose fermentation in 2.5 hours and 26 hours respectively. In both these studies by Fu et al., the inactivation time of the bacterium after the utilization of glucose was, however, not considered, therefore only presenting the effective fermentation productivities and not for the total batch fermentation. Furthermore, the de-activation of the first microorganism is logistically complicated at scale. Therefore, the separation of the two microorganisms, specifically *Z. mobilis* cells from *P. stipitis* cells is a prerequisite for a more successful and efficient co-utilization of sugars (Fu et al., 2009). In this thesis, using sequential batch culture of the high inoculum sizes of *Z. mobilis* and *P. stipitis* in one batch stirred tank reactor improved ethanol productivity to 1.35 g L^{-1} h^{-1} and 0.47 g g^{-1} for ethanol yield over available data in previous studies (inactivation time of glucose-fermenting microorganism is not considered). However, no improvement in ethanol yields and productivities were found on using immobilized cells in place of planktonic cells in stirred tank reactors because of mass transfer limitation. In this thesis no improvement in ethanol yields and productivities were found on using immobilized cells in place of planktonic cells in sequential reactors. The lower ethanol yields and productivity may be a result of mass transfer limitations for the substrate and product across the immobilized material within the setup of this experiment.

On investigations into improving ethanol yields and productivities, process engineering optimization (oxygen availability, inoculum sizes, immobilization), medium design, and microbial species selection are some significant factors that are easily manipulated and implemented. The presence of high inoculum sizes is known to reduce batch lag time in a batch and substantially increase productivity (Erten et al., 2006; Wanderley et al., 2014). Increasing the inoculum size of *P. stipitis* in this study significantly improved the fermentation efficiency for xylose conversion in the batch process. Obtained ethanol yield and productivity in this study (inoculum size of 50% v_{cell}/v_{ferm}) were 0.46 g g^{-1} and 0.95 g L^{-1} h^{-1} respectively which were better than other studies using 10% v_{cell}/v_{ferm} inoculum size (du Preez et al., 1986; Farias et al., 2014; Ferrari et al., 1992; Lee et al., 2000; Silva et al., 2011; Skoog et al., 1990). De Bari et al. (2013) indicated that immobilization increased the relative consumption rate of xylose-to-glucose from 2 to 6 times depending on the fermentation medium composition (De Bari et al., 2013). The combined optimization of yield and productivity was enhanced by achieving high biomass concentration through immobilization for biomass retention and by operating in a continuous process with a controlled dilution rate. Immobilization also allowed for the protection of the biomass from environmental stresses.

Combinations of *Z. mobilis* and *Pichia stipitis* showed good performance. Thus, this thesis presents a novel approach of a continuous process with immobilized *Z. mobilis* in fluidized bed and high inoculum of *P. stipitis* in CSTR using two reactors in series with *Z. mobilis* in the first (glucose consumption); and *P. stipitis* in the second (xylose fermentation) reactor. The overall ethanol productivity of this system was found to be 0.95 g L^{-1} h^{-1}, higher than achieved by Grootjen et al. (Grootjen et al., 1991a) i.e. 0.43 g L^{-1} h^{-1}

in the case of the two-reactor system using *S. cerevisiae* and *P. stipitis*, and 0.51 g L^{-1} h^{-1} in the three-reactor system with *P. stipitis* alone. The overall ethanol yield of this system was found to be 0.47 g g^{-1}, higher than achieved by Chaudhary et al. (Chaudhary and Ghosh, 2014) i.e. 0.46 g g^{-1} in the case of the two-reactor system using suspended *Z. mobilis* and suspended *P. stipitis*.

There was possibility to increase the ethanol yield and productivity in this study. Kinetic models had a positive impact on the optimization process, allowing for reduced amount of experiments to achieve the desired outcome. This thesis contributed to the kinetic modelling of bioethanol production from xylose by incorporating the oxygen term within the parameter sets. Batch fermentations were explored in this study, using sequential operations. It was found that although ethanol yields were kept relatively high, the time required for heat inactivation of the glucose-consuming organism reduced the productivities. Therefore, in continuous fermentations where simultaneous glucose and xylose conversions occur, allowed for both high yields and productivities of ethanol production. In implementing a continuous culture, strategies for cell retention were explored. Immobilization technique was used in these processes, and in combination of a fluidized bed, showed good cell retention and bioethanol production. This PhD study concludes that continuous fermentations would outcompete batch and fed-batch fermentations for higher bioethanol yields and productivities as under these conditions, the main bottlenecks of co-consumption of glucose and xylose are tackled – glucose repression, optimal oxygen requirements, and inhibition of products. Continuous fermentations may be more susceptible to quality issues and contamination, but in the context of bioethanol production, high returns remain the key factor for a feasible process. Therefore, further improvements and optimization on the cell retention immobilization technique, dilution rates, substrate concentrations and process conditions (i.e. oxygenation, mixing) are recommended.

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APPENDICES

Appendix A: Supplementary information for Chapter 2

Table I. Current studies of engineered culture for ethanol production from glucose and xylose							
Engineered culture system	**Fermentation mode**	**Fermentation condition**	**P_{max} (g L^{-1})**	**$Y_{p/s}$ (g g^{-1})**	**Q_p (g $L^{-1}h^{-1}$)**	**Other…**	**Reference**
Engineered *Saccharomyces cerevisiae*	Batch	Condition: Anaerobic, Initial xylose: 20 g/l, Strain: PUA6-9 (pRD1)	3.08	0.16	0.04	NA	(Kötter and Ciriacy, 1993)
	Batch	Retention time: 110 h, Condition: Anaerobic, Initial xylose: 30 g/l, Strain: H158 (pBXI)	1.3	0.13	0.01	Residual xylose: 65.3%	(Walfridsson et al., 1996)
	Batch	pH:5, Condition: Anaerobic, High inoculum, Initial xylose: 7.5 g/l, Initial glucose: 33.5 g/l, Initial galactose: 1 g/l, Initial arabinose:5 g/l, Strain: 1400 (pLNH32)	21	0.5	1.6	NA	(Moniruzzaman et al., 1997)
		pH:5, Condition: Anaerobic, High inoculum, Initial xylose: 40 g/l, Initial glucose: 80 g/l, Strain: 1400 (pLNH32)	52	0.44	3.44	NA	
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: 1400 (pLNH32)	16.0	0.3	0.33	NA	(Ho et al., 1998)
		Condition: Anaerobic, Initial Glucose: 90 g /l, Initial xylose: 40 g/l, Strain: 1400 (pLNH32)	NA	0.46	1.15	NA	
	Batch	Condition: Anaerobic, Initial Glucose: 20 g/l, Initial xylose: 50 g/l, Strain: H158 (fur1::LEU2 PY6)	5.5	0.1	NA	Residual sugars: 48%	(van Zyl et al., 1999)
	Batch	Condition: Anaerobic, Initial Glucose: 30 g/l, Initial xylose: 15 g/l, Strain: XYL1+XYL2	9.04	0.31	NA	NA	(Meinander et al., 1999)
		Condition: Anaerobic, Initial Glucose: 30 g/l, Initial xylose: 15 g/l, Strain: XYL1+XYL2+TAL1	10.28	0344	NA	NA	
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: H1691	3	0.09	0.03	NA	(Toivari et al., 2001)
		Condition: Microaerobic, Initial xylose: 50 g/l, Strain: H1691	NA	0.12	0.05	NA	

		Condition: Aerobic, Initial xylose: 50 g/l, Strain: H1691	NA	0.06	NA	NA	
	Batch	Condition: Aerobic, Initial glucose: 50 g/l, and Initial xylose: 50 g/l, Strain: TMB3001	23.3	0.23	NA	NA	(Zaldivar et al., 2002)
		Condition: Aerobic, Initial glucose: 50 g/l, and Initial xylose: 50 g/l, Strain: A	20.8	0.42	NA	NA	
		Condition: Aerobic, Initial glucose: 50 g/l, and Initial xylose: 50 g/l, Strain: A6	25.2	0.27	NA	NA	
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: TMB3255	NA	0.41	NA	NA	(Jeppsson et al., 2002)
	Batch	Condition: Aerobic, Initial xylose: 40 g/l, Strain: FPL-YSX3	1.94	0.12	NA	Residual xylose: 42%	(Jin et al., 2003)
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: TMB3255 (zwf1Δ)	NA	0.41	NA	NA	(Jeppsson et al., 2003)
	Continuous, D=0.06 1/h	Condition: Anaerobic, Initial glucose: 20 g /l, and Initial xylose: 20 g/L, Strain: TMB3255 (zwf1Δ)	NA	0.39	NA	NA	
	Batch	Condition: Anaerobic, Initial xylose: 10 g/l, Strain: TMB3001C1	NA	0.24	NA	NA	(Sonderegger and Sauer, 2003)
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: H2684	4.6	0.31	0.038	Residual xylose: 70%	(Verho et al., 2003)
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: TMB3113 (M-1021)	NA	0.43	NA	NA	(Lönn et al., 2003)
	Batch	Condition: Anaerobic, Initial xylose: 10 g/l, Strain: TMB3120	NA	0.46	NA	NA	(Träff-Bjerre et al., 2004)
	Batch	Condition: Anaerobic, Initial xylose: 20 g/l, Strain: RWB202-AFX	8.6	0.42	0.08	NA	(Kuyper et al., 2004)
	Batch	Condition: Anaerobic, Initial xylose: 20 g/l, Strain: RWB217	8.67	0.43	0.2	NA	(Kuyper et al., 2005a)
	Batch	Condition: Anaerobic, Initial xylose: 20 g/l, Strain: RWB218	7.52	0.41	NA	NA	(Kuyper et al., 2005b)
		Condition: Anaerobic, Initial glucose: 20 g/l, Initial xylose: 20 g/l, Strain: RWB218	15.7	0.4	NA	NA	

		Condition: Anaerobic, Initial glucose: 100 g/l, Initial xylose: 25 g/l xylose, Strain: RWB218	47.1	0.38	NA	NA	
	Batch	Condition: Anaerobic, 20 g/l xylose, 10 g/l xylose, Strain: FPL-YSX3P	8.4	0.29	0.12	NA	(Jin et al., 2004)
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: TMB3050	NA	0.29	NA	NA	(Karhumaa et al., 2005)
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: TMB3270	NA	0.36	NA	NA	(Jeppsson et al., 2006)
	Batch	Condition: Anaerobic, Initial xylose; 50 g/l, Strain: MT8-1/Xyl/BGL	18	0.37	0.25	NA	(Katahira et al., 2006)
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: TMB3057	13.3	0.33	0.13	NA	(Karhumaa et al., 2007)
	Batch	Condition: Anaerobic Initial xylose: 50 g/l, Strain: TMB3066	7.3	0.43	0.073	NA	
	NA	Condition: Oxygen-limited, Initial xylose: 15 g/l, Initial glucose: 5 g/l, Strain: Y-ARSdR	7.02	0.46	NA	NA	(Watanabe et al., 2007a)
	Batch	pH: 5.5, Temp: 30 °C, Stirring rate: 200 rpm, Retention time: 72 h, Condition: Aerobic, Initial xylose: 15 g/l, Initial glucose: 5 g/l, Strain: Y-R276H	5.94	0.43	NA	Xylose conversion: 59%	(Watanabe et al., 2007b)
	Batch	Condition: Anaerobic, Initial xylose: 50 g/l, Strain: INVSc1/pRS406XKS/ pILSUT1/ pWOXYLA	6.05	0.39	0.043	NA	(Madhavan et al., 2009b)
	Batch	Condition: Anaerobic, Initial xylose: 20 g/l, Strain: ADAP8	7.63	0.48	0.04	NA	(Madhavan et al., 2009a)
		Condition: Anaerobic, Initial Glucose: 50 g/l, Initial xylose: 20 g/l, Strain: ADAP8	33.2	0.48	NA	NA	
	Consolidated bioprocessing	Temp: 30 °C, Stirring rate: 200 rpm, Condition: Aerobic, Initial CMC: 5 g/l, glucose: 20 g/l, Strain: YPH499	3.45	0.34	NA	NA	(Hyeon et al., 2010)
	Simultaneous Saccharification and fermentation (SSF)	Retention time: 96 h, 10% dry weight of pretreated corn stover, Strain: K1-V1116	NA	NA	NA	Ethanol: 2.6 % v/v, Cellulose conversion: 63%	(Khramtsov et al., 2011)

	Closed 100-mL bottles equipped with a bubbling CO2 outlet	Temp: 30 C, Stirring rate: 500 rpm, Retention time: 48 h, Condition: Aerobic, Initial xylose: 40 g/l, Formic acid: 10 mM, Strain: BY4741	12.4	0.31	NA	NA	(Hasunuma et al., 2011)
	Batch	Condition: Anaerobic, Strain: *CpXylA*	NA	0.43	0.03	Xylitol production: 0.18 g/g	(Brat et al., 2009)
	Batch	Condition: Anaerobic, Strain: *BY4741*	NA	0.45	NA	NA	(de Figueiredo Vilela et al., 2015)(de Figueiredo Vilela et al., 2013)
	NA	Condition: Anaerobic, Strain: *ScXKS1*	0.5	NA	NA	NA	(Ota et al., 2013)
Engineered *Escherichia coli*	NA	Condition: Aerobic, Strain: TC4 (PLOI308-10)	15.5	NA	NA	NA	(Ingram and Conway, 1988) (Ingram et al., 1991)
		Condition: Anaerobic, Strain: TC4 (PLOI308-10)	22.17	NA	NA	NA	
	NA	Retention time: 80 h	40	NA	2	NA	(Reynen and Sahm, 1988)
	Batch	Initial glucose: 12% (120 g/l), Strain: ATCC 11303 (PLO1297), and ATCC 15224 (pLOI297)	58	0.48	1.4	Ethanol yield: 95% of the theoretical maximum	(Alterthum and Ingram, 1989) (Ingram et al., 1991)
		Initial xylose: 8% (80 g/l), Strain: ATCC 11303 (PLO1297), and ATCC 15224 (pLOI297)	42	0.52	0.64	Ethanol yield: 102% of the theoretical maximum	
	Batch	pH: 6.0, Temp: 30 °C, Initial glucose: 100 g/l (10%), Strain: ATCC11303(KO11)	52.8	0.54	1.7	Ethanol yield: 107% of the theoretical maximum	(Ohta et al., 1991a)
		pH: 6.0, Temp: 30 °C, Initial xylose: 80 g/l (8%), Strain: ATCC11303 (KO11)	41.6	0.53	1.3	Ethanol yield: 104% of the theoretical maximum	
	Batch	pH: 6.8, Temp: 30 °C, Retention time: 48 h, Inoculum: 330 mg dry cell weight/l, Initial	34.4	0.47	1.0	Ethanol yield: 93% of the theoretical maximum	(Beall et al., 1991)

		xylose: 80 g/l, Strain: ATCC 11303 (PLOI297)					
		pH: 6.8, Temp: 30 °C, Retention time: 36 h, Inoculum: 330 mg dry cell weight/l, Initial glucose: 80 g/l, Strain: ATCC 11303 (PLOI297)	34.4	0.48	1.7	Ethanol yield: 94% of the theoretical maximum	
		pH: 6.8, Temp: 30 °C, Retention time: 48 h, Inoculum: 330 mg dry cell weight/l, Initial mannose: 80 g/l, Strain: ATCC 11303 (PLOI297)	33.6	0.49	1.0	Ethanol yield: 96% of the theoretical maximum	
		pH: 6.8, Temp: 30 °C, Retention time: 48 h, Inoculum: 330 mg dry cell weight/l, Initial galactose: 80 g/l, Strain: ATCC 11303 (PLOI297)	32.0	0.45	1.0	Ethanol yield: 88% of the theoretical maximum	
		pH: 6.8, Temp: 30 °C, Retention time: 36 h, Inoculum: 330 mg dry cell weight/l, Initial fructose: 80 g/l, Strain: ATCC 11303 (PLOI297)	36.8	0.51	1.4	Ethanol yield: 101% of the theoretical maximum	
		pH: 6.8, Temp: 30 °C, Retention time: 48 h, Inoculum: 330 mg dry cell weight/l, Initial arabinose: 80 g/l, Strain: ATCC 11303 (PLOI297)	32.8	0.48	1.1	Ethanol yield: 93% of the theoretical maximum	
	Bath	Temp: 30 C, Initial glucose: 23 g/l, Strain: ATCC 11303 (PLOI297)	13	0.57	1.2	Ethanol yield: 112% of the theoretical maximum	(Lawford and Rousseau, 1991)
		Temp: 30 C, Initial glucose: 58 g/l, Strain: ATCC 11303 (PLOI297)	29	0.5	1.6	Ethanol yield: 98% of the theoretical maximum	
		Temp: 30 C, Initial xylose: 80 g/l, Strain: ATCC 11303 (PLOI297)	36	0.45	0.47	Ethanol yield: 88% of the theoretical maximum	
	Continuous	pH: 6.0, Temp: 30 C, Agitation rate: 150 rpm, Initial glucose: 38 g/l (complex lurla broth), Strain: ATCC 11303 (PLOI297)	12.9	0.45	2.58	Ethanol yield: 94% of the theoretical maximum	

		pH: 6.3, Temp: 30 C, Agitation rate: 150 rpm, Initial glucose: 50 g/l (complex lurla broth), Strain: ATCC 11303 (PLOI297)	16.6	0.45	1.56	Ethanol yield: 96% of the theoretical maximum	
		pH: 6.3, Temp: 30 C, Agitation rate: 150 rpm, Initial glucose: 50 g/l (Defined mineral salts), Strain: ATCC 11303 (PLOI297)	2.9	0.45	0.58	Ethanol yield: 88% of the theoretical maximum	
	Batch	Initial xylose: 72 g/l, Strain: *Pinus* sp. (softwood)	35	NA	NA	Ethanol yield: 91% of the theoretical maximum	(Barbosa et al., 1992)
	Batch	pH: 6.3, Temp: 30 C, Initial glucose: 32.2 g/l, Strain: B (PLO1297)	NA	0.55	NA	NA	(Lawford and Rousseau, 1994)
		pH: 6.3, Temp: 30 C, Initial xylose: 20.4 g/l, Strain: B (PLO1297)	NA	0.49	NA	NA	
		pH: 6.3, Temp: 30 C, Initial glucose: 28 g/l, Initial xylose: 14.7 g/l, Strain: B (PLO1297)	NA	0.5	NA	NA	
	Batch	Detoxified hydrolyzate	NA	0.54	1.32	NA	(Hahn-Hägerdal et al., 1994)
	Semi-continuous	Initial glucose: 20 g/l (2% (w/v)), Strain: ATCC 11303 (pLOI297) with antibiotic (tetracycline and ampiciline/ chloramphenicol)	NA	NA	NA	Ethanol yield: 90.2% of the theoretical maximum	(Lawford and Rousseau, 1995)
		Initial xylose: 20 g/l (2% (w/v)), Strain: ATCC 11303 (pLOI297) with antibiotic (tetracycline and ampiciline/ chloramphenicol)	NA	NA	NA	Ethanol yield: 92.2% of the theoretical maximum	
		Initial glucose: 20 g/l, Strain: ATCC 11303 (pLOI297) without antibiotic	NA	NA	NA	Ethanol yield: 90.2% of the theoretical maximum	
		Initial xylose: 20 g/l, Strain: ATCC 11303 (pLOI297) without antibiotic	NA	NA	NA	Ethanol yield: 94.1% of the theoretical maximum	
		Initial glucose: 20 g/l, Strain: KO11 with antibiotic (tetracycline and ampiciline/chloramphenicol)	NA	NA	NA	Ethanol yield: 96% of the theoretical maximum	

		Initial xylose: 20 g/l, Strain: KO11 with antibiotic (tetracycline and ampiciline/ chloramphenicol)	NA	NA	NA	Ethanol yield: 100% of the theoretical maximum	
		Initial glucose: 20 g/l, Strain: KO11 (pLOI297) without antibiotic	NA	NA	NA	Ethanol yield: 90.2% of the theoretical maximum	
		Initial xylose: 20 g/l, Strain: KO11 without antibiotic	NA	NA	NA	Ethanol yield: 90.2% of the theoretical maximum	
	Batch	pH: 6.0, Temp: 35 C, Retention time: 60 h, Initial xylose: 120 g/l, Strain: SL28, or SL40 (20% more ethanol than KO11)	60	NA	NA	NA	(Lindsay et al., 1995)
		pH: 6.0, Temp: 35 C, Retention time: 60 h, Initial xylose: 30 g/l, Initial glucose: 60 g/l, Strain: SL40	45	NA	NA	NA	
	Simultaneous Saccharification and Fermentation (SSF)	pH: 6.5, Temp: 37 C, Stirring rate: 150 rpm, Initial xylose: 40 g/l, Strain: ATCC 11303 (pLOI 297)	NA	NA	0.82	Ethanol yield: 90% of the theoretical maximum	(Padukone et al., 1995)
		pH: 6.5, Temp: 37 C, Stirring rate: 150 rpm, Initial glucose: 40 g/l, Strain: ATCC 11303 (pLOI 297)	NA	NA	1.066	Ethanol yield: 90% of the theoretical maximum	
	Batch	Initial arabinose: 23 g/l, Initial galactose: 11 g/l, Initial glucose: 27 g/l, Initial xylose: 39 g/l, Strain: KO11	41.7	NA	0.62	Ethanol yield: 90% of the theoretical maximum	(Asghari et al., 1996)
	Batch	Temp: 35 C, Stirring rate: 100 rpm, Retention time: 48 h, Initial xylose: 90 g/l, Strain: KO11	41	NA	0.85	Ethanol yield: 89.3% of the theoretical maximum	(Yomano et al., 1998)
		Temp: 35 C, Stirring rate: 100 rpm, Retention time: 48 h, Initial xylose: 90 g/l, Strain: LY01	42.4	NA	0.88	Ethanol yield: 91.9% of the theoretical maximum	
	Batch	pH: 6.5, Temp: 35 C, Retention time: 70-80 h	4.38-4.66% (w/v)	NA	NA	Ethanol yield: 90-91% of the theoretical maximum	(Dien et al., 1998)
	Batch	Initial xylose: 95 g/l, Strain: FBR5	41.5	NA	0.59	Ethanol yield: 90% of the theoretical maximum	(Dien et al., 2000)

		Initial arabinose: 15 g/l, Initial xylose: 30 g/l, Initial glucose: 30 g/l, Strain: FBR5	34	NA	0.92	Ethanol yield: 90% of the theoretical maximum	
	Batch	Condition: Anaerobic, Initial xylose: 100 g/l, Strain: KO11	50	NA	NA	NA	(Tao et al., 2001)
		Condition: Anaerobic, Initial xylose: 100 g/l, Strain: B (parent)	10	NA	NA	NA	
	Batch	Initial xylose: 74 g/l, Strain: FBR5	33.8	0.46	0.53	NA	(Nichols et al., 2001)
		Initial xylose: 73 g/l, Strain: FBR14	34.4	0.48	0.46	NA	
		Initial xylose: 73 g/l, Strain: FBR16	32.9	0.46	0.31	NA	
		Initial xylose: 83 g/l (Arabinose: 20 g/l, Glucose: 27 g/l, Xylose: 36 g/l), Strain: FBR5	37.8	0.46	0.91	NA	
		Initial xylose: 87 g/l (Arabinose: 17 g/l, Glucose: 35 g/l, Xylose: 35 g/l), Strain: FBR14	41.5	0.48	0.81	NA	
		Initial xylose: 92 g/l(Arabinose: 19 g/l, Glucose: 37 g/l, Xylose: 36 g/l), Strain: FBR16	40.7	0.45	0.75	NA	
	Batch	Retention time: 39 h, wheat straw, Strain: FBR5	NA	0.37	NA	NA	(Saha et al., 2005)
	Batch	Fermentation time: 47 h, Initial xylose: 90 g/l, Strain: FBR5	42.5	0.47	0.9	NA	(Qureshi et al., 2006)
	Batch	Retention time: 24 h, Initial sugar mixture: 37.5 g/l (75% of glucose), Strains: Recombinant E. coli with S. cerevisiae	NA	0.45	NA	NA	(Qian et al., 2006)
	NA	Initial sugar mixture: 22 g/l, Strain: FBR16	7.2	0.34	0.36	Residual sugar concentration: 4.54%, Ethanol yield: 66.5% of the theoretical maximum	(Dhabhai et al., 2012)
Engineered *Zymomonas mobilis*	NA	Temp: 30 C, Condition: Anaerobic, Initial xylose: 25 g/l, Strain: CP4 (pZB5)	NA	NA	NA	Ethanol yield: 86% of the theoretical maximum	(Zhang et al., 1995)
		Temp: 30 C, Condition: Anaerobic, Retention time: 30 h, Initial glucose: 25 g/l, Initial xylose: 25 g/l, Strain: CP4 (pZB5)	NA	NA	NA	Ethanol yield: 95% of the theoretical maximum	

		Temp: 30 C, Condition: Anaerobic, Initial glucose: 25 g/l, Strain: CP4 (pZB5)	NA	NA	NA	Ethanol yield: 94% of the theoretical maximum	
		Temp: 30 C, Condition: Anaerobic, Retention time: 22 h, Initial glucose: 25 g/l, Initial xylose: 25 g/l, Strain: CP4 (pZB186)	NA	NA	NA	Ethanol yield: 99% of the theoretical maximum	
		Temp: 30 C, Condition: Anaerobic, Initial glucose: 25 g/l, Strain: CP4 (pZB186)	NA	NA	NA	Ethanol yield: 97% of the theoretical maximum	
	NA	Temp: 37 C, Retention time: 72 h, Condition: Anaerobic, Initial arabinose 25 g/L, Strain: pZB206	11	NA	NA	Ethanol yield: 98% of the theoretical maximum	(Deanda et al., 1996)
		Temp: 37 C, Retention time: 48 h, Condition: Anaerobic, Initial arabinose: 25 g/l, Initial glucose: 25 g/l, Strain: pZB206	21	NA	NA	Ethanol yield: 84% of the theoretical maximum, Incomplete arabinose conversion	
		Temp: 37 C, Retention time: 24 h, Condition: Anaerobic, Initial glucose: 25 g/l, Strain: pZB206	18	NA	NA	Ethanol yield: 99% of the theoretical maximum, Incomplete arabinose conversion	
	Batch	pH: 5.0, Temp: 30 C, Retention time: 48 h, Initial glucose: 65 g/l, Initial xylose: 65 g/l, Strain: ZM4 (pZB5)	62	0.46	NA	NA	(Joachimsthal et al., 1999)
	Batch	Initial xylose: 60 g/l, Strain: CP4 (pZB5)	23	0.48	0.32	Ethanol yield: 94% of the theoretical maximum	(Lawford and Rousseau, 1999)
	Batch	pH: 5.5, Temp: 34° C, Retention time: 7 days	30	NA	NA	NA	(McMillan et al., 1999)
	Batch	Retention time: 48 h, Initial glucose: 53.7, Initial xylose: 21.8, Strain: CP4 (pZB5)	35.1	0.48	0.73	Residual sugars: 3.6%	(Krishnan et al., 2000)
	Continuous fluidized bed reactor	Initial glucose: 68.8, Initial xylose: 23.1, Dilution rate: 0.25, Strain: CP4 (pZB5)	34.5	0.45	8.6	Residual glucose: 1%, Residual xylose: 63.6%	

		Initial glucose: 68.8, Initial xylose: 23.1, Dilution rate: 0.5, Strain: CP4 (pZB5)	30.5	0.42	15.3	Residual glucose: 1.9%, Residual xylose: 77%	
	Continuous membrane bioreactor with cell recycling	Retention time: 50-60 h, Initial glucose: 50 g/l, Initial xylose: 50 g/l, Strain: Zm4 (pZB5), Dilution rate: 0.1 1/h	50	0.5	5	NA	(Joachimsthal and Rogers, 2000)
	Batch	pH:5.5, Retention time: 48 h, Initial arabinose: 20 g/l, Strain: AX101	5.63	0.26	0.04	Ethanol yield: 50o% of the theoretical maximum	(Lawford and Rousseau, 2002)
		pH: 5.5, Retention time: 50 h, Initial glucose: 40 g/l, Initial xylose: 40 g/l, Initial arabinose: 20 g/l, Strain: AX101	46.33	0.46	0.97	Ethanol yield: 91% of the theoretical maximum, Glucose and xylose conversion: 100%, Arabinose conversion: 75%	
		pH:5.0, Initial glucose: 60 g/l, Initial xylose: 30 g/l, Initial arabinose: 3.5 g/l, Strain: AX101	33.85	0.36	NA	Ethanol yield: 70.1% of the theoretical maximum, Residual xylose: 39%, Residual arabinose: 100%	
	Continuous	pH: 5.0, Initial glucose: 60 g/l, Initial xylose: 30 g/l, Initial arabinose: 3.5 g/l, Strain: AX101, Dilution rate: 0.068 1/h	43.7	0.463	2.97	, Ethanol yield: 90.7% of the theoretical maximum, Residual xylose: 12.3%, Residual arabinose: 54.3%	
		pH: 5.0, Initial glucose: 60 g/l, Initial xylose: 30 g/l, Initial arabinose: 3.5 g/l, Strain: AX101, Dilution rate: 0.78 1/h	43.01	0.457	3.35	Ethanol yield: 89.6% of the theoretical maximum, Residual xylose: 20%, Residual arabinose: 62.8%	
		pH: 5.0, Initial glucose: 60 g/l, Initial xylose: 30 g/l, Initial arabinose: 3.5 g/l, Strain: AX101, Dilution rate: 0.086 1/h	41.96	0.438	3.54	Ethanol yield: 85.9% of the theoretical maximum, Residual	

						xylose: 18%, Residual arabinose: 62.8%	
	Batch	Retention time: 50 h, Initial arabinose: 20 g/l, Initial glucose: 40 g/l, Initial xylose: 40 g/l, Strain: AX101	42	NA	0.61	Glucose and xylose conversion: 100%, Arabinose conversion: 75%, Ethanol yield: 84% of the theoretical maximum	(Mohagheghi et al., 2002)
	NA	Initial cellobios: 20 g/l, Strain: 29191 (pZAGFβg)	10.7	NA	0.44	Ethanol yield: 95% of the theoretical maximum	(Yanase et al., 2005)
Engineered *Klebsiella oxytoca*	Batch	pH: 6.0, Temp: 30 °C, Retention time; 48 h, Initial xylose: 10% (w/v), Strain: M5A1 (pLOI555)	46	0.48	2.1	Ethanol yield of glucose: 98% of the theoretical maximum, Ethanol yield of xylose: 94% of the theoretical maximum	(Ohta et al., 1991b)
	Two stage batch	Temp: 30 C, Retention time: 48 h, Initial glucose: 100 g/l, Strain: P2	46.4	0.46	1.6	NA	(Wood and Ingram, 1992)
		Temp: 30 C, Retention time: 48 h, Initial glucose: 100 g/l, Strain: M5A1 (pLOI555)	48	0.5	2.1	NA	
	Simultaneous Saccharification and fermentation (SSF)	Initial glucose: 50 g/l, Strain: P2	22.9	NA	NA	NA	(Zhou and Ingram, 1999)
		Initial cellobiose: 50 g/l, Strain: P2	22.7	NA	NA	NA	
	Batch	Initial arabinose: 20 g/l, Initial xylose: 40 g/l, Initial glucose: 20 g/l, Strain: P2	34.2	NA	0.35	Xylose conversion: 2/3, Ethanol yield: 84% of the theoretical maximum	(Bothast et al., 1994)
	Simultaneous Saccharification and	pH: 4.5-4.8, Temp: 45 C, Retention time: 7 days (168 h), Initial cellobiose (bagasse): 160 g/l, Strain: P2, Acid pretreatment	36.8	0.24	0.74	NA	(Doran et al., 1994)

	fermentation (SSF)	pH: 4.5-4.8, Temp: 45 C, Retention time: 7 days (168 h), Initial cellobiose (bagasse): 160 g/l, Strain: P2, Sigmacell pretreatment	42.1	0.43	0.76	NA	
	Simultaneous Saccharification and fermentation (SSF)	pH:5.0-5.2, Temp: 35 C, Retention time: 96 h, Initial MWOP: 120 g/l, Strain: P2, Water-Pulped MWOP and Spezyme CE	40.8	0.34	0.75	NA	(Brooks and Ingram, 1995)

NA: Not Available

Table II. Current studies of co-culture systems for ethanol production from glucose and xylose

Co-culture system	Fermentation mode	Fermentation condition	P_{max} (g L^{-1})	$Y_{p/s}$ (g g^{-1})	Q_p (g L^{-1} h^{-1})	Other…	Limitation	Reference
Pachysolen tannophilus (NRRL Y-2640) & *S. cerevisiae* (ATCC 24860)								
Free cells	Batch	pH: uncontrolled, Temp: 30 °C, Stirring rate:100 rpm, Working volume: 100 ml, Initial glucose: 46%, Initial xylose: 44%, Initial arabinose: 2%, Initial galactose: 4%	11.9	0.41	NA	Influent sugar concentration: 50 g/l	Catabolite repression on xylose by glucose, Consumption of xylose and ethanol after glucose conversion instead of ethanol production	(Beck et al., 1990)
			27	0.46	NA	Influent sugar concentration: 125 g/l		
			43.3	0.45	NA	Influent sugar concentration: 200 g/l		
E. coli S17-1 (pLOI308-10) & *S. cerevisiae* (ATCC 24860)								
Free cells	Batch	pH: uncontrolled, Temp: 30 °C, Stirring rate: 100 rpm, Working volume: 100 ml, Initial glucose: 46%, Initial xylose: 44%, Initial arabinose: 2%, Initial galactose: 4%	10.3	0.43	NA	Influent sugar concentration: 50 g/l	Low xylose conversion of xylose-fermenting microorganism, Catabolite repression on xylose by glucose, Consumption of xylose and ethanol after glucose conversion instead of ethanol production	(Beck et al., 1990)
			26.7	0.43	NA	Influent sugar concentration: 125 g/l		
			41.2	0.43	NA	Influent sugar concentration: 200 g/l		
	Batch	NA	NA	NA	NA	NA		(Hanly and Henson, 2011)
Pichia stipitis & *S. cerevisiae*								

Co-immobilized	Continuous	pH: 5.0, Temp: 30 °C, Working volume: 400 ml, Initial glucose: 40.9 g/l, Initial xylose: 10.5 g/l, *P. stipitis* (CBS 5773), suspended *S. cerevisiae* (CBS 8066)	NA	NA	NA	Dilution rate: 0.07 1/h, Residual glucose: 0.049%, Residual xylose: 62.8%	Low ethanol tolerance of xylose-fermenting microorganism in batch culture, Catabolite repression on xylose by glucose, Oxygen competition, Bead damage	(Grootjen et al., 1991)
	Batch	pH:5.5, Temp: 30 °C, Aeration level: 0.03 vvm, Initial glucose: 40 g/l, Initial xylose:10 g/l, *S. cerevisiae* (Baker's Yeast Type II, Sigma Aldrich), *P. stipitis* (NRRL Y-11544)	21.28	0.396	0.89	Residual sugars: 0.5%		(De Bari et al., 2004)
Immobilized *P. stipitis* (CBS 5773) & suspended *S. cerevisiae* (CBS 8066)	Continuous	pH: 5.0, Temp: 30 °C, Working volume: 400 ml, Initial glucose: 40.5 g/l, Initial xylose: 11.2 g/l	NA	NA	NA	Dilution rate: 0.11 1/h, Residual xylose: 89.3%	Catabolite repression on xylose by glucose, Diffusion limitation of oxygen, Bead damage	(Grootjen et al., 1991)
Free cells	Batch	pH: 5.0, Working volume: 1 l, Retention time: 40 h, Initial *P. stipitis*: 7.1 g/l, Initial *S. cerevisiae*: 1.5 g/l, q_{O2}: 66.7 mg/g cell/h for glucose consumption, q_{O2}: 14.3 mg/g cell/h for xylose consumption, Initial glucose: 50 g/l, Initial xylose: 25 g/l, *P. stipitis* (CBS 5773), *S. cerevisiae* (No.7)	29.4	0.39	0.74	NA	Low ethanol tolerance of xylose-fermenting microorganism, Catabolite repression on xylose by glucose, Oxygen competition	(M. Taniguchi et al., 1997; Taniguchi and Tanaka, 2004)
		Retention time: 68 h, Initial P. stipitis: 3.5 g/l, Initial S. cerevisiae: 0.75 g/l, q_{O2}: 66.7 mg/g cell/h for glucose consumption, q_{O2}: 14.3 mg/g cell/h for xylose consumption, Initial glucose: 50 g/l, Initial xylose: 25 g/l, *P. stipitis* (CBS 5773), *S. cerevisiae* (No.7)	26.2	0.35	0.39	NA		(Masayuki Taniguchi et al., 1997)

		pH: 4.5, Stirring rate: 100rpm, Working volume: 100 ml, Initial glucose: 30g/l, Initial xylose: 30 g/l, Initial mannose: 12 g/l, Initial galactose: 8 g/l, *P. stipitis* (CCUG18492), *S. cerevisiae* (from S. I. Lesaffre mareq france)	29.45	0.41	0.77	NA		(Hamidimotlagh et al., 2007)
		pH:5, Temp: 30 °C, Stirring rate: 150 rpm, Aeration level: 0.6 vvm, Initial glucose: 75 g/l, Initial xylose: 30 g/l, *P. stipitis* (NRRL Y-7124), *S. cerevisiae* (ITV-01)	30.3	0.4	1.26	Residual xylose: 20.4%		(Gutiérrez-Rivera et al., 2011)
		pH:5.5, Temp: 30 °C, Stirring rate: 100 rpm, Retention time: 72 h, Initial *P. stipitis*: 7.5 ml, Initial *S. cerevisiae*: 2.5 ml, Initial xylose: 24.59 g/l, Initial glucose: 4.52 g/l, Initial arabinose: 1.57 g/l, Initial mannose:1.35 g/l, and Initial galactose: 0.81 g/l, *P. stipitis* (NCIM 3498), *S. cerevisiae* (VS_3)	15.8±0.55	0.49±0.02	0.219±0.079	Using simulated synthetic medium, Residual sugars: 1.77%.		(Chandel et al., 2011)
			15.0±0.92	0.48±0.032	0.208±0.0142	Using S. spontaneum acid hydrolysate, Residual sugars: 4.8%		
		Temp:30 °C, Stirring rate: 80 rpm, Retention time: 108 h (12 h for glucose and 96 h for xylose conversion), *P. stipitis* (CBS 6054), *S. cerevisiae* Y5 (CGMCC 2660)	16.6	0.46	0.12	Without inhibitors		(Wan et al., 2012)
			15.8	0.43	0.11	With inhibitors		
			27.4	0.43	0.29	Non-detoxified hydrolysates		
		pH: 5.5, Temp: 30 °C, Stirring rate: 150 rpm, *S. cerevisiae* (Baker's Yeast Type II, Sigma Aldrich), *P. stipitis* (NRRL Y-11544)	NA	0.40±0.01	0.48±0.01	Total sugars: 40 g/l, Ratio of *P. stipitis* to *S. cerevisiae*: 2		(De Bari et al., 2013)
			NA	0.455±0.009	0.38±0.02	Total sugars: 40 g/l, Ratio of *P. stipitis* to *S. cerevisiae*: 4		
			NA	0.314±0.01	0.72±0.03	Total sugars: 60 g/l, Ratio of *P. stipitis* to *S. cerevisiae*: 2		

			NA	0.337 ±0.02	0.43± 0.02	Total sugars: 60 g/l, Ratio of *P. stipitis* to *S. cerevisiae*: 4		
			NA	0.276 ±0.01	0.60± 0.01	Total sugars: 80 g/l, Ratio of *P. stipitis* to *S. cerevisiae*: 2		
			NA	0.286 ±0.02	0.57± 0.02	Total sugars: 80 g/l, Ratio of *P. stipitis* to *S. cerevisiae*: 4		
	NA	NA, *S. cerevisiae* (NRRL Y-12632), *P. stipitis* (NRRL Y-7124)	NA	0.45	0.45	NA		(Dhabhai et al., 2013)
	Batch	pH: 5.5, Temp: 28 °C, Working volume: 150 ml, Retention time: 96 h, Aeration level of P. stipitis: 1% v/v, Initial glucose: 35 g/l, Initial xylose:15 g/l, *P. stipitis* (CCY 39501), *S. cerevisiae* (V30)	15	0.39	0.318	Residual glucose: 0.29 %, Residual xylose: 73.33 %	Low ethanol tolerance of xylose-fermenting microorganism in batch culture, Catabolite repression on xylose by glucose	(Kordowska-Wiater and Targonski, 2001)
P. stipitis & respiratory deficient mutant of *S. cerevisiae*	Batch	pH: 5.0, Working volume: 1 l, Retention time: 40 h, Initial *P. stipitis*: 7.1 g/l, Initial *S. cerevisiae*: 1.5 g/l, Initial glucose: 50 g/l, Initial xylose: 25 g/l, *P. stipitis* (CBS 5773), *S. cerevisiae* (No.7)	37.5	0.5	0.94	NA	Low ethanol tolerance of xylose-fermenting microorganism in batch culture, Catabolite repression on xylose by glucose	(M. Taniguchi et al., 1997; Taniguchi and Tanaka, 2004)
	Continuous	pH:6, Temp: 30 °C, Stirring rate: 800 rpm, Working volume:1.5 l, Inoculum size: 3% v/v, Aeration level: 0.005 vvm, Oxygen transfer rate (OTR): 1.7 mmol/l/h, Initial glucose: 35 g/l, Initial xylose:15 g/l, *P. stipitis* (NRRL Y7124), *S. cerevisiae* (NF)	19	0.43	2	Dilution rate=0.1 1/h, Residual xylose: 40%		(Delgenes et al., 1998)

	Batch	pH: 5.5, Temp: 28 °C, Working volume: 150 ml, Retention time: 96 h, Aeration level of *P. stipitis*: 1% v/v, Respiratory deficient mutant *S. cerevisiae*: 2% v/, Initial glucose: 35 g/l, Initial xylose:15 g/l, *P. stipitis* (CCY 39501), *S. cerevisiae* (V30)	18.80	0.38	0.264	Reduced xylose: 0.33%		(Kordowska-Wiater and Targonski, 2001)
	Batch	pH: 5, Temp: 30 °C, Stirring rate: 500 rpm, Inoculum size of *P. stipitis*: 0.9 g/l, Inoculum size of *S. cerevisiae*: 0.1 g/l, K_La: 10.1 1/h, Microaerobic (K_La<10 1/h), Initial glucose: 16 g/l, Initial xylose: 8 g/l, *S. cerevisiae* (311), *P. stipitis* (NF)	9.07	NA	0.469	NA		(Hanly and Henson, 2013)
Restricted catabolite repressed mutant P. stipitis (CCY 39501) & respiratory deficient mutant of *S. cerevisiae* (V30)	Batch	pH: 5.5, Temp: 28 °C, Working volume: 150 ml, Retention time: 120 h, Aeration level of *P. stipitis*: 1% v/v, Respiratory deficient mutant S. cerevisiae: 1% v/v, Initial glucose: 35 g/l, Initial xylose:15 g/l	20.30	0.45	0.169	Residual xylose: 32%		(Kordowska-Wiater and Targoński, 2002)
Immobilized *S. cerevisiae* (NRRL Y-12632) & suspended *P. stipitis* (NRRL Y-7124)	NA	NA	NA	0.45	0.45	NA	Low ethanol tolerance of xylose-fermenting microorganism, Catabolite repression on xylose by glucose, Bead damage	(Dhabhai et al., 2013)
P. stipitis & Sacchromyces diastaticus								

P. stipitis (NRRL Y7124) & respiratory deficient mutant of *S. diastaticus* (NCYC 625)	Continuous	pH: 5.0, Temp: 30 °C, Stirring rate: 800 rpm, Working volume: 1.5 l, Inoculum size: 3% v/v, Oxygen transfer rate (OTR): 1.75 mmol/l/h, Initial glucose: 35 g/l, Initial xylose:15 g/l	27.5	0.45	NA	Dilution rate: 0.015 1/h	Low ethanol tolerance of xylose-fermenting microorganism, Catabolite repression on xylose by glucose	(Jean M. Laplace et al., 1993a)
		pH: 5.0, Temp: 30 °C, Inoculum size: 3% v/v, Initial glucose: 35 g/l, Initial xylose:15 g/l	21.5	0.45	4.3	Dilution rate: 0.20 1/h		(J.M. Laplace et al., 1993)
Candida shehatae & S. cerevisiae								
C. shehatae & S. cerevisiae	Batch	pH: 5.0, Temp: 30 °C, Stirring rate: 800 rpm, Working volume: 1.5 l, Inoculum size of each yeast strain: 1.5% v/v, Aeration level: 0.005 vvw, Oxygen transfer rate (OTR): 1.75 mmol/l/h, Initial glucose: 14 g/l, Initial xylose:6 g/l, *S. cerevisiae* (CBS 1200), *C. shehatae* (ATCC 22984)	14.5	0.39	NA	NA	Low xylose conversion of xylose-fermenting microorganism, Low ethanol tolerance of xylose-fermenting microorganism, Catabolite repression on xylose by glucose, Oxygen competition	(Jean M. Laplace et al., 1993b)
	Continuous	pH: uncontrolled, Temp: 30 °C, Stirring rate: 300 rpm, Working volume: 700ml, Retention time: 48 h, OD_{600}=5, Aeration level: 0.01 vvm, Initial glucose: 5% w/v, Initial xylose: 4% w/v, Initial cellobiose: 5% w/v, *S. cerevisiae* (NBRC 0224), *C. shehatae* (D45-6)	60	0.43	0.86	NA		(Guan et al., 2013)
C. shehatae (ATCC 22984) & respiratory deficient mutant of *S.*	Batch	pH: 5.0, Temp: 30 °C, Stirring rate: 800 rpm, Working volume: 1.5 l, Inoculum sixe of each yeast strain: 1.5% v/v, Aeration level: 0.005 vvw, Oxygen transfer rate (OTR): 1.75 mmol/l/h, Initial glucose: 14 g/l, Initial xylose:6 g/l	14.7	0.4	NA	NA	Low xylose conversion of xylose-fermenting microorganism, Low ethanol tolerance of xylose-fermenting microorganism,	(Jean M. Laplace et al., 1993b)

cerevisiae (CBS 1200)	Continuous	pH: 5.0, Temp: 30 °C, Stirring rate: 800 rpm, Working volume: 1.5 l, Inoculum size of each yeast strain: 1.5% v/v, Aeration level: 0.005 vvw, Oxygen transfer rate (OTR): 1.75 mmol/l/h, Initial glucose: 14 g/l, Initial xylose:6 g/l	14.5	0.39	NA	NA	Catabolite repression on xylose by glucose	
P. stipitis (CCUG18492) & *K. marxianus* (NF)								
Free cells	Batch	pH: 4.5, Stirring rate: 100 rpm, Working volume:100 ml, Initial glucose: 30g/l, Initial xylose: 30 g/l, Initial mannose: 12 g/l, Initial galactose: 8 g/l	31.87	0.36	1.08	NA	Catabolite repression on xylose by glucose, Oxygen competition	(Hamidimotlagh et al., 2007)
P. stipitis (CBS 5773) & *Z. mobilis* (ATCC 10988)								
Free cells	Batch	pH: uncontrolled, Temp: 30°C, Stirring rate: 150 rpm, Working volume: 800 ml, Aeration level: 80 cm3/min, Inoculum size of P. stipitis: 50% v/v, Initial glucose: 30 g/l, Initial xylose: 20 g/l,	NA	0.43	0.518	Retention time: 35 h, Inoculum size of Z. mobilis: 50 % v/v	Low ethanol tolerance of xylose-fermenting microorganism, Catabolite repression on xylose by glucose, Oxygen competition, Utilization of the intermediate metabolites of xylose fermentation by Z. mobilis, Bead damage	(Fu et al., 2009a)
Immobilized *Z. mobilis* and suspended *P. stipitis*	Normal batch		NA	0.44	0.869	Retention time: 24 h, Inoculum size of Z. mobilis: 50 % v/v		
	Modified batch		NA	0.5	1.126	Retention time: 22 h, Inoculum size of Z. mobilis: ¼ batch	Low ethanol tolerance of xylose-fermenting microorganism	
Kluyveromyces marxianus (DMKU 3-1042) & *S. cerevisiae* (M30)								
Free cells	Batch	Stirring rate: 150 rpm, Retention time: 72 h, Initial sugarcane juice: 220 g/l, Ratio of *S. cerevisiae* to *K. marxianus*: 1,	At Temp: 37 °C: 74.9	0.4	1.04	NA	Low xylose conversion of xylose-fermenting microorganism, Catabolite repression on xylose by glucose, Oxygen competition	(Eiadpum et al., 2012)
			At Temp: 40 °C: 66.2	0.43	0.92	NA		
Alginate-loofa-matrix-			At Temp: 37 °C: 66.5	0.39	0.92	NA	Low xylose conversion of xylose-fermenting	

co-immobilized cells			At Temp: 40 °C: 73.2	0.41	0.98	NA	microorganism, Catabolite repression on xylose by glucose	
Thin-shell silk cocoon-immobilized cells			At Temp 37 °C: 81.4	0.41	1.10	NA		
			At Temp 40 °C: 77.3	0.44	1.07	NA		
P. stipitis (CBS6054) & *Issatchenkia orientalisY4* (CGMCC 2159)								
Free cells	Batch	Temp:30 °C, Stirring rate: 80 rpm, , Initial glucose: 23.5 g/l, Initial xylose: 13 g/l	NA	NA	NA	Not successful after 144 h, only 20% of xylose was consumed	Xylose metabolism of strain P. stipitis was interfered with the metabolites of strain I. orientalis	(Wan et al., 2012)

NA: Not Available

Table III. Current studies of sequential culture for ethanol production from glucose and xylose

Sequential culture system	Fermentation mode	Fermentation condition	P_{max} (g L^{-1})	$Y_{p/s}$ (g g^{-1})	Q_p (g L^{-1} h^{-1})	Other…	Reference
S. cerevisiae (ATCC 24860) & *E. coli* (S17-1 with pLOI308-10)	Batch	pH: uncontrolled, Temp: 30 °C, Stirring rate: 100 rpm, Addition of *E. coli* at 3rd day	10.6	0.41	NA	Initial sugar concentration: 50 g/l	(Beck et al., 1990)
			28.3	0.51	NA	Initial sugar concentration: 125 g/l	
			40	0.41	NA	Initial sugar concentration: 200 g/l	
S. cerevisiae (ATCC 24860) & *P. tannuphilus* (NRRL Y-2460)	Batch	pH: uncontrolled, Temp: 30 °C, Stirring rate: 100 rpm, Addition of *P. tannuphilus* at 3rd day	10.5	0.38	NA	Initial sugar concentration: 50 g/l	(Beck et al., 1990)
			27.9	0.42	NA	Initial sugar concentration: 125 g/l	
			44.4	0.42	NA	Initial sugar concentration: 200 g/l	
S. cerevisiae (NRRL Y-2034) & *P. tannuphilus* (NRRL Y-2460)	Batch	pH: 6(4-6), Temp: 30(20-40 °C), Stirring rate: 92(50-150 rpm), Inoculum size of *P. tannuphilus*: 5.0 % v/v, Inoculum size of *S. cerevisiae*: 2.5% v/v, Ratio of glucose to xylose: 7/3	22.24	0.448	NA	NA	(Kocher and Uppal, 2013)
S. cerevisiae (No. 7) & *P. stipitis*	Batch	pH: 5.0, Working volume: 1 l, Retention time: 40 h, Initial *P. stipitis*: 7.1 g/l, Initial *S. cerevisiae*: 1.5 g/l,	25.5	0.34	0.28	NA	(M. Taniguchi

(CBS 5773) in two reactors		qo2: 14.3 mg/g cell/h for xylose consumption, qo2: 66.7 mg/g cell/h for glucose consumption, Initial glucose: 50 g/l, Initial xylose: 25 g/l					et al., 1997)
P. tannophilus & *Z. mobilis* (Not given)	Batch	pH: uncontrolled, Temp: 30 °C, Working volume: 900 ml, Condition of Glucose fermentation: Anaerobic, Aeration level of xylose fermentation: 1 mmol/l/h, Initial glucose: 60 g/l, Initial xylose: 40 g/l, Batch process	NA	0.33	2.32	$X_{max,\ Z.\ mobilis}$: 5.1 *10^7 , $X_{max,\ P.tannophilus}$: 5.7*10^7	(Fu and Peiris, 2007)
Z. mobilis (ATCC 10988) & *P. stipitis* (CBS 5773)	Batch	pH: uncontrolled (between 4 and 7), Temp: 30°C, Stirring rate: 150 rpm, Working volume: 800 ml, Aeration level: 80 cm3/min, Inoculum size of both cells: 50% v/v, Initial glucose: 30 g/l, Initial xylose: 20 g/l, Batch process	NA	0.47	0.83	Retention time: 28.5 h (2.5 h for glucose conversion and 26 h for xylose conversion)	(Fu et al., 2009b)
C. shehatae (ATCC 22984) & *B. bruxellensis* (NBRC 1586)	Batch	pH: uncontrolled, Temp: 30 °C, Stirring rate: 300 rpm, working volume: 700ml, Aeration level: 0.01 vvm	58	0.43	NA	Retention time: 192 h	(Guan et al., 2013)
C. shehatae (ATCC 22984) & *S. cerevisiae* (NBRC 0224)	Batch	pH: uncontrolled, Temp: 30 °C, Stirring rate: 300 rpm, working volume: 700ml, Aeration level: 0.01 vvm, Initial glucose: 5% w/v, Initial xylose: 4% w/v, Initial cellobiose: 5% w/v	56	0.42	NA	Retention time: 92.5 h	(Guan et al., 2013)
C. tropicalis (IEC5-ITV) & *S. cerevisiae* (ITV01-RD)	Batch	Temp: 30°C, Stirring rate:150 rpm, Working volume: 250 mL, Aeration level: 0.5 vvm, Inoculum size of *S. cerevisiae*: 6 × 10^6 cells/mL, Inoculum size of *P. stipitis*: 6×10^6–15×10^6 cells/ mL, Adding *C. tropicalis* after 24 h of *S. crevisiae* growth	NA	0.37	0.72	NA	(Castañón-Rodríguez et al., 2014)
Co-immobilized of *C. shehatae* (ATCC 22984) & *S. cerevisiae* (CBS 1200) in two chambered	Batch	pH: 5.0, Temp: 30, Retention time: 230 h, Magnetic stirring, Initial *C. shehatae*: 0.65 mg dry wt/ml, Initial *S. cerevisiae*: 5.00 mg dry wt/ml, Without gas bubbling, Initial glucose: 35 g/l, Initial xylose: 15 g/l	NA	0.47	7.5	NA	(Lebeau et al., 1997)
S. cerevisiae (CBS 8066) & *P. stipitis* (CBS 5773) in two reactors	Continuous	pH: 5.0, Temp: 30 °C, Flow rate: 0.05 l/h, Working volume of first reactor (*S. cerevisiae*): 0.5 l, Working volume of second reactor (*P. stipitis*): 1 l and after 34 h change to 1.5 l	11.6	0.28	0.38	Residual xylose: 79%	(Grootjen et al., 1991)

S. cerevisiae (No. 7) & *P. stipitis* (CBS 5773) in two reactors	Continuous	Initial *P. stipitis*: 7.0 g/l, Initial *S. cerevisiae*: 0.75 g/l, Aeration level of first reactor: 0.2 vvm, Nitrogen level of second reactor: 0.2 vvm, Retention time: 56 h, Working volume of both reactor: 1 l, Initial glucose: 50 g/l, Initial xylose: 25 g/l, q_{O2} for glucose consumption: 66.7 mg/g cell/h, q_{O2} for xylose consumption: 14.3 mg/g cell/h	For *P. stipitis* in fermentor A: 33.1	0.44	0.59	NA	(Masayuki Taniguchi et al., 1997)
			For *S. cerevisiae* in fermentor B: 33.7	0.45	0.60	NA	
Z. mobilis (MTCC 91) & *P. stipitis* (CBS 6054) in two reactors	Continuous	Initial glucose: 80 g/l, Initial xylose:40 g/l	At flow rate of 100 ml/h, Reactor 1	0.49	2.22	NA	(Chaudhary and Ghosh, 2014)
			At flow rate of 100 ml/h, Reactor 2	0.46	0.9	NA	
			At flow rate of 400 ml/h, Reactor 1	0.49	2.28	NA	
			At flow rate of 400 ml/h, Reactor 2	0.33	4.58	NA	

NA: Not Available

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Appendix B: Aspen custom Modeller code of Chapter 6

```
Model glucose

//~~~~~~~~~~~~~~~~~~~PORTS~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Feed as input MoleFractionPort(Description:"feed inlet port");

Liquid as output MoleFractionPort(Description:"liquid outlet");

Vapour as output MoleFractionPort(Description:"vapour outlet");


//~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

//=================ALL CONSTANTS==========================================

//========================================================================

//*********************REACTOR VARIABLES*******************************

T as temperature (description:"temperature in deg C", value:30, spec:Fixed);

P as pressure (description:"pressure in bar",value: 1.01325, spec:Fixed);

Rvol as volume (description:"reactor volume m3", value:700, spec:Fixed);

VFrac as vapfraction (description:"vapour molefraction", value:0.5, spec:Free);

Q as enthflow (description:"enthalpy of vapour",value:72, spec:Free);


//*********************FEED PORT VARIBALES*****************************

Tin as temperature (description:"temperature in deg C", value:30, spec:Free);

Pin as pressure (description:"pressure in bar",value: 1.01325, spec:Free);

F as flow_mol (description:"Feed molar flow kmol/hr",value: 1, spec:Free);

z(componentlist) as molefraction (description:"inlet molefraction", value:1/size(componentlist),
spec:Free);

hin as enth_mol (description:"activity cofficients of liquid", spec:Free);

rhol as dens_mass (description:"liquid density");

rhov as dens_mass (description:"vapour density");


//*********************ACTUAL PRODUCT PORT VARIABLES********************

Fn as flow_mol (description:"intermediate flow kmol/hr",value:1, spec:Free);

Vn as flow_mol (description:"vapour molar flow kmol/hr",value: 0.01, spec:Free);
```

Ln as flow_mol (description:"liquid molar flow kmol/hr",value:0.99, spec:Free);

zn(ComponentList) as molefraction (description:"intermediate molefraction", lower:-10000000, value:1/size(componentlist), spec:Free);

xn(ComponentList) as molefraction (description:"liquid molefraction", value:1/size(componentlist), spec:Free);

yn(ComponentList) as molefraction (description:"vapour molefraction", value:1/size(componentlist), spec:Free);

hl as enth_mol (description:"enthalpy of liquid", spec:Free);

hv as enth_mol (description:"enthalpy of vapour", spec:Free);

//**********PRODUCT PORT VARIABLES**********

V as flow_mol (description:"vapour molar flow kmol/hr",lower:-1000000, value: 0.01, spec:Free);

L as flow_mol (description:"liquid molar flow kmol/hr",value:0.99, spec:Free);

x(ComponentList) as molefraction (description:"liquid molefraction", lower:-10000000, value:1/size(componentlist), spec:Free);

y(ComponentList) as molefraction (description:"vapour molefraction", lower:-1000000000, value:1/size(componentlist), spec:Free);

//**********INTERMEDIATE VARIABLES**********

//----------CONCENTRATIONS----------

c(ComponentList) as conc_mass (description:"liquid mass concentration", spec:Free);

cM(ComponentList) as conc_mole (description:"molar concentration, kmol/m3", spec:Free);

//----------MOLAR MASSES----------

Mwf as molweight (description:"feed molar weight");

Mwl as molweight (description:"liquid molar weight");

Mwv as molweight (description:"vapour molar weight");

Mwc(ComponentList) as molweight (description:"molar weight", spec:Free);

//----------VAPOUR PRESSURES----------

Pvap(componentlist) as pressure (description:"vapour pressures bar");

//----------ACTIVITY COEFFICIENTS----------

gamma(componentlist) as act_coeff_liq (description:"activity cofficients of liquid");

```
//----------------------PSEUDO MOLE FRACTIONS OF LIQUID-------------------

px(ComponentList) as RealVariable (description:"pseudo mole fractions", value:1/(size(componentlist)-
2), spec:Free);

pMolComp(ComponentList) as flow_mol (description:"component molar flow", lower:-100000000,
value:0.1, spec:Free);

pMolTot as flow_mol (description:"liquid without biomass ", value:1, spec:Free);


//**********************REACTION CONSTANTS*******************************


mumaxInv as time_ (description:"inverse mumax hr", value: 10/5, spec:fixed);

Ksx1 as RealVariable (description:"kvalue mmol/g/hr", value: 0.48,spec:fixed);

Pix1 as RealVariable (description:"kvalue mmol/g/hr", value: 24,spec:fixed);

Pmx1 as RealVariable (description:"something", value: 97.24, spec:fixed);

Kix1 as RealVariable (description:"kvalue mmol/g/hr", value: 600,spec:fixed);

qpmaxInv as time_ (description:"inverse mumax hr", value: 1/10, spec:fixed);

Ksp1 as RealVariable (description:"kvalue mmol/g/hr", value: 2.1,spec:fixed);

Pip1 as RealVariable (description:"kvalue mmol/g/hr", value: 35.5,spec:fixed);

Pmp1 as RealVariable (description:"something", value: 128.18, spec:fixed);

Kip1 as RealVariable (description:"kvalue mmol/g/hr", value: 558,spec:fixed);

Pi1 as RealVariable (description:"inibition constant", value:1,spec:free);

Pi2 as RealVariable (description:"inubiyion constant", value:1, spec:free);

Ki1 as RealVariable (description:"inibition constant", value:1,spec:free);

Ki2 as RealVariable (description:"inubiyion constant", value:1, spec:free);

Xglucolig as RealVariable (description:"fractional conversion of glucolig", value: 1,spec:fixed);

Xisobutanol as RealVariable (description:"fractional conversion of glucolig", value: .01,spec:fixed);

Xglycerol as RealVariable (description:"fractional conversion of glucolig", value: 0.03165,spec:fixed);

XaceticAcid as RealVariable (description:"fractional conversion of glucolig", value: 0.0178,spec:fixed);

XisoamylAlcohol as RealVariable (description:"fractional conversion of glucolig", value:
0.025,spec:fixed);

F1 as flow_mol (description:"Feed molar flow kmol/hr",value: 1, spec:Free);
```

```
z1(componentlist) as molefraction (description:"inlet liquid molefraction adjusted",
value:1/size(componentlist), spec:Free);

Xc(ComponentList) as flow_mol (description:"converted molar flows kmol/hr", lower:-1000000000,
value:0, spec:Free);

Fg as flow_mol (description:"intermediate glucose flows kmol/hr", value:0, spec:Free);

alpha as RealVariable (value: 0.5,spec:fixed);

//**********************REACTION RATES***********************************

rrx as reaction (description:"reaction rate kmol/hr/m3", value:10, spec:Free);

rrE as reaction (description:"reaction rate kmol/hr/m3", value:10, spec:Free);

rrxA(ComponentList) as reaction (description:"component reactions rates kmol/hr/m3", value:10,
spec:Free);

xg(ComponentList) as molefraction (description:"liquid molefraction", lower:-100000000,
value:1/size(componentlist), spec:Free);

//====================ACCOUNTING FOR NO FEEDPORT==========================

//==========================================================================

//==========================================================================

//********************FEEDPORT PRESENT***********************************


F1=Feed.F;

z1=Feed.z;

Tin=Feed.T;

Pin=Feed.P;


//********************FEEDPORT NOT PRESENT*******************************

/*F1=4496.57;

for i in componentlist do

if i=="S" then

z1(i)=0.0203522;

elseif i=="NH3" then

z1(i) =0.000429937;

elseif i=="H2O" then
```

```
z1(i) = 0.9692535;
elseif i=="XYLOSE" then
z1(i)=0.00597597;
elseif i=="FURFURAL" then
z1(i) =2.69267E-05;
elseif i=="H3PO4" then
z1(i) =9.8965E-06;
elseif i=="H2SO4" then
z1(i)=0.000205535;
elseif i=="HMF" then
z1(i)=0.000646428;
elseif i=="MINERALS" then
z1(i)=0.000130849;
elseif i=="SALTS" then
z1(i)=0.00131767;
elseif i=="XYLOLIG" then
z1(i)=0.000354963;
elseif i=="ACETATE" then
z1(i)=0.00129609;
else
z1(i)=0;
endif
endfor
Tin = 30;
Pin = 1.01325;
*/
//=========================================================================
//=========================================================================
//=========================================================================
```

```
//=====================NON-IMPORTANT EQUATIONS============================
//*********************ACTIVITY COEFFIEINTS*******************************
for i in componentlist do
if rrxA(i)==0 then
xg(i)=0.1;
else
xg(i)=x(i);
endif
endfor
Call (gamma) = pAct_Coeff_Liq(T,P,xg) ComponentList;

//*********************VAPOUR PRESSURES***********************************
Call (Pvap) = pVap_Pressures(T);

//***********************PSEUDO MOLE FRACTIONS*****************************
pMolComp = x*L;
pMolTot = sigma(pMolComp) - pMolComp("X");
if pMolTot < 0.0001 then
px = x;
else
px = pMolComp/pMolTot;
endif

//***********************MOLECULAR WEIGHTS**********************************
CAll (Mwf)= pMolWeight(z) Componentlist;
CAll (Mwl)= pMolWeight(x) Componentlist;
Call (Mwv) = pMolWeight(y) componentlist;
call (rhol) = pDens_Mass_Liq(T,P,x) componentlist;
Call (rhov) = pDens_Mass_Vap(T,P,y) componentlist;
Call (Mwc) = pMolWeights() ComponentList;
```

```
//**********************LIQUID CONCENTRATIONS*****************************
/*if c("H2O")==9.85 then
for i in componentlist do
if i=="S" then
c(i)=10;
elseif i=="E" then
c(i) =20;
elseif i=="X" then
c(i)=5;
elseif i=="NH3" then
c(i)=4;
elseif i=="CO2" then
c(i)=0.5;
elseif i=="H2O" then
c(i) = 900;
else
c(i)=0;
endif
endfor
else*/
c=xn*Mwc/Liquid.V;
 // endif
cM = c/Mwc;

//===============APPROXIMATE METHOD FOR VAPOUR-LIQUID
EQUILIBRIUM=========
//=============================================================================
//=============================================================================
for i in componentlist do
```

```
if (i=="X" OR i=="S" OR i=="NH3") then
y(i)=0;
elseif i=="CO2" then
y(i)*P=2000*x(i);
elseif rrxA(i)==0 then
y(i)=0;
else
y(i)*P=x(i)*gamma(i)*Pvap(i);
endif
endfor

//=======================MASS AND ENERGY
BALANCE============================
Eqn_MB: z*F+(rrxA*Rvol)=y*V+x*L;
Eqn_YSum: sigma(y)=1;
Eqn_XSum: sigma(x)=1;
Fn = V+L;
Fn*zn=x*L+y*V;

//================ACCURATE METHOD FOR VAPOUR-LIQUID
EQUILIBRIUM============
//=============================================================================
//=============================================================================
Call (yn, xn, VFrac, hv, hl) = pFlash(T,P,zn) ComponentList;
Vn=Vfrac*Fn;
Ln=Fn-Vn;
//---------------------ENERGY BALANCE ----------------------------------
Eqn_enthin: Call (hin) = pEnth_Mol(Tin,Pin,z) ComponentList;
Eqn_EB: hin*F+Q=Ln*hl+Vn*hv;

//=====================REACTIONS=========================================
```

```
//**********************INHIBITION TERMS**********************************

if (c("s")) <= 100 then
Ki1 = 1;
Ki2 = 1;
else
Ki1 = Kix1/(Kix1+c("S"));
Ki2 = Kip1/(Kip1+c("S"));
endif
Pi1 = 1-(c("E")-Pix1)/(Pmx1-Pix1);
Pi2 = 1-(c("E")-Pip1)/(Pmp1-Pip1);
//**********************MAIN REACTIONS**********************************

if c("X") <=0.1 then
rrx = ((1/mumaxInv)*c("S")/(Ksx1+c("S"))*0.1)*Pi1*Ki1/Mwc("X");
else
rrx = ((1/mumaxInv)*c("S")/(Ksx1+c("S"))*c("X"))*Pi1*Ki1/Mwc("X");
endif
rrE = ((1/qpmaxInv)*c("S")/(Ksp1+c("S"))*c("X"))*Pi2*Ki2/Mwc("E");

//**********************RECTION RATES**********************************
for i in componentlist do
if i=="X" then
 rrxA(i) = rrx;
 elseif i=="S" then
 rrxA(i) = -2*rrE*0.2557;
 elseif i=="NH3" then
 rrxA(i) = -48/40*rrx;
 elseif i=="E" then
 rrxA(i) = rrE;
```

```
 /* elseif i=="H2O" then
 rrxA(i) = 108/40*rrx;
 elseif i=="CO2" then
// rrxA(i) = rrE+12/40*rrx;
rrxA(i)=((0.31/0.6107))*(1/alpha)*rrE;
elseif i=="ISOBUTOH" then
rrxA(i) = 0.01*(1/alpha)*rrE;
elseif i=="Glycerol" then
rrxA(i) = 0.0633*rrE; *///*(46.06844/92.09382);
/*  elseif i=="Acetate" then
rrxA(i) = 0.0356*rrE;*///*(60.05/92.09382);
else
rrxA(i)=0;
endif
endfor

//=========================================================================
Fg = (z1("S")+Xglucolig*z1("GLUCOLIG"))*F1;
for i in componentlist do
 if (i=="X" OR i=="S" OR i=="E" OR i=="NH3") then
 Xc(i)=rrxA(i)*Rvol;
 elseif i=="GLUCOLIG" then
 Xc(i)=-Xglucolig*z1(i)*F1;
 Elseif i=="ISOBUTOH" then
Xc(i)=Xisobutanol*Fg;
elseif i=="GLYCEROL" then
Xc(i)=Xglycerol*Fg*12/7;
elseif i=="ISOAMIL" then
Xc(i)=XisoamylAlcohol*Fg*4/5;
elseif i=="H2O" then
```

```
Xc(i)=(Xisobutanol-6/7*Xglycerol+6/5*XisoamylAlcohol+(0.02925*2.13))*Fg;
elseif i=="CO2" then
Xc(i)=(2*Xisobutanol+6/7*Xglycerol+10/5*XisoamylAlcohol+2*0.9-(0.02925*0.6545))*Fg;
else
Xc(i)=0;
endif
endfor
z1*F1+Xc=z*F;
sigma(z)=1;

//=====================ASSIGNING STREAM VARIABLES=========================
Ln=Liquid.F;
Vn=Vapour.F;
xn=Liquid.z;
yn=Vapour.z;
Vapour.T=T;
Liquid.T=T;
Liquid.P=P;
Vapour.P=P;
Liquid.h=hl;
Vapour.h=hv;
Liquid.V*rhol=Mwl;
Vapour.V*rhov=Mwv;

End
```