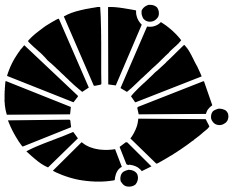


UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

REMIGIUS REINERUS MARIA ZAUTSEN

FERMENTAÇÃO ALCOÓLICA E EXTRAÇÃO LÍQUIDO-LÍQUIDO SIMULTÂNEA DE ETANOL E INIBIDORES PROVENIENTES DE CALDO HIDROLÍTICO DE BIOMASSA LIGNOCELULÓSICA

TESE DE DOUTORADO APRESENTADA À FACULDADE DE ENGENHARIA DE ALIMENTOS UNICAMP PARA OBTENÇÃO DO TÍTULO DE DOUTOR EM ENGENHARIA DE ALIMENTOS

PROF DR FRANCISCO MAUGERI FILHO
ORIENTADOR

Este exemplar corresponde à versão final da tese defendida por Remigius Reinerus Maria Zautsen, aprovada pela comissão julgadora em 14/12/2011 e orientado pelo Prof. Dr. Francisco Maugeri Filho.

_____
Orientador

CAMPINAS, 2011

FICHA CATALOGRÁFICA ELABORADA POR
CLAUDIA AP. ROMANO DE SOUZA – CRB8/5816 - BIBLIOTECA DA FACULDADE DE ENGENHARIA DE ALIMENTOS – UNICAMP

Z19f

Zautsen, Remigius Reinerus Maria, 1977-
Fermentação alcoólica e extração líquido-líquido simultânea de etanol e inibidores provenientes de caldo hidrolítico de biomassa lignocelulósica / Remigius Reinerus Maria Zautsen. -- Campinas, SP: [s.n], 2011.

Orientador: Francisco Maugeri Filho.
Tese (doutorado) – Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos.

1. Fermentação. 2. Lignocelulose. 3. Etanol. 4. Inibidores. 5. Extração. I. Maugeri Filho, Francisco. II. Universidade Estadual de Campinas.Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em inglês: Ethanol fermentation and simultaneous liquid-liquid extraction of ethanol and inhibitors present in hydrolyzed lignocellulosic biomass
Palavras-chave em inglês (Keywords):
Fermentation
Lignocellulose
Ethanol
Inhibitors
Extraction
Área de concentração: Engenharia de Alimentos
Titulação: Doutor em Engenharia de Alimentos
Banca examinadora:
Francisco Maugeri Filho [Orientador]
Luciano Armiliato
Daniel Ibraim Pires Atala
Antonio Maria Francisco Luiz José Bonomi
Carlos Eduardo Vaz Rossell
Data da defesa: 14/12/2011
Programa de Pós Graduação: Engenharia de Alimentos

BANCA EXAMINADORA

PROF DR FRANCISCO MAUGERI FILHO

ORIENTADOR – DEA/FEA/UNICAMP

DR LUCIANO ARMILIATO

TITULAR – P&D-ETHANOL/CTC

DR DANIEL IBRAIM PIRES ATALA

TITULAR – BIOFUELS/BP

PROF DR ANTONIO MARIA FRANCISCO LUIZ JOSÉ BONOMI

TITULAR – Programa de Avaliação Tecnológica/CTBE

PROF DR FERNANDO ANTONIO CABRAL

SUPLENTE – FEA/DEA/UNICAMP

DRa FÁTIMA APARECIDA DE ALMEIDA COSTA

SUPLENTE – FEA/DEA/UNICAMP

PROF DR ANTONIO JOSÉ de ALMEIDA MEIRELLES

SUPLENTE – FEA/DEA/UNICAMP

PROF DR CARLOS EDUARDO VAZ ROSSELL

TITULAR – Programa Industrial/CTBE

"The value of a budget is equal to the inverse of its amount when it is not available."

"The conservative, blind and careless can end a work according to any plan. The creative, watchful and persistent face Murphy's law."

"The average tax payer can be easily fooled as long as the quality of academic work is measured by quantifications of the same."

DEDICATÓRIA

Dedico este trabalho aos meus pais, Riek e Will, que o fruto deste trabalho seja uma consolação para minha ausência contínua.

À minha esposa, Danielle, expressando o meu apreço e gratidão pela sua paciência imensa.

A quem acredita nos poderes positivos e construtivos de cada um de nós e todos nós. Que este trabalho seja inspirador frutífero; enquanto andamos juntos para o mesmo destino, estaremos no caminho certo.

AGRADECIMENTOS

Agradeço muito ao meu orientador, Francisco Maugeri Filho, por seus conselhos, motivação, apoio, liberdade, paciência e impaciência sempre quando eu precisava.

Ao Carlos E. Vaz Rossell pelo acolhimento, iniciativas e apoio profissionais e amigáveis; sempre lembro, com muito carinho, de nossas conversas e acompanhamentos.

À Gabriella Macedo, por sua dedicação e iniciativas vitais e indispensáveis no lançamento deste projeto.

Aos demais membros da banca pela correção e enriquecimento do trabalho.

A Maria Isabel Rodrigues por seu apoio e conhecimento nos momentos chaves da pesquisa. Que o entrelaçamento dos nossos conhecimentos ainda dê muitos frutos.

Gerben, Richard e Rodolfo por suas contribuições científicas e substanciais a este trabalho.

Luuk van der Wielen, Adrie Straathof e Jan de Bont, pela inspiração, bolsa e viagens. Gostaria que as circunstâncias tivessem deixado espaço para uma cooperação mais intensa e frutífera.

A meus pais, Riek e Will, a quem devo algumas palavras em Holandês: dank jullie wel voor jullie steun, liefde, vertrouwen en geduld. De interesse die jullie steeds toonden over de inhoud en voortgang van dit werk waren absoluut de inspiratie die ik nodig had om steeds met frisse moed door te kunnen gaan.

A Danielle, minha esposa, por sua paciência, torcida, amor e de novo por sua paciência.

A minha família Brasileira, Josefa, Sebastião e Fabio, pelo acolhimento, torcida e confiança.

Ao pessoal do Laboratório de Engenharia de Bioprocessos (LEB) que encheram a ‘minha casa’ todos os dias com momentos de alegria, energia e companheirismo.

Aos funcionários e Professores da Faculdade de Engenharia de Alimentos.

E a todos que contribuíram para a realização deste trabalho.

Índice

ÍNDICE ..... **IX**

ÍNDICE DE FIGURAS ..... **XIII**

ÍNDICE DE TABELAS ..... **XVII**

RESUMO ..... **XIX**

ABSTRACT ..... **XXI**

INTRODUÇÃO **1**

1.1 INTRODUÇÃO E JUSTIFICATIVA 2

1.1.a Contexto *2*

1.1.b Inibidores provenientes de hidrólise de biomassa lignocelulósica *3*

1.1.c Aplicação de fermentação extrativa líquido-líquido *3*

1.1.d Quadro do trabalho *4*

1.2 OBJETIVOS 5

1.2.a Objetivo geral *5*

1.2.b Objetivos específicos *5*

1.3 DESCRIÇÃO DOS CAPÍTULOS 6

REVISÃO BIBLIOGRÁFICA **9**

2.1 INTRODUÇÃO NA PRODUÇÃO DE ETANOL 10

2.1.a História *10*

2.1.b Futuro *12*

2.2 MATÉRIAS-PRIMAS CONVENCIONAIS 14

2.2.a Cultivos mundiais para produção de etanol *14*

2.2.b Cana-de-açúcar *15*

2.2.c Milho *16*

2.2.d Uma comparação *16*

2.2.e Processo de preparo de cana-de-açúcar *17*

2.2.f Processo de preparo de milho *19*

2.3 LIGNOCELULOSE COMO MATÉRIA-PRIMA 20

2.3.a Fontes *20*

2.3.b Composição *22*

2.3.c Vantagens de utilização de biomassa lignocelulósica *25*

2.4 HIDRÓLISE DE BIOMASSA LIGNOCELULÓSICA E GERAÇÃO DE AÇÚCARES E INIBIDORES 27

2.4.a Pré-tratamento *28*
2.4.b Hidrólise enzimática *30*
2.4.c Hidrólise catalisada por ácido *33*
2.4.d Combinação de pré-tratamento organosolv e hidrólise ácida *35*
2.4.e Formação de inibidores *36*
2.4.f Efeitos de inibidores na fermentação *39*
2.4.g Métodos de detoxificação de hidrolisados *47*
2.5 PROCESSOS DE FERMENTAÇÃO 49
2.5.a Regimes de fermentação *51*
2.5.b Fermentação extrativa líquido-líquido *54*
2.6 ESCOLHA DE SOLVENTE 58
2.6.a Coeficientes de partição *59*
2.6.b Seletividade e remoção de água *61*
2.6.c Biocompatibilidade *62*
2.6.d Valor log P_{ow} *63*
2.6.e Ponto de ebulição *63*
2.6.f Estrutura molecular *64*
2.6.g Óleo de mamona e biodiesel *65*
2.7 REFERÊNCIAS 66

LIQUID-LIQUID EXTRACTION OF FERMENTATION INHIBITING COMPOUNDS IN LIGNOCELLULOSE HYDROLYZATE 77

3.1 ABSTRACT 77
3.2 INTRODUCTION 79
3.3 MATERIAL AND METHODS 82
3.3.a Partition coefficients *82*
3.3.b Biocompatibility *84*
3.3.c Feasibility of the approach *86*
3.4 RESULTS 87
3.4.a Partition coefficients *87*
3.4.b Biocompatibility *87*
3.4.c Feasibility *89*
3.5 DISCUSSION 91
3.5.a Partition coefficients *91*
3.5.b Biocompatibility *93*
3.5.c Feasibility *93*
3.6 ACKNOWLEDGEMENT 95

3.7 REFERENCES 95

COMPARISON OF VEGETABLE OIL AND VEGETABLE OIL BASED BIODIESEL AS ORGANIC SOLVENT FOR *IN-SITU* EXTRACTION OF FERMENTATION INHIBITORS IN HYDROLYSED BAGASSE 97

4.1 ABSTRACT 97
4.2 INTRODUCTION 98
4.3 MATERIAL AND METHODS 101
4.3.a Biocompatibility 101
4.3.b Water and ethanol absorption 101
4.3.c Partition coefficients 102
4.3.d Viscosity 103
4.3.e Fermentations 104
4.3.f Used chemicals 107
4.4 RESULTS AND DISCUSSION 108
4.4.a Biocompatibility 108
4.4.b Water and ethanol absorption 108
4.4.c Partition coefficients 110
4.4.d Viscosity 111
4.4.e Fermentations 113
4.4.f Further observations 118
4.5 CONCLUSION 119
4.6 ACKNOWLEDGEMENT 120
4.7 REFERENCES 120

KINETICS OF ETHANOL FERMENTATION AND INHIBITION BY HYDROLYZED LIGNOCELLULOSIC BIOMASS 123

5.1 ABSTRACT 123
5.2 INTRODUCTION 124
5.2.a Context 124
5.2.b Fermentation inhibitors 125
5.2.c Biochemical and modeling complexity 125
5.2.d Scope of this work 126
5.3 MATERIALS AND METHODS 127
5.3.a Used materials 127
5.3.b Experimental design 127
5.3.c Inoculum preparation 130
5.3.d Shaked flasks fermentations 131

5.3.e Modeling *132*
5.4 RESULTS AND DISCUSSION 139
5.4.a Experimental Design *139*
5.4.b Modeling and parameter estimations *140*
5.5 CONCLUSION 149
5.6 NOMENCLATURE 150
5.7 REFERENCES 151
5.8 APPENDIX 1 154

MODELING AND SIMULATION OF EXTRACTIVE ETHANOL FERMENTATION COMBINING *IN-SITU* PRODUCT RECOVERY, MEDIUM DETOXIFICATION AND COOLING 157

6.1 ABSTRACT 157
6.2 INTRODUCTION 158
6.3 PROCESS DESCRIPTION 162
6.4 MODEL INPUTS AND OPTIMIZATION OBJECTIVES 164
6.5 MODEL DEVELOPMENT 166
6.5.a Fermentation kinetic model *167*
6.5.b Partition coefficients *170*
6.5.c Heat production *171*
6.5.d Dimensions and assumptions *173*
6.5.e Calculation method *175*
6.6 RESULTS AND DISCUSSION 175
6.6.a Conventional fermentation *176*
6.6.b Extractive fermentation *177*
6.7 CONCLUSION 181
6.8 NOMENCLATURE 183
6.9 REFERENCES 186

CONCLUSÕES E SUGESTÕES 189

7.1 CONCLUSÕES PRINCIPAIS 190
7.2 SUGESTÕES PARA PESQUISAS POSTERIORES 192

Índice de figuras

Capítulo 1

Figura 1: Principais inibidores gerados na hidrólise de material lignocelulósico. 3

Capítulo 2

Figura 1: Produção mundial de bioetanol em bilhões de litros por ano (BP Statistical Review of World Energy 2010)..... 11
Figura 2: Previsão de rendimento de cana-de-açúcar convencional no estado de São Paulo em toneladas de cana úmida por hectare. (fonte: elaboração própria a partir de dados fornecidos por Tadeu, 2008)..... 13
Figura 3: Potencial estimado de bagaço disponível em 2008 (em milhões toneladas em base seca; mapa gerado com dados fornecidos por World Energy Council 2010). 15
Figura 4: Comparação de composição de massa seca de cana-de-açúcar e milho (fontes: Aguilar et al., 2002, Mantelatto 2005, Belyea et al., 2004, Lee et al., 2007, Cordova 1998) 17
Figura 5: Diagrama de fluxo simplificado do processo de tratamento de caldo misto (Rossell, 2006, Nolasco, 2005)..... 18
Figura 6: Diagrama de fluxo simplificado do processo de preparo de glicose a partir de amido de milho com moagem úmida (Raminez et al., 2008 e Veen et al., 2006)..... 20
Figura 7: Diversas fontes de matérial lignocelulosico: a) cana-de-açúcar, b) milho, c) sorgo sacarino, d) Miscanthus, e) sorgo, f) switchgrass. (fontes imagens: a) elaboração própria, b) http://www.gettyimages.pt/detail/82877391/Stone#, c) (http://www.icrisat.org/what-we-do/SASA/sasaindex.htm, d) http://www.ceres.net/products/Products-Miscanthus.html, e) http://images.businessweek.com/ss/08/10/1007_green_tech/13.htm, f) (http://www.jgi.doe.gov/sequencing/why/50008.html)..... 21
Figura 8: Estrutura da lignocelulose (reproduzida de Rubin, 2008)..... 22
Figura 9: Estrutura da celulose, polímero de dímeros de glicose (Ogeda et al., 2010) 24

Figura 10: Redução da distância máxima (a) e média (b), dos campos até a usina, a ser percorrida para entregar matéria-prima á usina: 29,3 e 27,1%. A distância total para os caminhões percorrerem é conseqüentemente reduzida em 63,6%. 25
Figura 11: Mecanismo de hidrólise da celulose catalise ácida (Ogeda et al., 2010) 34
Figura 12: Diagrama de fluxo de hidrólise organosolv (Olivério et al., 2007) 36
Figura 13: Inibidores provenientes de hidrólise de lignocelulose: aldeídos, cetonas e fenóis (adaptado de Liu 2011) 37
Figura 14: Degradação de xilose em furfural e ácido fórmico (Almeida et al., 2009) 38
Figura 15: Degradação de glicose em HMF, ácido levulínico e ácido fórmico (Almeida et al., 2009) 38
Figura 16: Ácidos orgânicos provenientes da hidrólise de lignocelulose (adaptado de Liu 2011) 39
Figura 17: Mecanismos de resposta da levedura diante de inibidores: múltiplos complexos envolvendo expressão de genes (repressão e indução), danos e reparos de membrana, enzimas e código genético, equilibrio do pH interno. Repressão e indução estimulam a utilização de via das pentoses fosfato e o ciclo TCA, aumentando a produção de NAD(P)H e ATP necessários para reduzir aldeídos e para o funcionamento de enzimas de proteção contra estresse oxidativo como GSR e TR. (fontes: Liu 2011, Almeida 2007, 2008, 2009, Modig et al., 2002, Horváth et al., 2003, Larsson et al., 2001) 46
Figura 18: Relação entre temperatura e taxa de crescimento de Saccharomyces cerevisiae (Atala et al., 2001,Rivera et al., 2006) 50
Figura 19: Princípio da fermentação extrativa líquido-líquido 54
Figura 20: Diagrama de fluxo de fermentação extrativa líquido-líquido 55
Figura 21: Aumento de conversão total de substrato no sistema com solvente. Com a extração seletiva de água da fase aquosa para a fase orgância, o volume aquoso diminui para sistema B. Porém, as concentrações finais de substrato são iguais para sistema A e B. 62
Figura 22: Álcool oléico (cis-9-octadecen-1-ol) 64
Figura 23: (1) Triglicéride de ácido ricinoléico ou óleo de mamona, (2) éster etílico de ácido ricinoléico e (3) éster metílico de ácido ricinoléico. Foto ao lado direito: mamona (fonte: imagem própria, Paraíba 2008) 65

Capítulo 3

Figure 1: Chemical structure of investigated furans and phenolic compounds 81
Figure 2: A. Blank experiment; the quotient of the slope of second part and the first part equals 1. The slope does not increase during the first 60 min, thus no growth of the yeast takes place. B. Addition of hexane after 30 minutes 86
Figure 3: Biocompatibility during 1 h fermentation of alkanes, alkanols and oleic acid according to Log P value 88
Figure 4: Volumetric ethanol production rate after 4.5 h of fermentation in the presence or absence of organic solvents in the media, for different furfural concentrations 90
Figure 5: Ethanol concentration during fermentation with 6 g/L vanillin in the media, in the presence or absence of organic solvents 90
Figure 6: Volumetric ethanol production rate after 4.5 h of fermentation in the presence or absence of organic solvents in the media, for different vanillin concentrations 91

Capítulo 4

Figure 1: Castor oil, triglyceride of ricinoleic acid(a), ethyl ester of ricinoleic acid (b) and methyl ester of ricinoleic acid (c) 100
Figure 2: Biocompatibility of castor oil and biodiesel demonstrated by carbon dioxide production during fermentation without organic phase (dotted line), with castor oil (white triangle) and with biodiesel (black circle) 108
Figure 3: Water and ethanol absorption in water-biodiesel and water-castor oil two-phase systems for different final ethanol concentrations in the aqueous phase 109
Figure 4: Viscosity comparison between castor oil and biodiesel for various temperatures 112
Figure 5: Fermentation without inhibitors 114
Figure 6: Fermentation with inhibitors 114
Figure 7: Fermentation with inhibitors and with biodiesel 115
Figure 8: Concentration profiles of inhibitors during fermentation without biodiesel 117
Figure 9: Concentration profiles of inhibitors during fermentation with biodiesel in the organic phase (org) and aqueous phase (no suffix). 118

Capítulo 5

Figure 1: Profiles of state variables for different initial concentrations of furfural and vanillin at 35 °C by experimental data (symbols) and model (solid lines) 142
Figure 2: Profiles of state variables for different initial concentrations of furfural and vanillin at 32.5 °C by experimental data (symbols) and model (solid lines) 143

Capítulo 6

Figure 1: Process schematics with streams and processing units of a continuous extractive fermentation system. Process steps within the dotted area are modeled 162
Figure 2: Volumetric production rate (A) inverse of substrate cost per volume produced ethanol (B) for fermentation without organic solvent 176
Figure 3: Fermentation without organic solvent; optimal fermentation temperatures. 177
Figure 4: Volumetric production rate (A) inverse of substrate cost per volume produced ethanol (B) for fermentation with organic solvent 178
Figure 5: Fermentation with organic solvent; optimal fermentation temperatures. 178
Figure 6: Volumetric production rate (A) and inverse of substrate cost per volume produced ethanol (B) for juice and hydrolyzate ratios fixed at 0.7 180
Figure 7: Volumetric production rate (A) and product yield (B) for a solvent dilution rate fixed at 0.3 181

Índice de tabelas

Capítulo 2

Tabela 1: Principais matérias-primas cultivadas para a produção de bioetanol em vários países (Sánchez et al., 2008) 15
Tabela 2: Rendimentos por hectare para milho e cana-de-açúcar (Mejean et.al., 2010) 16
Tabela 3: Aplicações para a lignina e seus derivados (resumido de Doherty et al., 2011 e Ogeda et al., 2010) 23
Tabela 4: Diversas fontes comuns de material lignocelulósico e celulose (Sanchez et al., 2008, completado) 26
Tabela 5: Métodos aplicados para pré-tratamento de lignocelulose (Olsson et al., 1996, Holzapple et al., 1991, Cardona et al., 2010, Sanchez et al., 2008, Gírio et al., 2010) 28
Tabela 6: Vantagens e desvantagens de várias técnicas de pré-tratamento, segundo Gírio et al. (2010) 30
Tabela 7: Enzimas envolvidas na hidrólise de xilana, o principal polímero na hemicelulose (Gírio et al., 2010) 31
Tabela 8: Ácidos orgânicos em ordem de toxicidade e fatores que podem explicar o grau de toxicidade de cada ácido: pK_a (logaritmo da constante de acidez), TPSA (topological polar surface área), $logP_{ow}$ (logaritmo de coeficiente de partição octanol/água), fato de haver uma dupla ligação na estrutura molecular, volume e PM (peso molecular), Nenhuma constante é consistente com a toxicidade. (Almeida et al., 2007,Klinke et al., 2004, Erdemgil et al., 2007, Pow: http://logkow.cisti.nrc.ca/logkow/, volume, TPSA: http://www.molinspiration.com/cgi-bin/properties) 43
Tabela 9: Métodos de detoxificação de caldos hidrolíticos 47
Tabela 10: Comparação de regimes de fermentação alcoólica (Sanchez e Cardona, 2008, *Atala 2004,** Daugulis et al., 1994) 51

Capítulo 3

Table 1: Partition coefficients of fermentation inhibiting solutes (g solute in organic phase per g solute in aqueous phase) 89

Capítulo 4

Table 1: Partition coefficients of various compounds for Biodiesel (BD) and Castor Oil (CO) in the presence of different concentrations of ethanol in the aqueous phase 111
Table 2: Initial inhibitor concentrations and fermentation parameters in the following order: maximal specific growth-, furfural and vanillin reduction- and ethanol production rates, maximal volumetric ethanol production rate, ethanol, glycerol and biomass yields. 113

Capítulo 5

Table 1: Factor level values, real values (in brackets) and results of the Plackett-Burman screening design 128
Table 2: Factor level values, real values (in brackets) and results of the Plackett-Burman screening design 129
Table 3: Standardized effects (t-values at 8 degrees of liberty) and p values. Significant effects are marked bold ($p<0.2$). 139
Table 4: Regression of model per state variable and per experiment. 145
Table 5: Model parameter values per state differential. Estimated values are marked as bold. 149

Capítulo 6

Table 1: Composition of untreated cane juice and hydrolyzate in mass percentages. 165
Table 2: Values of the fermentation kinetic model parameters per state variable differential 169
Table 3: Linear constants used for the calculation of partition coefficients and regression to experimental data..... 170

Resumo

Na fermentação de produtos como etanol, utilizando biomassa lignocelulósica como matéria-prima, existem dois fatores principais que limitam a produtividade e eficiência do processo: inibição pelo produto e inibição por substâncias no caldo hidrolítico provenientes da hidrólise. Neste trabalho, é proposta a remoção simultânea de ambos os fatores para eliminar seus efeitos negativos na fermentação alcoólica.

Produtos de fermentação prejudicam muitas vezes a integridade da membrana celular do micro-organismo utilizado como fermento. Portanto, a toxidez do produto não permite que a fermentação ocorra de forma ilimitada, uma vez que o produto está presente no meio em certa concentração. O crescimento do micro-organismo, a produtividade e o rendimento são prejudicados pela presença do mesmo. Compostos como furfural, hidroximetil furfural, compostos fenólicos e ácidos, que são produzidos durante o pré-tratamento ou hidrólise da biomassa lignocelulósica, introduzem outros efeitos inibidores, como a extensão da fase lag da levedura, prejudicam o crescimento e a produção. Esta tese propõe empregar um solvente orgânico na dorna do biorreator, com o fim de extrair o produto inibidor e todos os componentes inibidores existentes no substrato, de tal forma que o processo de fermentação não seja prejudicado.

Com esse objetivo, primeiramente foi definida a relação entre o tamanho molecular de agentes extrativos, bio-compatibilidade e propriedades extrativas dos mesmos. Em seguida, um solvente foi escolhido, sendo o biodiesel à base de óleo de mamona, através de características como biocompatibilidade, coeficientes de partição, seletividade, alta disponibilidade e reutilização. Foram feitas fermentações em regime batelada em fermentadores de bancada, utilizando o biodiesel como agente extrativo, demonstrando os efeitos positivos no desempenho da fermentação de um licor hidrolítico. Adicionalmente, o comportamento de uma cepa de levedura industrial foi estudado na presença de inibidores e foi construído um modelo matemático que descreve as taxas de conversão dos principais inibidores e as condições em que a levedura, ao invés de manter uma fase lag, inicia a

produção de biomassa e etanol. Finalmente, foi elaborado, como exemplo da utilização da tecnologia proposta, um modelo do sistema contínuo de fermentação alcoólica com a extração líquido-líquido, incluindo a recuperação do produto e resfriamento do meio de fermentação pelo próprio solvente orgânico. Por meio desta modelagem e uma série de simulações, foram determinadas as faixas ideais das principais variáveis na produção de etanol pelo sistema bifásico, sendo elas a fração de licor hidrolítico no mosto, concentração de substrato, temperatura de fermentação, e taxa de diluição do solvente.

Assim, o trabalho demonstra as vantagens, efeitos positivos e os limites da utilização de extração líquido-líquido na fermentação de substrato da segunda geração. Entre as vantagens se destacam: maior tolerância de caldo hidrolítico no mosto, elevada produtividade, maior rendimento e maior custo-benefício do substrato.

Abstract

There are two main factors that limit fermentation productivity and eficiency during the production of chemicals like ethanol when using lignocelulosic biomass as raw material: product inhibition and inhibition by substances in hydrolitic liquor generated during hydrolyzis. In this work, the simultaneous removal of both factors is proposed to eliminate their negative effects on ethanol fermentation.

Fermentation products often damage the cellular wall of the micro-organism that is used as ferment. As a result, the toxicicity of the product does not permit that the fermentation continues unhindered once the product concentration has reached a certain level; growth of the micro-organism, productivity and yield are effected. Substances like furfural, hydroximetil furfural, phenolic compounds and organic acids, that are produced or released during pre-treatment or hydrolyzis of ligno-celulosic biomass, introduce other inhibiting effects, like the extension of the lag phase of the ferment or decreasing growth and production. This thesis proposes the use of an organic solvent as a second liquid phase in the bioreactor, to extract both the inhibiting product and all inhibiting compounds present in the substrate, such that the fermentation process remains unhindered.

With this objective, first the relation between the molecular size of an extractive agent and its biocompatibility and extractive properties was determined. Next, a solvent was chosen, being biodiesel based on castor oil, by prioritizing characteristics as biocompatibility, partition coeficients, selectivity, availability and possibilities for recycling and reuse. Batch fermentations were executed in bench-scale, using biodiesel as extractive agent, demonstrating the improvements of fermentation of hydrolytic liquor. Aditionaly, the performance of an industrial yeast strain was studied in the presence of inhibitors and a mathematical model was constructed that descibes the conversion rates of the main inhibitors and conditions at which the yeast, instead of maintaining a lag phase, starts production of biomass and ethanol. Finally, as a practical example of the proposed technology, simulations were performed for an integrated process including continuous

ethanol fermentation with liquid-liquid extraction, product recovery and cooling of the fermentation broth by the extractive agent itself. The simulation results reveiled the optimal ranges for the most important variables of the two-phase ethanol production process, i.e. fraction of hydrolitic liquor in the must, substrate concentration, fermentation temperature and dilution rate of the solvent.

In all, the work shows the advantages, positive effects of and limits to the use of liquid-liquid extraction in fermentation of second-generation substrate. Advantages are, among others, higher tolerance of hydrolyzate in the must, higher yield, higher productivity and higher return on investment of raw-material.

Capítulo 1

Introdução

1.1 Introdução e justificativa

Neste trabalho foi proposto um método de extração *in-situ* de etanol e de seus principais inibidores na fermentação alcoólica, provenientes de hidrolisado de bagaço de cana-de-açúcar. As vantagens da aplicação deste método foram avaliadas, destacando seu impacto na produção de etanol.

1.1.a Contexto

Observando-se as mudanças causadas por elevadas concentrações de CO_2 na atmosfera, com conseqüências danosas a médio e longo prazo ao meio ambiente, estima-se que, no Brasil, o setor de transporte é responsável por mais de 41% da emissão de CO_2 (Timilsina e Shrestha, 2008). Os preços do petróleo têm aumentado sistematicamente durante a última década e os governos estão interessados em diminuir a dependência do mercado de petróleo, buscando meios alternativos para cumprir o protocolo de Kyoto, seguido pelo Acordo de Copenhague. O desenvolvimento de tecnologia de transformação de biomassa em etanol oferece mercados alternativos para agricultores e benefícios macro-econômicos para a sociedade, diminuindo as emissões de CO_2 e outros gases que promovem o efeito estufa. Esta linha de pesquisa resultará em estímulo ao uso de fontes renováveis e criará uma indústria de processos baseada em carboidratos. A tecnologia proposta neste estudo promove a produção de etanol, deixando-o mais competitivo como combustível, tanto no mercado nacional como em mercados internacionais.

Como o Brasil pretende ser um grande exportador de bioetanol, o preço competitivo é de vital importância. A produção de etanol a partir de carboidratos depende principalmente dos custos da matéria-prima, capital e da utilização de energia, deixando outros fatores como de importância secundária. Processos fermentativos de alta produtividade são importantes para reduzir custos, se não aumentarem consumo de energia ou reduzirem o rendimento. A fermentação extrativa, como proposta nesta tese, tem o potencial de aumentar a produtividade, reduzir consumo de energia e aumentar o rendimento.

1.1.b Inibidores provenientes de hidrólise de biomassa lignocelulósica

Os principais inibidores gerados na hidrólise de bagaço de cana ou outros compostos lignocelulósicos são os furanos, compostos fenólicos e ácidos carboxílicos. A Figura 1 apresenta alguns componentes destes grupos que provocam maior impacto na cinética da levedura.

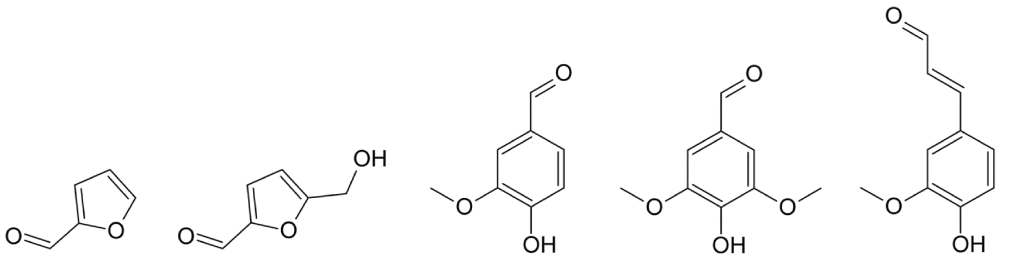


Figura 1: Principais inibidores gerados na hidrólise de material lignocelulósico.

A presença destes inibidores no meio de fermentação é inevitável com a utilização de caldo hidrolítico como matéria-prima, prejudicando a produtividade da levedura e a eficiência do processo.

1.1.c Aplicação de fermentação extrativa líquido-líquido

Até hoje, a fermentação extrativa foi sugerida somente para remoção do etanol durante a fermentação, visando concentrar o produto, diminuir os efeitos de inibição e diminuir custos de destilação. Assim, a fermentação extrativa já mostrou, na teoria, ser economicamente viável, aumentando a eficiência da fermentação e diminuindo o consumo de energia (Daugulis *et al*., 1991). Porém, na fermentação extrativa líquido-líquido é possível também retirar os inibidores provenientes de caldo hidrolítico.

Neste trabalho foram estudadas as vantagens da fermentação extrativa em relação ao aumento de eficiência de fermentação, aumento da produtividade volumétrica, viabilidade

da levedura, estabilidade do processo e estudo do sistema de resfriamento do meio pelo próprio solvente ao invés de utilização de sistemas dispendiosos de resfriamento. Dentre as vantagens pode-se destacar também: a eliminação de oscilações na fermentação contínua a fim de reduzir perdas de substrato e custos de recuperação de etanol, a possibilidade de utilização de concentrações elevadas de substrato, subproduto de destilação (vinhaça) mais concentrado com custos inferiores de transporte desta vinhaça, sistemas de centrifugação mais compactos, formação de uma fase superior rica em solvente que poderia diminuir o volume e aumentar a pureza do gás de saída. A camada orgânica, com elevada concentração de inibidores provenientes do caldo hidrolítico, poderia formar uma barreira para micro-organismos, resultando em menor risco de infecção e menor uso de antibióticos. A tecnologia pode ser aplicada em processo contínuo ou em batelada, para produção de etanol, acetona, butanol, ácido acético ou outros produtos obtidos via fermentação. Do conjunto de todas estas plausíveis vantagens, foi apenas elaborado um subdomínio nesta tese.

1.1.d Quadro do trabalho

A tese foi elaborada visando tanto uma contribuição científica de aprofundamento de conceitos de fermentação na presença de inibidores e solventes, quanto uma aplicação prática da tecnologia, utilizando insumos amplamente disponíveis no território nacional. Portanto, foi considerado o uso de um etil-éster de ácido ricinoléico como solvente (na prática, biodiesel feito a partir de óleo de mamona) por ser disponível e, comparado com solventes derivados de petróleo, de baixo custo, renovável, biocompatível e biodegradável. Como micro-organismo, foi utilizada uma cepa de levedura com bom desempenho e amplamente aprovada no ambiente industrial de várias usinas brasileiras.

A partir dos resultados dos experimentos, focados principalmente na superação de problemas bioquímicos e tecnológicos, modelagem e pesquisa bibliográfica, criou-se uma tecnologia apropriada para ser testada em escala de planta piloto.

1.2 Objetivos

1.2.a Objetivo geral

Desenvolvimento de um processo de fermentação alcoólica, a partir de caldos hidrolíticos, empregando um solvente biocompatível para extração simultânea de produto e inibidores do meio de fermentação.

1.2.b Objetivos específicos

- Verificar experimentalmente a relação entre os coeficientes de partição de diversos inibidores e o tamanho de cadeia de carbonos de diversos álcoois e alcenos;
- Verificar experimentalmente a relação entre a biocompatibilidade e valor log P_{ow} (coeficiente de partição em sistema bifásico octanol/água) e o tamanho de cadeia de carbonos de diversos álcoois e alcenos;
- Verificar experimentalmente a viabilidade de extração *in-situ* de componentes inibidores com uma seleção de solventes, em escala de frascos agitados.
- Comparar experimentalmente os dois melhores solventes em termos de biocompatibilidade, viscosidade, absorção de água e coeficientes de partição para álcool, substratos e diversos inibidores;
- Verificar experimentalmente a viabilidade técnica de remoção simultânea de inibidores e etanol em processo de fermentação extrativa líquido-líquido em escala de bancada;
- Verificar experimentalmente quais são os fatores mais importantes entre temperatura, pH e concentração de composto furano (furfural), composto fenólico (baunilha) e ácido carboxílico (ácido acético) descrevendo qualitativamente a inibição da fermentação alcoólica;
- Realizar um planejamento experimental e modelar a cinética de fermentação através dos dados obtidos experimentalmente;

- Modelar o processo de fermentação extrativa líquido-líquido empregando os conhecimentos previamente obtidos em termos de coeficientes de partição e cinética do processo biológico;
- Otimizar por simulação o rendimento e a produção volumétrica de um processo contínuo de fermentação extrativa líquido-líquido, em termos de fluxos de substrato e solvente, concentração de substrato no fluxo de alimentação, temperatura da fermentação e solvente.

1.3 Descrição dos capítulos

Capítulo 1. Introdução geral e justificativa.

Capítulo 2. Revisão bibliográfica.

Este capítulo aborda uma revisão bibliográfica relatando a literatura recente e mais relevante sobre o tema proposto neste trabalho.

Capítulo 3. Liquid–liquid extraction of fermentation inhibiting compounds in lignocellulose hydrolysate.

Neste capítulo foi realizado um estudo da relação entre o tamanho de cadeia de carbonos de diversos álcoois e alcenos e os coeficientes de partição de diversos inibidores, biocompatibilidade e valor log P_{ow}. Também foram realizadas fermentações em frascos agitados com quatro solventes e diferentes concentrações de furanos (furfural) e compostos fenólicos (baunilha) para comprovar a viabilidade da ideia de extração *in-situ* dos mesmos, aumentando a produtividade da fermentação.

Capítulo 4. Comparison of vegetable oil and vegetable oil based biodiesel as organic solvent for *in-situ* extraction of fermentation inhibitors in hydrolyzed bagasse.

Neste capítulo foi realizada uma comparação de dois solventes (óleo de mamona e biodiesel a partir deste óleo) em termos de biocompatibilidade, viscosidade, absorção de água e coeficientes de partição para álcool, substratos e diversos inibidores. Também foi verificada experimentalmente a viabilidade técnica de remoção simultânea de inibidores e etanol em processo de fermentação extrativa líquido-líquido em escala de bancada.

Capítulo 5. Kinetics of ethanol fermentation and inhibition of hydrolyzed lignocellulosic biomass.

Neste capítulo foram verificados por meio de experimentos quais os fatores mais importantes dentre: temperatura, pH, concentração de um composto furano (furfural) e um composto fenólico (baunilha) e um composto ácido carboxílico (ácido acético) para descrever qualitativamente a inibição da fermentação alcoólica. Em seguida foram realizados experimentos de acordo com um planejamento experimental completo (DCCR) e a partir dos resultados foi construído um modelo descrevendo a cinética da fermentação alcoólica na presença de inibidores.

Capítulo 6. Modeling and simulation of extractive ethanol fermentation combining *in-situ* product recovery, medium detoxification and cooling.

Neste capítulo foi elaborado um modelo de um processo contínuo de fermentação extrativa líquido-líquido. Por meio de simulações foram introduzidos vários fluxos de solvente em diferentes condições de concentração de substrato na alimentação da dorna e temperatura do solvente. Com estas simulações foi construído um banco de dados para visualizações de rendimento e produtividade obtidos com várias combinações destes fatores.

Capítulo 7. Conclusões e sugestões.

Neste capítulo, as principais conclusões sobre os resultados foram relatadas.

Capítulo 2

Revisão bibliográfica

2.1 Introdução na produção de etanol

2.1.a História

A utilização de etanol como combustível era comum na Europa e nos Estados unidos no início de século XX (Balat 2009). Comparado à gasolina, o bioetanol tem um maior índice de octano e gera menos componentes tóxicos na sua combustão. Por outro lado, o bioetanol tem maior entalpia de vaporização (Balabin 2007), densidade energética menor, maior corrosividade, e afinidade com água. Com a abundância de petróleo e baixo custo de produção de gasolina, o bioetanol perdeu importância como combustível alternativo, até a crise de petróleo no início dos anos 70. Os elevados preços e baixa disponibilidade do petróleo incentivaram o Brasil a iniciar o Programa Nacional do Álcool, o Proálcool. Este programa, lançado em 1975, estimulou a produção de etanol aumentando a produção de cana-de-açucar, ampliando destilarias e instalando novas unidades produtoras. Depois da introdução de automóveis movidos exclusivamente a álcool em 1978, um segundo aumento substancial do preço de petróleo incentivou o governo brasileiro a implementar o Proálcool definitivamente. A partir de 1985 o preço de petróleo caiu e o preço de bioetanol no mercado brasileiro subiu, diminuindo o interesse do consumidor pelo bioetanol durante aproximadamente duas décadas. Com preços de combustíveis flutuantes ao longo da vida útil do veículo e a baixa confiança do consumidor no bioetanol ao longo prazo, os veículos movidos exclusivamente a álcool eram cada vez menos aceitos no mercado. No entanto, a introdução do motor 'Flex' no mercado em 2003 coincidiu com um novo incremento significante do preço de etanol. O motor Flex possibilita a escolha de tipo de combustível, bioetanol ou gasolina, ou uma mistura destes, tornando mais econômico o uso do combustível. Em poucos anos, uma alta porcentagem de carros vendidos passaram a possuir tecnologia do motor Flex, dando novo impulso para o mercado nacional de bioetanol.

Enquanto isso, houve internacionalmente um crescimento da sensibilização sobre o efeito estufa e sua importância, e cada vez mais ocorriam incentivos concretos para o desenvolvimento e produção de bio-combustíveis visando a diminuição de gases como

dióxido de carbono. Isto, aliado ao desejo de tornar-se independente da importação de petróleo, resultou em um crescimento mundial da produção de bioetanol como combustível conforme mostra a Figura 1.

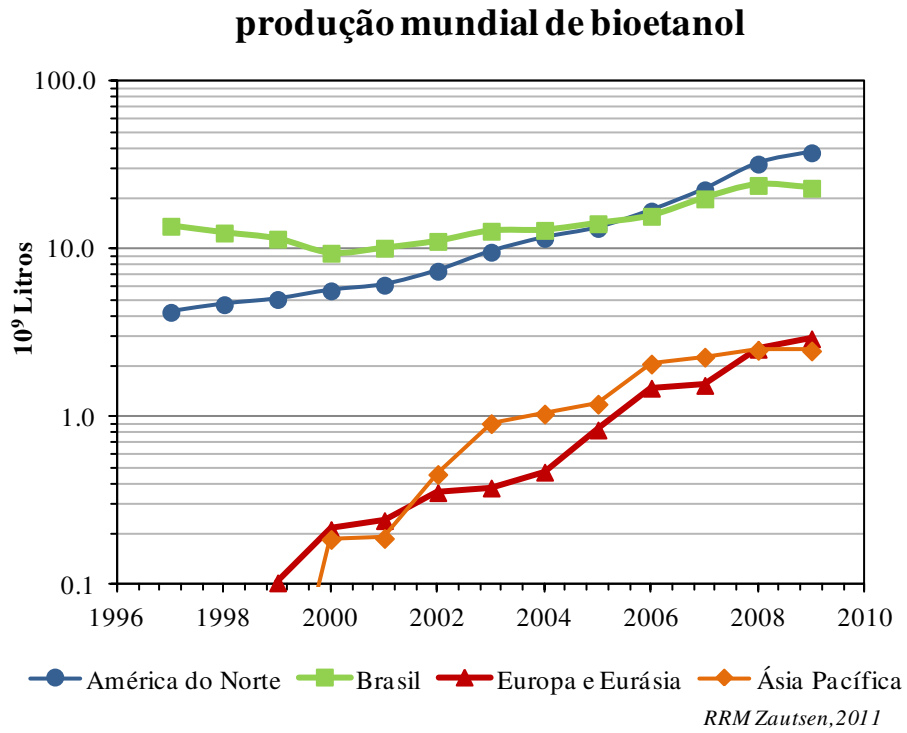


Figura 1: Produção mundial de bioetanol em bilhões de litros por ano (BP Statistical Review of World Energy 2010)

Convém frisar que o produto não é limitado ao uso como combustível: atualmente, 73% de etanol produzido mundialmente é usado como combustível, 17% para indústria de bebidas e 10% como etanol industrial (Sanchez 2008).

Nos últimos 15 anos, o Brasil manteve inicialmente a liderança no volume de bioetanol produzido, mas perdeu sua posição para os Estados Unidos, que incrementaram sua produção com o incentivo à ampliação do cultivo de milho como a matéria-prima para o bioetanol. Em 2009, o Brasil era responsável por 92,3% da produção total das Américas Central e do Sul. A produção de bioetanol na Europa e Eurásia teve um aumento de 3000% em uma década, e nesta região a França liderou em 2009 com 31% da produção total. Na Ásia Pacífica, onde a China manteve a liderança com 73,3% da produção total, aconteceu

um crescimento semelhante. Em comparação ao Brasil e aos Estados Unidos, a produção total nestas regiões ainda é modesta, mas o forte crescimento porcentual na última década ressalta a importância do bicombustível e a dedicação para o desenvolvimento do produto e sua implementação como substituto de gasolina nos países envolvidos.

2.1.b Futuro

Um estudo realizado por Özbek e Özlale (2010) mostra que o preço de petróleo é cada vez mais determinado pela demanda global. Tendo em vista as economias crescentes em países com grandes populações como a China e a Índia, e a duplicação mundial de veículos nos próximos 20 anos (Balat e Balat 2009), esta demanda continuará aumentando, resultando em preços de petróleo cada vez maiores. Existem então importantes forças econômicas que podem aumentar o uso de biocombustíveis como etanol em detrimento de combustível a partir de petróleo.

A cana-de-açúcar tem alguns pontos importantes a favor de sua utilização como fonte vegetal preferida, ajudando a cumprir o objetivo em nível global, como descrito em item 2.2. A Figura 2 mostra, por exemplo, a evolução de rendimentos médios de cana-de-açúcar no estado de São Paulo durante uma década. Méjean *et al.* (2010) preveem para 2030, custos de produção de bioetanol de cana-de-açúcar no Brasil até 4,8 vezes menor do que a partir de milho nos Estados Unidos, baseando-se em um progresso previsível da tecnologia e na evolução da qualidade da terra em cada país.

A maior necessidade de recursos como água e área para o plantio do milho, comparado com o cultivo de cana-de-açúcar, também são fatores que apontam para a cana-de-açúcar como fonte mais adequada para a produção de bioetanol, economicamente viável e altamente competitiva.

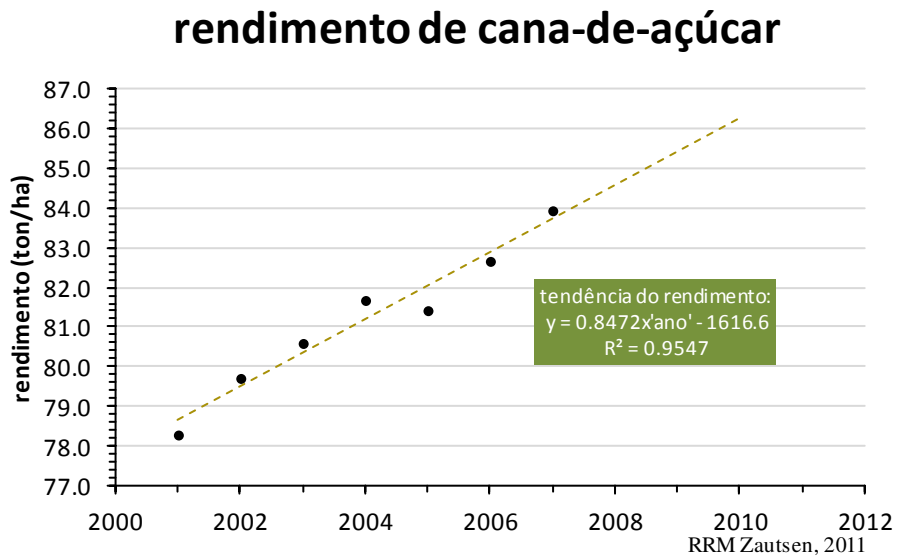


Figura 2: Previsão de rendimento de cana-de-açúcar convencional no estado de São Paulo em toneladas de cana úmida por hectare. (fonte: elaboração própria a partir de dados fornecidos por Tadeu, 2008)

Porém, a extensão necessária para satisfazer o futuro mercado de álcool, sem comprometer o mercado de açúcar, exige uma mudança na integração de produção de açúcar e álcool nas usinas. Uma das possibilidades é a produção de álcool a partir de outras fontes de substratos como hemiceluloses e celuloses, que se encontram no bagaço e na palha da cana-de-açúcar. Assim o bagaço, que já está presente nas usinas de cana, se torna, por meio de hidrólise, uma fonte alternativa para a produção de álcool.

Observa-se que os rendimentos máximos de toneladas de cana-de-açúcar por hectare utilizado no estudo de previsão em 2030 por Méjean et al (2010), 67,8 ton/ha, são números modestos, já que na literatura podem ser encontrados rendimentos obtidos no passado, de 79,5 toneladas/ha/ano, em massa seca (assumindo a massa seca ser 30% da massa total, Mantelatto 2005), já em 1975 (Giamalva *et al.*, 1984), ou mesmo mais do que 95 toneladas/ha/ano, em massa seca (Legendre *et al.*, 1995). Porém, uma maior massa seca da cana leva a uma mudança na composição da planta, devido a maior incorporação de (ligno) celulose necessária para fortalecer sua estrutura. Consequentemente, os açúcares redutores totais (ART) que podem ser obtidos da cana sofreram uma diminuição relativa ao peso seco total, diminuindo a qualidade do suco (Legendre *et al.*, 1995). Mesmo com maior ART, em

valores absolutos, que pode ser obtido por hectare através deste tipo de cana, a economia de transporte, tratamento e moagem da cana se torna favorável para uma planta mais convencional, desde que se desenvolvam tecnologias voltadas à conversão simultânea de material lignocelulósico em produtos de valor agregado, como proposto nesta tese. Uma planta convencional rende menos massa seca por hectare, mas rende maior teor relativo de ART, o que é importante levando em consideração que o ART é a única fonte de carbono para a fermentação convencional. Com a possibilidade da utilização de material (hemi) celulósico para a fermentação, o equilíbrio destas considerações mudará profundamente. Com maior valor da massa seca total da planta em relação ao conteúdo relativo de açúcares, os rendimentos atuais podem ser dobrados, e tornarem-se maiores que as 80 toneladas de massa seca por hectare, relatados por Giamalva *et al.*, (1984). Considerando que a massa seca da cana que é convertível em etanol (açúcares solúveis, celuloses e hemiceluloses), 50% são, atualmente, holoceluloses, tudo isto significando um aumento teórico de rendimento de etanol por área de campo de cerca de quatro vezes do atual.

2.2 Matérias-primas convencionais

2.2.a Cultivos mundiais para produção de etanol

As principais matérias-primas tradicionalmente utilizadas para a produção de bioetanol são cana-de-açúcar, milho, trigo, e beterraba. A cana-de-açúcar (*Saccharum* spp.) é cultivada no Brasil, China e Índia, enquanto que o milho é a principal matéria-prima nos Estados Unidos. Na Europa, Eurásia e Canadá, o trigo e a beterraba são os mais usados, embora no Canadá o milho também tenha sido utilizado (Kline *et al.*, 2007). A Tabela 1 mostra as principais matérias-primas utilizadas em vários países. Para produção de bioetanol, de todas essas fontes, cana-de-açúcar e milho são as mais utilizadas em termos absolutos, portanto o foco deste parágrafo é na comparação destes cultivos.

Tabela 1: Principais matérias-primas cultivadas para a produção de bioetanol em vários países (Sánchez et al., 2008)

País	principais matérias-primas
Brasil	cana-de-açúcar
Estados Unidos	milho
Canadá	milho, trigo, cevada
Colômbia	cana-de-açúcar
Espanha	trigo, cevada
França	beterraba, trigo, milho
Suécia	trigo
China	milho, trigo
Índia	cana-de-açúcar
Tailândia	mandioca, cana-de-açúcar, arroz

2.2.b Cana-de-açúcar

As áreas globais que permitem crescimento viável de cana-de-açúcar de clima tropical chuvoso (150 cm por ano, 25-30°C), estimulando a germinação e desenvolvimento com crescimento de até 2,5 cm por dia, inverno seco, estimulando maturação e acúmulo de sacarose, são limitadas à América do Sul e América Central, Austrália, Índia e China, e várias regiões da África, como ilustrado na Figura 3 a partir de disponibilidade estimada de bagaço. Nos Estados Unidos, a cana somente cresce em regiões do sul como Luisiana e Flórida. A duração da safra depende da localização geográfica e pode demorar de 10 a 24 meses. (Legendre *et al.*, 1995, World Energy Council 2010).

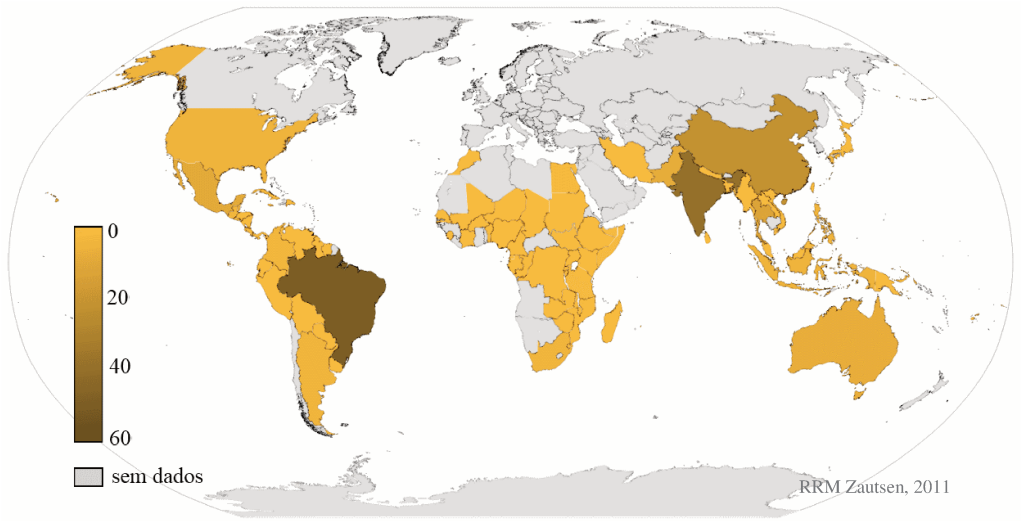


Figura 3: Potencial estimado de bagaço disponível em 2008 (em milhões toneladas em base seca; mapa gerado com dados fornecidos por World Energy Council 2010).

2.2.c Milho

O milho, também uma planta de origem tropical, foi adaptado ao clima moderado, com menores temperaturas e outros padrões de chuva, ao longo do tempo. Este desenvolvimento e o acúmulo de conhecimentos importantes continuam (Sacks *et al.*, 2011), possibilitando seu cultivo viável até no Canadá. Atualmente os Estados Unidos, o maior produtor de bioetanol, gera a maioria do seu etanol a partir de milho (Méjean *et al.*, 2010), seja com diversos estímulos governamentais, como isenção de impostos para misturas de bioetanol com gasolina, acumulando US$ 3 bilhões em 2007 (EIA, 2008), e gerando motivadas discussões sobre prejuízos para o mercado nacional de cultivos de alimentos, possibilitando ironicamente que preços mais altos do petróleo tenham impacto nos preços dos alimentos (Martin *et al.*, 2010).

2.2.d Uma comparação

Em termos de energia, é comum se obter em torno de 355 GJ a partir de cana ou somente 260 GJ a partir de milho, por hectare e por ano. A densidade energética de cana-de-açúcar não é muito diferente do milho, estimada respectivamente em 17,5 e 16,5 GJ/tonelada seca (Mejean et. al., 2010). Portanto, a diferença no rendimento em tonelada por hectare é significativa, como mostra a Tabela 2. A quantidade de massa seca que pode ser colhida, em condições ideais, por hectare de cana-de-açúcar é 67,8 comparado com 27,1 para o milho.

Tabela 2: Rendimentos por hectare para milho e cana-de-açúcar (Mejean et.al., 2010)

	massa seca (tonelada/hectare)		**energia (GJ/hectare)**	
	atual	*máxima*	*atual*	*máxima*
milho	15,1	27,1	258	464
cana de açúcar	20,3	67,8	355	1188

Observa-se que estes números representam valores calóricos da planta inteira, e não necessariamente valores que refletem o potencial em termos de produção de volumes do produto, etanol. Nem toda energia capturada na planta pode ser transformada em produto, uma vez que a fermentação é conduzida somente em base aos substratos consumíveis pelo micro-organismo. Por isto é importante entender a composição da planta. A Figura 4 mostra a comparação da composição de cana-de-açúcar e do milho. De todas estas substâncias, o fermento pode ser diretamente alimentado somente com açúcares sacarose, glicose e frutose, ou glicose obtido de amido através de um processo de hidrólise.

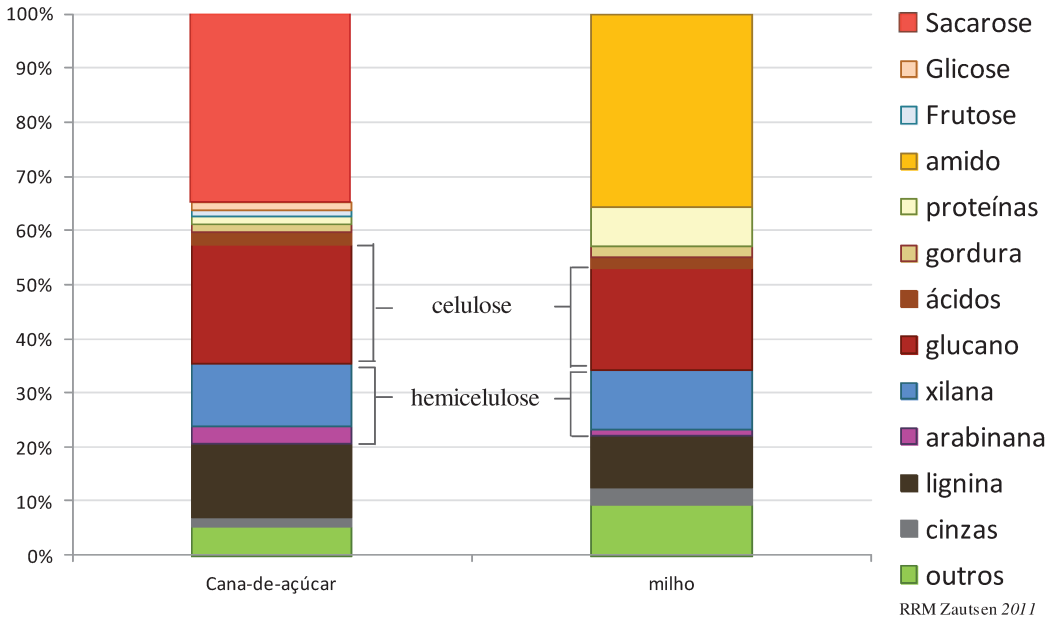


Figura 4: Comparação de composição de massa seca de cana-de-açúcar e milho (fontes: Aguilar et al., 2002, Mantelatto 2005, Belyea et al., 2004, Lee et al., 2007, Cordova 1998)

2.2.e Processo de preparo de cana-de-açúcar

A cana permite, por meio de processo com força mecânica (moagem), obter-se um suco (caldo misto) contendo sacarose (~15%), glicose (~0,6%) e frutose (~0,6%). Este caldo turvo contém impurezas suspensas que prejudicam o desempenho da fermentação e complicam os processos de separação (*downstream*). Em sistema de fermentação contínua, por exemplo, muitas destas impurezas são dificilmente retiradas do meio por centrifugação,

acumulam no reciclo e constituem um veículo de bactérias contaminantes, que prejudicam a fermentação, promovendo floculação, diminuindo o pH, consumindo substrato ou produto e ainda gerando metabólitos inibidores para o fermento (Nolasco-Junior 2005, Rossell 2006). Problemas no processo *downstream* inclui a formação de incrustações nas superfícies do equipamento necessário para aquecimento de mosto (Rossell 2006). Portanto, é necessário conduzir o caldo a uma série de tratamentos (como peneiramento, evaporação, decantação, esterilização), antes de introduzir os açúcares na dorna (Mantellato 2005). Na indústria sucro-alcooleira, a produção de etanol é integrada com a produção de açúcar, que gera melaço (mel), um fluxo com alto teor de açúcar (38,5% sacarose, 3,18% glicose e 4,54% frutose) (Zautsen 2004). Por ter uma qualidade inferior para produção de açúcar, este melaço é usado para produção de etanol e misturado com o caldo tratado (xarope), assim formando o mosto final a ser alimentado à dorna da fermentação. A Figura 5 mostra um diagrama esquemático deste processo de tratamento.

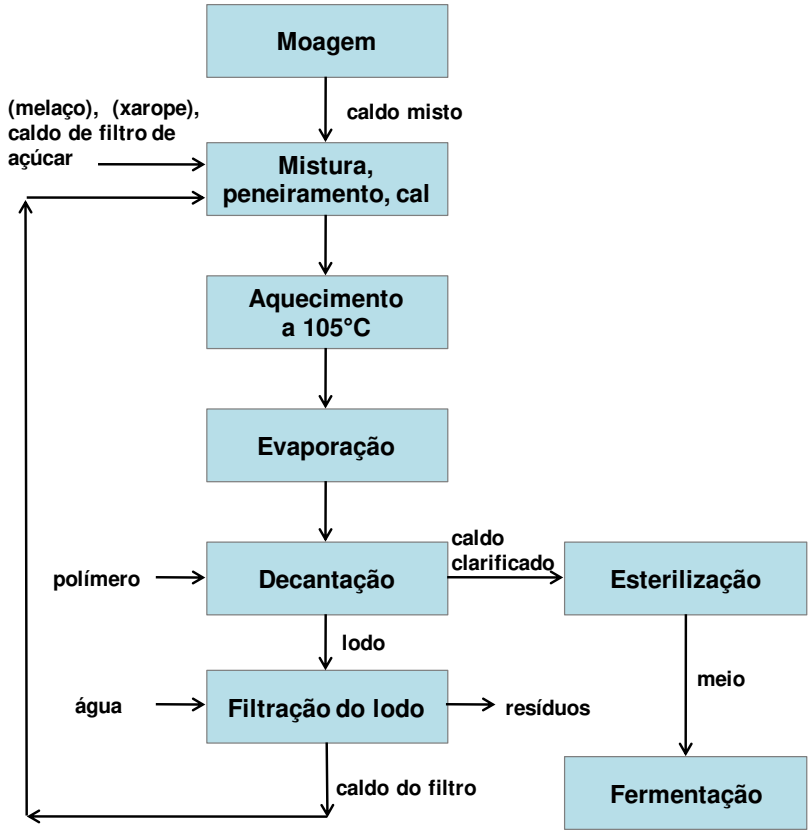


Figura 5: Diagrama de fluxo simplificado do processo de tratamento de caldo misto (Rossell, 2006, Nolasco, 2005)

2.2.f Processo de preparo de milho

No caso do milho, o amido é utilizado como fonte de carbono para a fermentação. Existem dois processos de pré-tratamento para recuperação e purificação de amido (e recuperação de subprodutos): moagem a seco ('dry milling') e moagem úmida ('wet milling'). Com moagem a seco, o milho é moído em partículas de composição heterogênea, contendo, além de amido, glútem, farelo de glúten e germe. Os últimos não são separados e ficam, em sua maioria, inclusos no processo de purificação. No processo de moagem úmida, no entanto, estes compostos são recuperados (Raminez *et al.*, 2008). Glútem e germe são usados na indústria de alimentos ou como forragem animal. Farelo de glútem também, e tem, além disto, valor agregado como fertilizante, supressor de plantas daninhas (Liu *et al.*, 1996) ou na produção de plásticos biodegradáveis (Corradini *et al.*, 2011).

O processo de moagem úmida é mais caro do que moagem a seco, porém a recuperação destes subprodutos justifica sua aplicação. De qualquer modo, para gerar glicose de amido, como substrato da fermentação, o fluxo resultante é sujeito a um processo de hidrólise em dois passos: liquefação e sacarificação. A sacarificação foi tradicionalmente conduzida por hidrólise ácida, mas o uso de enzimas amilases é mais comum, devido a vantagens em especificidade, condições leves de reação e a ausência de reações secundárias da hidrólise enzimática (Sanchez *et al.*, 2008). As duas principais enzimas nestes processos são α-amilase para liquefação e glicoamilase para hidrólise (Veen *et al.*, 2006). A

Figura 6 mostra uma esquematização deste processo de moagem úmida.

Há também tecnologias em desenvolvimento para fermentação e sacarificação simultânea (Kroumov *et al.*, 2006, Yamada *et al.*, 2009), que mostram a possibilidade da fermentação direta de amido, utilizando α-amilase e glicoamilase produzidas pelo próprio fermento geneticamente modificado.

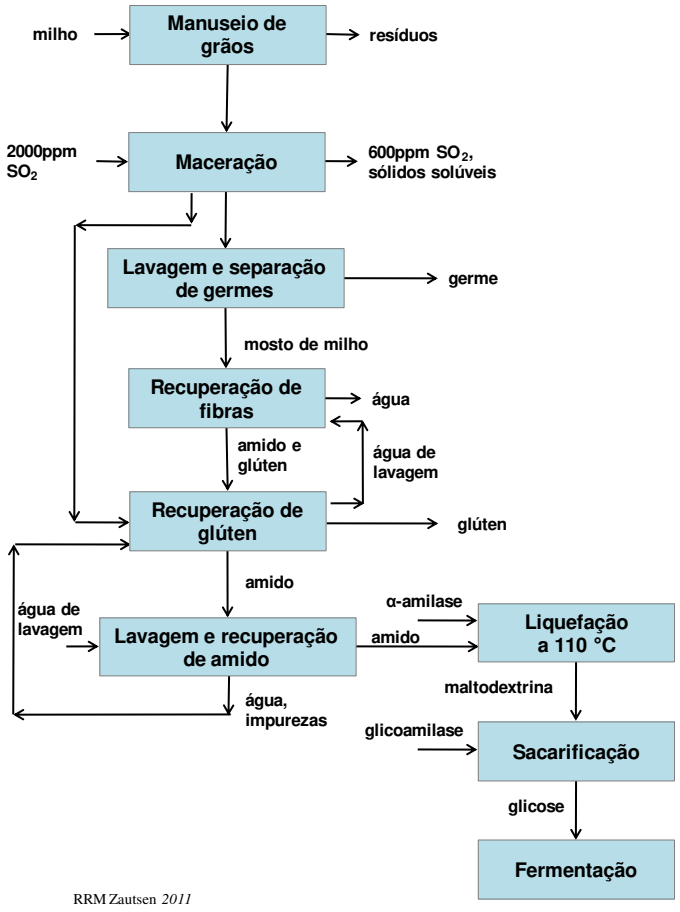


Figura 6: Diagrama de fluxo simplificado do processo de preparo de glicose a partir de amido de milho com moagem úmida (Raminez et al., 2008 e Veen et al., 2006)

2.3 *Lignocelulose como* matéria-prima

2.3.a Fontes

Uma interessante observação a ser feita na comparação de composição de cana-de-açúcar e milho (Figura 4 do item 2.2.d) é que as proporções de celulose (glucano), hemicelulose (arabinana e xilana) e ácidos são muito parecidas. Proporções semelhantes podem ser encontradas em outras plantas, como, por exemplo, *switchgrass* (Lee *et al.*,

2007). Estes componentes têm sido avaliados como fontes de carbono para a fermentação com interesse crescente: como o amido, estes componentes também podem ser convertidos para seus respectivos monômeros sacarinos. A Figura 7 mostra vários exemplos de cultivos populares de fontes de material lignocelulósico como a própria cana-de-açúcar, milho e cultivos herbáceos como *Miscanthus*, *switchgrass*, sorgo e sorgo sacarino. Em termos de biomassa, os rendimentos de várias fontes herbáceas são muito semelhantes à cana-de-açúcar ou até superiores. Por exemplo, um estudo por Vasilakoglou *et al.* (2011) revelou rendimentos de até 37,4 ton/ha massa seca com um cultivo de sorgo sacarino. Outro relato interessante com relação a estas fontes herbáceas foi feito por Fenske *et al.* (1998), que relatam que as concentrações de inibidores fenólicos da fermentação, gerados no hidrolisado desta matéria-prima, são relativamente baixas, fato que permite sua fermentação.

Figura 7: Diversas fontes de matérial lignocelulosico: a) cana-de-açúcar, b) milho, c) sorgo sacarino, d) Miscanthus, e) sorgo, f) switchgrass. (fontes imagens: a) elaboração própria, b) http://www.gettyimages.pt/detail/82877391/Stone#, c) (http://www.icrisat.org/what-we-do/SASA/sasaindex.htm, d) http://www.ceres.net/products/Products-Miscanthus.html, e) http://images.businessweek.com/ss/08/10/1007_green_tech/13.htm, f) (http://www.jgi.doe.gov/sequencing/why/50008.html)

2.3.b Composição

A lignocelulose é a composição tri-dimensional da celulose, hemicelulose e lignina, formando o material estrutural da planta, seja de cana, milho ou outras plantas e é convencionalmente visto como subproduto (bagaço) da produção de açúcar e álcool. A Figura 8 mostra a estrutura lignocelulósica no seu contexto da planta; a parede celular de células estruturais da planta contém macro-fibrilas, um conjunto estrutural de micro-fibrilas compostos de celulose e hemicelulose interligado por lignina. Da massa seca total de cana-de-açúcar, aproximadamente 22% é celulose e 15% é hemicelulose (deduzido de dados de Cordova *et al.*, 1998, Aguilar *et al.*, 2002), como do milho 19% é celulose e 12% é hemicelulose (deduzido de dados de Lee *et al.*, 2007). Juntos, celulose e hemicelulose são denominadas 'holocelulose' (Cândida Rabelo, 2010).

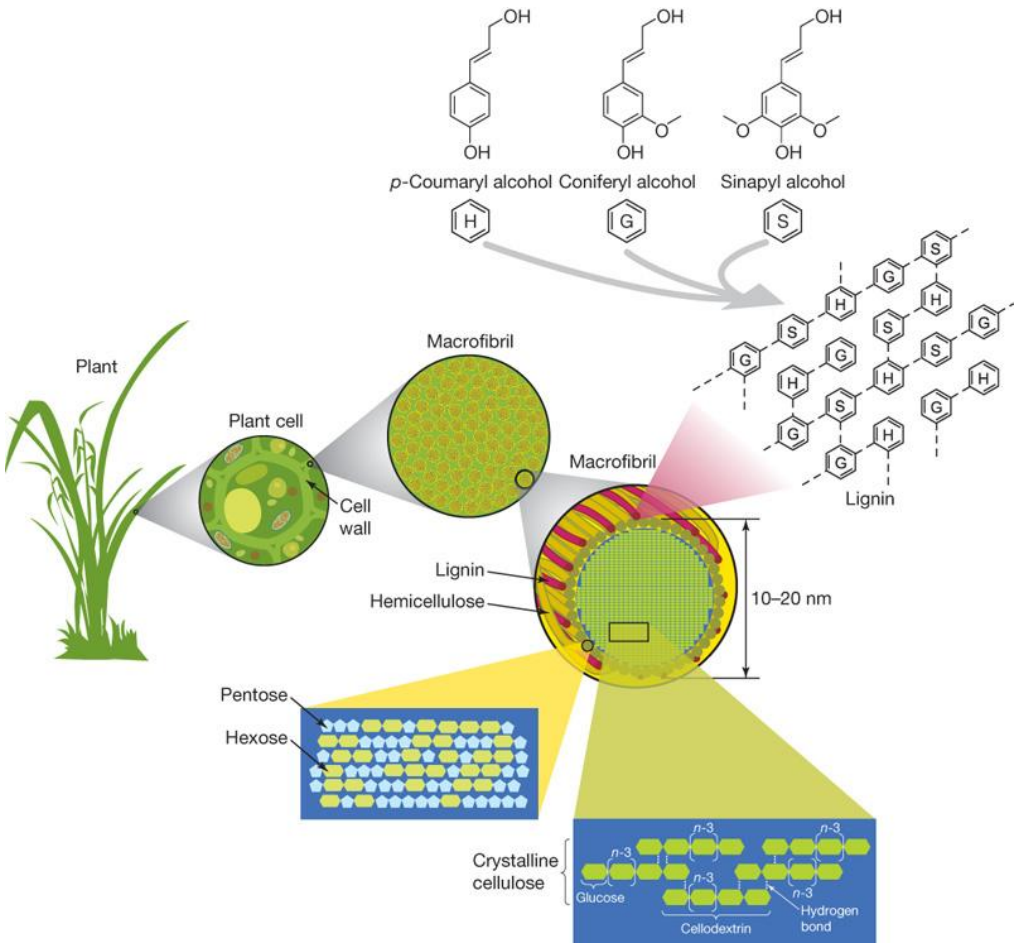


Figura 8: Estrutura da lignocelulose (reproduzida de Rubin, 2008)

Lignina

A lignina (8,5 a 14,2 % da massa seca total da planta, segundo Lee *et al.*, 2007) é uma estrutura polifenólica, composta de unidades fenilpropanóides derivadas de p-hidroxicinamil-álcoois, como p-coumaril álcool, coniferil-álcool e sinapil álcool (Martinez *et al.*, 2005, Doherty *et al.*, 2011, Rubin, 2008). Na planta, a matriz de lignina e hemicelulose em volta da celulose funcionam como um cimento entre as micro-fibrilas, aumentando a resistência mecânica e micro-biológica da mesma (Martínez *et al.* 2005). A lignina não tem valor como substrato para um processo fermentativo, pois seus componentes e produtos de sua degradação, durante o processo de pré-tratamento ou hidrólise, têm propriedades tóxicas para o micro-organismo (Olsson 1996). Porém, lignina tem um valor energético significante e sua queima se torna interessante para geração de energia calórica com fins de prover vapor e eletricidade à planta. A Tabela 3 resume outras aplicações para a lignina e/ou seus derivados que têm, potencialmente, um valor econômico ainda maior.

Tabela 3: Aplicações para a lignina e seus derivados (resumido de Doherty et al., 2011 e Ogeda et al., 2010)

baixo valor	**valor agregado**	**misturas com**
aditivo de concreto	baunilha	proteina
componente rígido na pastilha de forragem animal	pesticidas	amido
processamento de minérios metálicos	dispersante para carvão	polihidroxialcanoates
lamas para perfuração de poço de petróleo	corantes e pigmentos	ácido polilático e -poliglicólico
controle de poeira	placa de gipsita	resinas de epóxi
fonte de fenol e etileno	tratamento de água	resinas de fenol-formaldeido
	inibidores de escala	poliolefinas
	detergentes industriais	polímero de vinil
	emulsificantes	poliéster
	matriz para fertilizadores	poliuretano
	preservação de madeira	borracha
	expansores de bateria	copolímeros de enxerto
	quelantes especiais	
	tijolos refratários e materiais cerâmicos	
	agentes de retenção na fabricação de papel	
	fibras de carbono	

Celulose

A celulose, ou glucano, é um polissacarídeo linear e cristalino de celobiose, um dímero da glicose, unidos através de ligações β-1,4 (Rubin 2008, Ogeda *et al.*, 2010). Pontes de hidrogênio entre as moléculas de glicose fortalecem a estrutura do polímero como demonstrado na Figura 9.

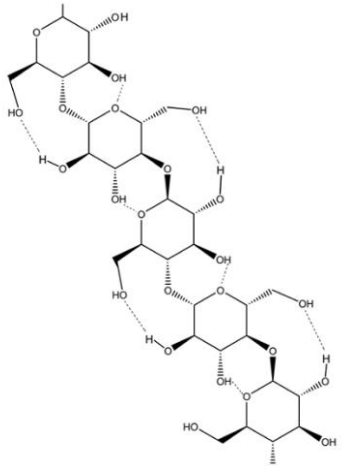


Figura 9: Estrutura da celulose, polímero de dímeros de glicose (Ogeda et al., 2010)

Hemicelulose

A hemicelulose é um composto de polímeros de vários açúcares, de 5 ou 6 carbonos. A xilose é o monômero principal, mas encontram-se também quantidades de arabinose, galactose, glicose e manose (Rubin 2008) além de ácido glucurônico, ácidos galacturonicos e ácido manurônico (Ogeda *et al.*, 2010, Gírio *et al.*, 2010). A estrutura, por exemplo, da xilana é linear com resíduos de d-xilopiranose ligados através de ligações -1,4, que podem ser substituídos com por resíduos acetílico, arabinosílico e glicuronosílico (Spiridon *et al.*, 2008).

2.3.c Vantagens de utilização de biomassa lignocelulósica

Tendo em vista que a celulose e a hemicelulose são basicamente compostas de açúcares, é possível recuperar estes açúcares para utilização como substrato da fermentação. Definimos aqui, 'material útil' da cana-de-açúcar ou milho como a fração total de compostos que são conversíveis em produto, ou seja, sacarose, frutose, glicose, amido, celulose e hemicelulose; deste material útil, celulose e hemicelulose representam em torno de 49,6% (calculado com dados de Lee *et al.*, 2007). Para a produção de certa quantidade fixa de produto, como etanol, por exemplo, isto significa uma redução de metade da área agrícola plantada. A Figura 10 demonstra a conseqüência que tal redução traria, por exemplo, na distância necessária para o transporte da cana, um fator importante no cálculo de custos variáveis da produção. A redução de 50% da área reduz a distância média em 27,1%. A distância total para os caminhões percorrerem, que é uma função linear da distância média multiplicada pela metade da área, é consequentemente reduzida em 63,6%.

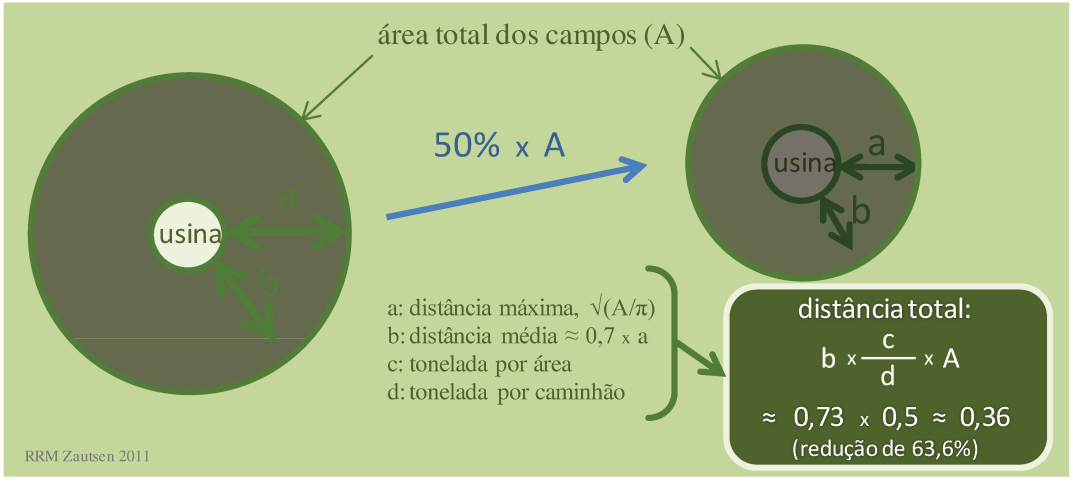


Figura 10: Redução da distância máxima (a) e média (b), dos campos até a usina, a ser percorrida para entregar matéria-prima á usina: 29,3 e 27,1%. A distância total para os caminhões percorrerem é conseqüentemente reduzida em 63,6%.

Além disso, para o transporte da cana para a usina e da vinhaça para os campos, com fins de fertilização, existem outras consequências significantes para os custos envolvidos

como: preparação do campo, rega e drenagem, colheita, e, na usina, custos envolvidos com o tratamento da cana e o caldo misto, como extração, aquecimento e clarificação, filtração e esterilização.

Um fator importante para melhorar o desempenho da fermentação convencional é a qualidade da cana geralmente estimada pelo tempo entre queima ou corte e processamento da mesma (Rossell 2006). Embora o método para estimativa da qualidade ainda esteja sujeito a melhorias e padronização, deduz-se diretamente a importância de que o período a partir do momento da colheita até a utilização da cana esteja menor possível. Sendo que período é reduzido praticamente linearmente com a redução da distância média entre os campos e a usina, é possível obter uma melhoria da qualidade da cana no momento da entrega e consequentemente do desempenho da fermentação.

Outra vantagem da utilização de material lignocelulósico como o bagaço de cana é a recuperação do açúcar do bagaço, que, caso contrário, seria perdido pela falta de tecnologia viável de extração do mesmo: foi estimado que 23,6% das perdas totais do açúcar no processo de produção de etanol convencional são devidos às perdas no bagaço (Finguerut, 2006).

Para se ter uma melhor concepção do potencial de utilização de material lignocelulósico, é importante lembrar que sua fonte não se limita ao bagaço de cana. A Tabela 4 resume diversas fontes comuns do material.

Tabela 4: Diversas fontes comuns de material lignocelulósico e celulose (Sanchez et al., 2008, completado)

resíduos agrícolas	**biomassa herbácea**	**madeira**	**resíduos de celulose**
bagaço de cana	alfafa	choupo	papel de jornal
palha de milho	switchgrass	salgueiro	papel resídual de escritório
palha de trigo	Capim-amarelo	pinheiro	lodo de papel reciclado
palha de arroz	grama Timóteo	abetos	lodo de filtração de xarope
casca de arroz	Cynodon	eucalipto	resíduos sólidos urbanos
palha de cevada	Sorgo (Sacarino)		
caroço	Miscanthus		
polpa			

Com relação ao uso de resíduos de diversas culturas agrícolas, biomassa herbácea e madeira, nota-se que seus cultivos rotativos no mesmo terreno agrícola em combinação com o plantio direto traz vantagens importantes em termos de, entre outros, menor uso de água e herbicidas, maior rendimento, menor perda de nutrientes e menor erosão do campo (Chauvel *et al.*, 2001, Kelley *et al.*, 2003).

2.4 Hidrólise de biomassa lignocelulósica e geração de açúcares e inibidores

A lignocelulose pode ser fermentada por uma mistura de micro-organismos, porém, o rendimento ainda é muito baixo (ex. 0,11 g g^{-1}), com taxa de produção de 16 mg $L^{-1}h^{-1}$ (Lin *et al.*, 2011). Para se obter um processo bioquímico mais viável, é necessário quebrar ligações covalentes na lignina e hemicelulose, desfazer as estruturas cristalinas da celulose e quebrar os polissacarídeos, gerando monômeros de açúcar que podem ser convertidos em produto com maior facilidade pelo micro-organismo.

A hidrólise de material lignocelulósico é feita por diferentes clivagens para geração de glicose a partir de glucano (celulose) e xilose, arabinose e outros açúcares através de xilana e arabinana e outros polímeros que formam a hemicelulose. A fermentação destes açúcares pode ser obtida por leveduras adaptadas para conversão de pentoses, como demonstrado por Martin *et al.* (2007). A maioria das tecnologias de hidrólise envolve uma etapa de pré-tratamento ou pré-hidrólise para quebrar a estrutura densa da lignocelulose. Nesta etapa a hemicelulose é liquefeita gerando uma mistura de mono- e oligossacarídeos, e a lignina e hemicelulose podem ser removidas (Sun *et al.*, 2002). O processo facilita a segunda etapa, a própria hidrólise (Olsson *et al.*, 1996, Olivério *et al.*, 2005), que pode ocorrer com tratamento ácido ou através de reação enzimática. Também é possível hidrolisar bagaço/palha moída em uma só etapa, utilizando, por exemplo, ácido clorídrico concentrado (Herrera *et al.*, 2004) ou um processo *organosolv* com ácido sulfúrico e uma solução aquosa de solvente como etanol (Olivério *et al.*, 2005).

2.4.a Pré-tratamento

O pré-tratamento é a operação mais importante para o processo de hidrólise enzimática. A estrutura cristalina da celulose e o empacotamento da mesma em estruturas densas de hemicelulose e lignina faz com que a celulose seja praticamente impermeável às enzimas. A lignina é parcialmente ligada à hemicelulose através de ligações covalentes, impedindo o acesso destas enzimas hidrolíticas à celulose (Cardona *et al.* 2010). Mesmo com a celulose exposta, a estrutura cristalina da celulose é o fator de barreira mais importante para a conversão eficiente da mesma (Szjiarto *et al.*, 2008). É por isso que processos biológicos de conversão ou pré-tratamento de lignocelulose, que têm benefícios energéticos e condições de processos mais leves, são tão demorados (Yu *et al.*, 2009). Portanto, é importante aplicar um pré-tratamento para quebrar muitas destas ligações covalentes entre lignina, hemicelulose e celulose, remover a lignina da hemicelulose, introduzir e aumentar a porosidade do material e reduzir a cristalinidade de celulose. Assim, numa etapa posterior, as enzimas terão acesso direto a uma maior superfície de celulose (Cardona *et al.* 2010). Isto também vale para a hidrólise ácida: o pré-tratamento facilita a hidrólise pela maior porosidade, maior superfície de contato e quebra de ligações covalentes entre a lignina, hemicelulose e celulose (Olivério *et al.*, 2005). A Tabela 5 mostra vários métodos de pré-tratamento que têm sido desenvolvidos.

Tabela 5: Métodos aplicados para pré-tratamento de lignocelulose (Olsson et al., 1996, Holzapple et al., 1991, Cardona et al., 2010, Sanchez et al., 2008, Gírio et al., 2010)

físico	químico	biológico
hidrotérmico, explosão a vapor	ácido diluido ou concentrado (dióxido de enxofre ou ácido clorídico, fosfórico, peracético, nítrico ou sulfúrico)	fungos de podridão branca (*Basidiomycota*): *Pleurotus sajor-caju, Penicillium echinulatu, Phanerochaete chrysosporium, Phanerochaete sordida, Ceriporia sp.*
estilhaçamento, trituração, moagem	tratamento alcalino (hidróxido de sódio, amônia)	fungos marinhos: *Phlebia sp*
explosão com amônia pressurisada (congelada)	solventes orgânicos, organosolv (metanol, etanol, etileno glicol, trietileno glicol, acetona)	enzimático: celulase e hemicelulase; fermentação em estado sólido
água quente	oxidação úmida, T > 120 °C	bio-organosolv; *Ceriporiopsis subvermispora* seguido por etanolise
fluidos supercríticos, p. ex. explosão com dióxido de carbono	ácido sulfúrico diluido com aquecimento por micro-ondas	
líquidos iônicos	ozonólise (pressão e temperatura ambiental)	
	deslignificação oxidativa, peroxidase	
	líquidos iônicos	

Atualmente, o tratamento com ácido ou explosão a vapor recebem o maior foco industrial, devido a altos rendimentos de açúcar (Cardona *et al.*, 2010) e a relativa facilidade de implantação, eficiência energética, rapidez e viabilidade econômica em processo industrial (Leibbrandt *et al.*, 2011, Olivério *et al.*, 2005).

Um pré-tratamento ácido, convencionalmente com ácido sulfúrico na concentração entre 0,5-1,5% e temperaturas entre 121 e 160 °C, despolimeriza e quebra a estrutura da lignocelulose e ligações covalentes entre lignina e hemicelulose. O tratamento promove a exposição de hemicelulose e celulose à ação protônica (Olivério *et al.*, 2005) ou ação enzimática, na etapa seguinte de hidrólise. O processo é eficaz e rápido, com baixos custos de investimento e resulta em alto rendimento de recuperação de celuloses e hemicelulose (maior que 90%), mas a formação de produtos de degradação é o maior problema associado a esta técnica, devido ao poder inibidor destes componentes, além de problemas com corrosão e a utilização de grandes quantidades de ácido, necessitando uma etapa de neutralização antes da fermentação (Gírio *et al.*, 2010, Domínguez, 2003). O pré-tratamento com ácidos orgânicos, como ácido maleico, é mais específico para celulose e requer menor temperatura ou maior pH para efeito semelhante ao do ácido sulfúrico, resultando em maior eficiência em menor produção de componentes inibidores (Lee e Jeffries, 2011).

A explosão a vapor funciona com vapor saturado a 160-290 °C e 0,7- 4,9 MPa durante alguns segundos ou minutos, seguido por descompressão rápida até a pressão atmosférica. Desta maneira, com o líquido acumulado e pressurizado dentro das fibras evapora em fração de segundos, ocorre um desarranjo e ruptura das fibras de lignocelulose, paralelamente à despolimerização e ruptura de ligações intra-moleculares por auto-hidrólise, devido ao calor e à formação de ácido acético, proveniente de resíduos acetil da hemicelulose (Domínguez, 2003). O processo é capaz de tratar grandes quantidades de sólidos com alta eficiência de hidrólise de hemicelulose, menor consumo de energia comparada, por exemplo, com a moagem. As desvantagens são: baixo rendimento de xilose (45-65%), formação de inibidores e distribuição de lignina, dificultando sua remoção (Sanchez *et al.*, 2008, Ramos *et al.*, 2003, Domínguez 2003).

A Tabela 6 mostra uma comparação das várias técnicas de pré-tratamento (Gírio *et al.* 2010). Outras técnicas muito promissoras neste ramo são: processo *organosolv* alcalino (Koo *et al.*, 2011), oxidação úmida (Klinke 2002) e ozonólise (García-Cubero *et al.*, 2009).

Tabela 6: Vantagens e desvantagens de várias técnicas de pré-tratamento, segundo Gírio et al. (2010)

Características desejáveis	Ácido concentrado	Ácido diluído	Explosão a vapor	Auto-hidrólise	Organosolv	Sólidos super-ácidos	Alcalino	Líquidos iônicos	Fluidos supercríticos
alta solubilização de hemicelulose	++	++	++	++	+	+	+	++	+
alta produção de monossacarídeos	++	++	0	-	-/0	+	-/0	0/+	-
baixa produção de oligossacarídeos	+	+	0	-	-/0	+	-/0	0/+	-
alto rendimento de celulose	++	++	++	++	-	++	+	+	++
digestibilidade alta de celulose	++	++	+	+	-	+	++	+	0/+
qualidade alta de lignina	-	-	0/+	+	+	-/0	-	+	+
reciclo de químicos de processo	-	-	0	n.r.	-	+	-/0/+	+	n.r./+
formação baixa de inibidores	-	-	0	0	+	0	+	-	0
poucos problemas de corossão	-	-	0	0	-	0	-	-/0	-/0
baixa dependência de químicos	-	-	0	++	-	0/+	-/0	+	++
baixa necessidade de neutralização	-	-	0	n.r.	+	-/0	-/0	0	n.r.
baixos custos de investimento	+	+	-	+	0	0	0/+	+	-
baixos custos de operação	-	0	++	+	-	0	-/0	-	-
uso baixo de energia	0	-	0	0	0	+	+	++	+

+: vantagem, -: desvantagem, 0: neutro, n.r.: não-relevante.

2.4.b Hidrólise enzimática

Para cada polímero de hexoses ou pentoses existem várias enzimas necessárias para sua conversão em seus respectivos monômeros. Assim, celulose é convertida com celulases: endoglicanases, exoglicanases, glicosidases e celodextrinases. A conversão de hemicelulose é conduzida por hemicelulases: xilanases, xilosidases, glicuronidases, endoarabinases, α-L-arabinofuranosidases, esterases, manases, manosidases e outras (Yeoman *et al.*, 2010). O mecanismo de conversão de cada polímero é bastante complexo e envolve uma série de processos que devem ocorrer em certa sequência. Por exemplo, a conversão de celulose envolve um componente 'CBM' (cellulose binding molecule), que, uma vez ligado à superfície do polímero de celulose, tem a função de concentrar e envolver enzimas glicosil-hidrolases para hidrólise e/ou desprendimento de uma única cadeia celulose da estrutura cristalina, disponibilizando-a à área catalítica das enzimas, empregando exo- e endo-glicanases, que, por sua vez, mostram efeitos sinérgicos (Szjiarto *et al.*, 2008). A função de concentração de enzimas por 'CBM' foi também observado em resíduos de lignina (Adsul *et al.*, 2005). Exoglicanases são somente ativas nas pontas

terminais de cadeia celulósica, clivando unidades de glicosil ou celobiosil e produzindo celobiose ou glicose. Endoglicanases, por outro lado, clivam a celulose em pontos arbitrários na cadeia, aumentando o número de cadeias menores e o número de terminais, para serem utilizados pelas exoglicanases. Cadeias pequenas, oligômeros com propriedades mais solúveis liberados pelas endoglicanases, e de preferência dímeros (celobiose), serão convertidos por β-glucosidase (Kent *et al.*, 2007).

No caso de hemicelulose, com mais enzimas envolvidas, o processo é mais complexo. A Tabela 7 mostra uma coleção de enzimas que são utilizadas na hidrólise enzimática de xilana e suas respectivas funções. Para outros polímeros na hemicelulose como arabinana, manana, etc., as mesmas enzimas são empregadas. Para cada enzima de cada sistema são relatadas cada vez mais alternativas e micro-organismos produtores, ampliando a variação em estabilidade, taxas de conversão e especificidade disponíveis para o mercado.

Tabela 7: Enzimas envolvidas na hidrólise de xilana, o principal polímero na hemicelulose (Gírio et al., 2010)

enzima	**função**
endo-1,4-β-xilanase	cliva as ligações glicosídicos na cadeia principal de xilana
β-xilosidase	hidrólise de oligômeros e xilobiose, liberando resíduos de β-D-xilopiranosil da extremidade não-redutor
α-D-glicuronidase	clivando ligações α-1,2 entre resíduos de ácido glicuronico e unidades de β-D-xilopiranosil da cadeia principal
α-L-arabinofuranosidase	remoção de resíduos de L-arabinose ligados nas posições 2 e 3 de β-D-xilopiranosil
Acetil-xilan esterase	remoção de grupos O-acetil nas posições 2 e/ou 3 nos resíduos β-D-xilopiranosil de acetil xilana
ácido ferúlico e ácido p-coumarico - esterases	hidrólise de ligações éster na xilana, liberando ácidos fenólicos ligados a resíduos arabinofuranosídicos

O desenvolvimento de enzimas efetivas e capazes de converter os polímeros de açúcar do material lignocelulósico tem se intensificado (Doherty *et al.*, 2011), e em geral chega-se a resultados muito positivos, com altos rendimentos e baixa produção de

componentes inibidores. Novas enzimas e organismos produtores de enzimas têm sido descobertos, exibindo maior estabilidade térmica e maior tolerância a pH, pré-requisitos importantes para seu uso industrial, onde altas temperaturas de processo reduzem a viscosidade da solução de substrato, aumentando a taxa de conversão, com maior eficiência energética e menor risco de contaminação microbiológica (Szijárto *et al.*, 2008).

Permanecem como desafios importantes: a baixa taxa volumétrica da hidrólise, os altos custos associados à necessidade de quantidades suficientes de enzimas para um processo em larga escala e, se possível, a recuperação das mesmas.

A velocidade reduzida do processo, em comparação com hidrólise ácida, requer grandes volumes de reatores e altos custos de investimento em equipamento (Olivério *et al.*, 2005). Para maximizar o desempenho das enzimas e minimizar o tempo necessário para a hidrólise é indispensável apresentar a maior superfície possível de hemicelulose e celulose às enzimas, ou seja, o ideal é que o processo de pré-tratamento resulte em partículas extremamente pequenas, porosas e sem estrutura cristalina da celulose ou ligações covalentes entre lignina, hemicelulose e celulose, pois podem atrapalhar a ação das enzimas nas ligações entre os monômeros sacarinos (Gírio *et al.*, 2010). Tal requisito em larga escala, com recuperação máxima de hemicelulose e celulose, e com a mínima presença de componentes inibidores, é outro fator oneroso ao processo.

As enzimas industriais e os conjuntos complexos das mesmas, entram em fase de mercado somente após um período longo de pesquisa avançada e especializada, preferencialmente com misturas de enzimas otimizadas para cada (tipo de) matéria-prima. Apesar de grandes volumes necessários para o processo hidrolítico, a requisição destas enzimas em termos de compra e importação, torna o custo alto por tonelada de material lignocelulósico processado, sendo que a produção das mesmas dificilmente é permitida na própria planta do produtor de etanol, devido à cautela dos produtores das enzimas pela segurança de direitos intelectuais. Contudo, a possibilidade de unir os processos de produção de enzimas, hidrólise e fermentação (bioprocessamento consolidado) pode se tornar uma abordagem interessante neste ramo (Lynd *et al.*, 2005).

2.4.c Hidrólise catalisada por ácido

A maior diferença entre pré-tratamento ácido e a hidrólise catalisada por ácido é a elevada temperatura empregada no processo de hidrólise (Lee *et al.*, 2011) e maior concentração de ácido (Ogeda *et al.*, 2010).

A hemicelulose tem maior sensibilidade para hidrólise ácida do que a celulose. Para evitar a degradação dos seus monômeros como xilose e galactose, que diminuiria o rendimento final da hidrólise e introduziria compostos inibidores para a fermentação *a posteriori*, é necessário empregar duas etapas: pré-tratamento (hidrólise de hemicelulose) e hidrólise de celulose (Ogeda *et al.*, 2010, Domínguez 2003). Durante o pré-tratamento, a hidrólise de hemicelulose é completada utilizando ácido diluído (até 1%) e temperatura menor (160 - 190 °C). Os produtos desta hidrólise são removidos do meio, junto com a lignina. Na segunda etapa, a celulose é hidrolisada em condições mais severas com temperaturas entre 200 e 230 °C e ácido mais concentrado até 2,5% (Iranmahboob *et al.*, 2002, Domínguez 2003, Ogeda *et al.*, 2010).

A Figura 11 mostra o mecanismo de hidrólise da celulose. O ácido funciona meramente como um catalisador, sendo que o processo ocorreria também espontaneamente em água, mas com uma velocidade inviável. O ácido protonisa a ligação covalente entre os monômeros diretamente no átomo de oxigênio (a), resultando em um grupo hidroxílico, para um monômero, e quebra de ligação C-O para o outro monômero (b). Este último monômero, agora com carbono estabilizado pelo par de elétrons do oxigênio adjacente, acaba ganhando um grupo hidroxílico através de ataque nucleofílico da água (c, d), liberando o próton (e) possibilitando a catalisação da próxima reação.

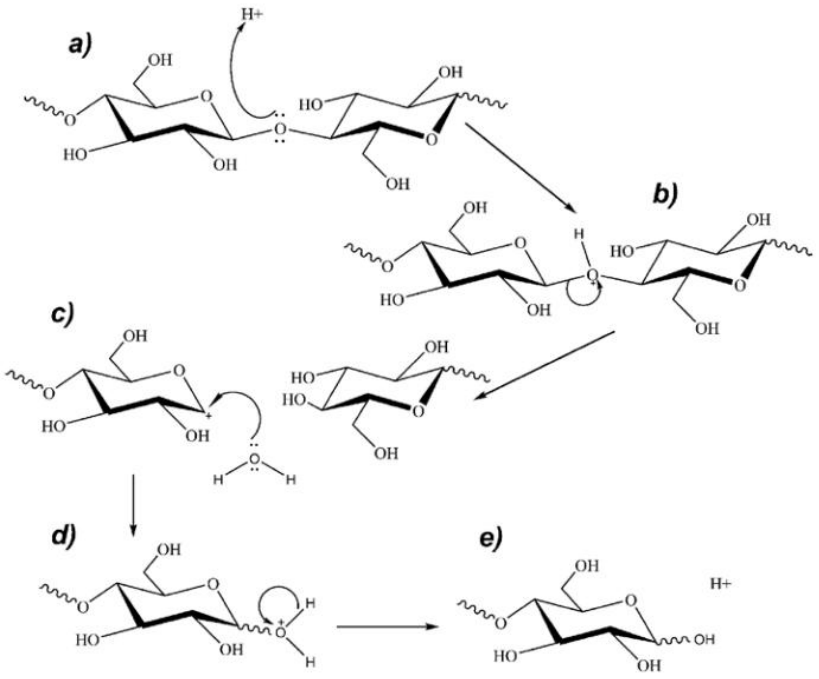


Figura 11: Mecanismo de hidrólise da celulose catalise ácida (Ogeda et al., 2010)

Maiores concentrações de ácido possibilitam altos rendimentos e baixas temperaturas do processo. Iranmahboob et al (2001), por exemplo, usaram 26% m/m de ácido sulfúrico consegundo rendimentos de até 82% do valor teórico em 2 horas de tratamento. No entanto, a utilização destas quantidades de ácido, a necessidade de neutralização alcalina antes da etapa de fermentação (Gírio *et al.*, 2010), formação de gipsita a partir desta neutralização alcalina (Mosier *et al.*, 2005) e problemas de corrosão, toxicidade e perigos inerentes (Sun *et al.*, 2002) são fatores adversos, o que favorece processos com concentrações mais diluídas de ácido. Assim, com ácido a 1%, temperaturas e pressões mais elevadas, com limites físicos e operacionais (Almeida *et al.*, 2009), é possível executar a hidrólise com tempos de residência muito curtos (na faixa dos segundos). Porém, a temperatura alta nesta etapa de hidrólise ácida ainda provoca corrosão de equipamentos, a degradação de açúcares e formação de inibidores, diminuindo o rendimento de glicose (Domínguez 2003, Iranmahboob *et al.*, 2002), muito embora o curto tempo de residência nesta faixa de temperatura favoreça mais a hidrólise do que a degradação de açúcar (Almeida *et al.*, 2009). Não é necessário descrever maiores detalhes para entender que a melhor escolha

deste processo não é trivial e, apesar de mais de cem anos de pesquisas nesta área, existem ainda opiniões bastante controversas.

2.4.d Combinação de pré-tratamento organosolv e hidrólise ácida

É possível combinar pré-tratamento organosolv e hidrólise em uma só etapa. Neste caso, a hidrólise catalisada por ácido conta com a remoção de lignina e hemicelulose por solvente orgânico. A ação do solvente, sob alta temperatura, possibilita a remoção de lignina e hemicelulose da celulose em tempo curto, dando acesso imediato a uma grande superfície da hemicelulose da celulose para ação do ácido empregado. Devido à alta temperatura, o ácido pode ser bastante diluído sem diminuir desempenho da hidrólise, eliminando a necessidade de grandes quantidades de substâncias alcalinas neutralizantes após do processo (Olivério *et al.*, 2005, Ogeda *et al.*, 2010) e evitando a formação de gipsita a partir desta neutralização alcalina (Mosier *et al.*, 2005).

O processo tem sido desenvolvido em escala de planta piloto. Um exemplo brasileiro é o processo DHR (Dedini Hidrólise Rápida), em Pirassununga-SP (usina São Luiz), gerando caldo hidrolítico a partir de bagaço por meio de hidrólise ácida sob alta pressão e utilização de ácido sulfúrico de forma diluída e álcool como solvente de lignina e hemicelulose (Olivério *et al.*, 2005). Um sistema de evaporação rápida (flash) após a hidrólise possibilita a interrupção abrupta da hidrólise através de menor pressão e temperatura, evitando a formação desnecessária de componentes inibidores. O processo é integrado com sistema de fermentação alcoólica e destilação com produção de 5000 litros de etanol por dia. Atualmente o rendimento de hidrólise em termos de açúcares redutores totais (ART) está entre 60 e 70% (Olivério *et al.*, 2007). A Figura 12 mostra um diagrama simplificado de fluxo deste processo.

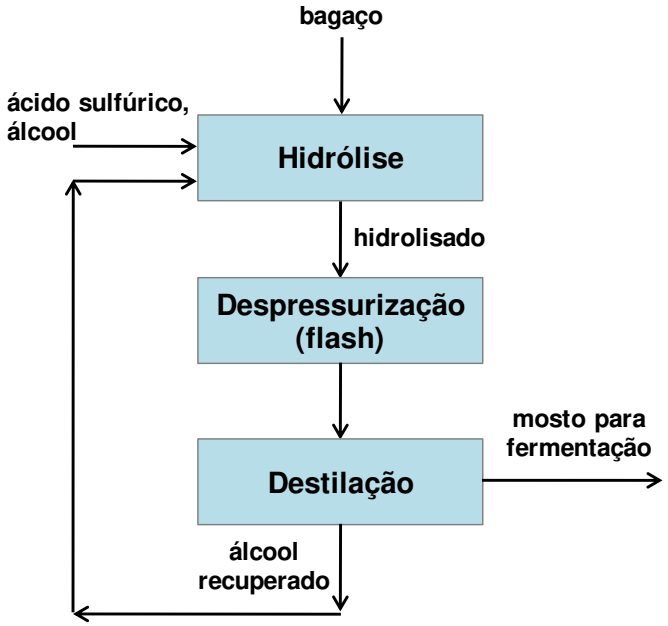


Figura 12: Diagrama de fluxo de hidrólise organosolv (Olivério et al., 2007)

2.4.e Formação de inibidores

Na hidrólise de lignocelulose, especialmente quando é empregado um processo de hidrólise catalisada por ácido, são gerados ou liberados alguns componentes que têm um efeito inibidor na fermentação. Para hidrólise enzimática, o processo de pré-tratamento de lignocelulose também causa a geração destas substâncias. Isto ocorre inclusive no pré-tratamento físico com explosão a vapor, uma vez que a hidrólise de resíduos acetil produz ácido acético que, por sua vez, provoca degradação de monômeros sacarinos, principalmente de hemicelulose (Oliva *et al.*, 2003, Domínquez 2003). Estes componentes são convencionalmente divididos em três classes (Larsson *et al.*, 1999, Oliva *et al.*, 2003 e 2004, Klinke *et al.*, 2002 e 2004): furanos (furfural, 5-hydroxyl-methyl furfural), compostos fenólicos (hidroxibenzaldeido, aldeído coniferil), ácidos carboxílicos (ácidos acético, levulínico e fórmico). No entanto, avanços recentes no entendimento de mecanismos microbiológicos de inibição e desintoxicação levam a sugerir uma classificação por substituintes moleculares: partes da molécula inibidora são responsáveis

pela inibição, e cuja modificação na molécula geralmente transforma a mesma em um componente menos tóxico (Liu 2011):

- aldeídos, R-(CH=O), como furfural, 5-hydroxyl-methyl furfural (HMF), seringaldeído e baunilha
- cetonas, R-(C=O)-R , com 4-hidroxiacetofenone, aceto-baunilha, aceto-seringone
- fenóis, anel aromático, como fenol, catechol, eugenol, hidroquinone
- ácidos orgânicos, R-(C=O)-OH, como ácidos acético, levulínico, fórmico e ferúlico

A Figura 13 mostra vários inibidores (aldeídos, cetonas e fenóis) e suas respectivas estruturas químicas, nesta nova classificação. Os inibidores mais estudados são os aldeídos furfural e HMF.

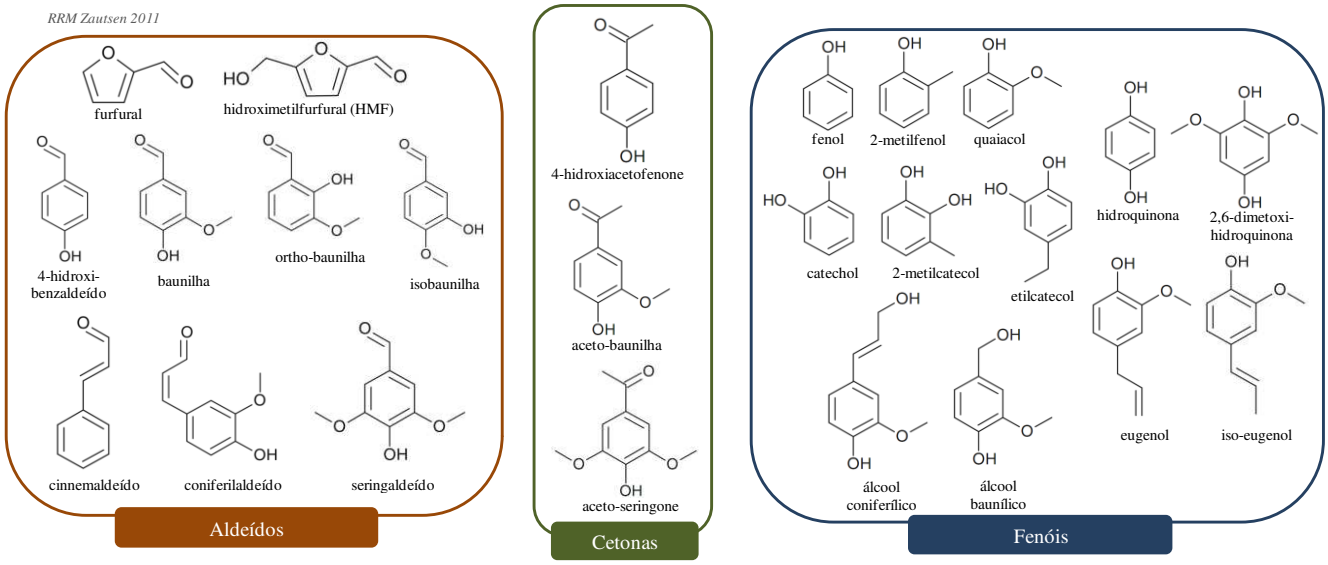


Figura 13: Inibidores provenientes de hidrólise de lignocelulose: aldeídos, cetonas e fenóis (adaptado de Liu 2011)

O furfural é gerado através da degradação das pentoses xilose e arabinose, principais produtos da hidrólise de hemiceluloses. Certa fração de furfural pode ainda degradar-se formando ácidos orgânicos como ácido levulínico ou ácido fórmico, como mostra a Figura 14.

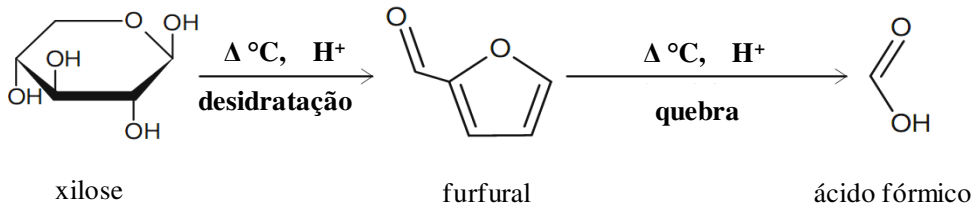


Figura 14: Degradação de xilose em furfural e ácido fórmico (Almeida et al., 2009)

O HMF é gerado através da degradação de glicose, produto da hidrólise de celulose, ou de outras hexoses como galactose e manose, produtos da hidrólise de hemicelulose. HMF pode ainda ser degradado formando ácido levulínico e ácido fórmico. A Figura 15 mostra uma esquematização deste processo.

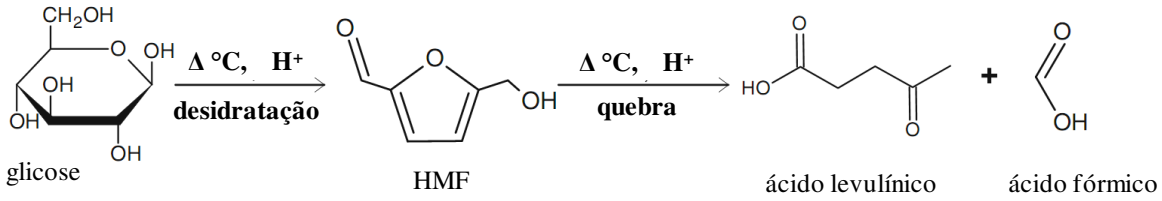


Figura 15: Degradação de glicose em HMF, ácido levulínico e ácido fórmico (Almeida et al., 2009)

Através da quebra do complexo de lignina, são gerados outros aldeídos, como baunilha, 4-hidroxibenzaldeído e seringaldeído, cetonas, como 4-hidroxiacetofenone, aceto-baunilha, aceto-seringone, e fenóis, como fenol, catechol, eugenol, hidroquinone (Klinke *et al.* 2004, Delgenes *et al.*, 1996). Catechol também é formado a partir de pentoses e ácido hexurônico (Larsson *et al.*, 2000). Todos estes produtos são do tipo aromático e poli-aromático, contendo uma grande variedade de substituintes como ilustrado na Figura 13.

A Figura 16 mostra vários ácidos orgânicos provenientes da hidrólise de lignocelulose. Ácido acético é formado através a hidrólise de resíduos acetil de hemicelulose (Domínguez 2003). Outros ácidos são resultados da degradação de substituintes na lignina (ex. oxidação de aldeídos), ou então, da degradação (oxidação) de furfural e HMF (Almeida *et al.*, 2009).

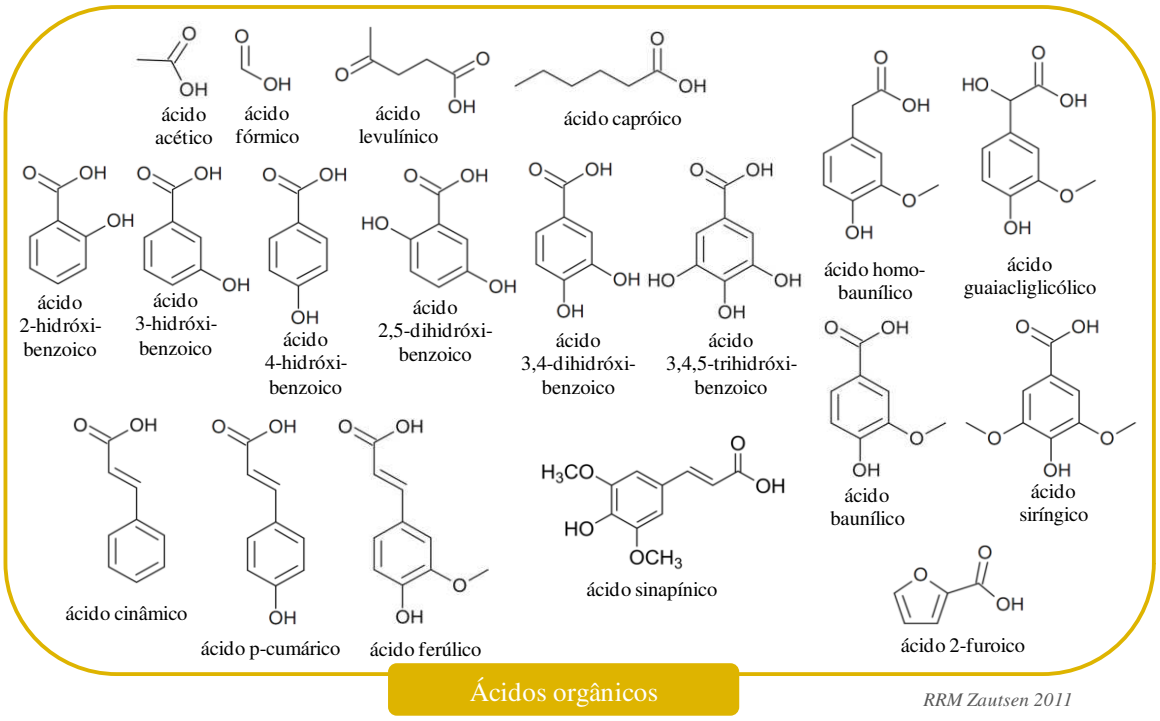


Figura 16: Ácidos orgânicos provenientes da hidrólise de lignocelulose (adaptado de Liu 2011)

2.4.f Efeitos de inibidores na fermentação

Destes compostos, efeitos inibidores foram observados para produção de etanol utilizando *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis, Candida shehatae* (Delgenes *et al.*, 1996) e *Kluyveromyces marxianus* (Oliva *et al.*, 2004) como também para produção de acetona, butanol e etanol (ABE) utilizando *Clostridium beijerinckii* (Ezeji *et al.*, 2007). Em muitos casos, o efeito inibidor pode ser neutralizado pela levedura, mas não sem prejuízo à taxa de crescimento, produtividade e até viabilidade (Almeida *et al.*, 2007).

Aldeídos - furfural

Para a levedura empregada na fermentação, o furfural tem um efeito negativo na taxa específica de crescimento, rendimento de massa celular sobre ATP e produção volumétrica e específica de etanol. Além disso, sua presença diminui a viabilidade da levedura e causa uma fase lag no seu crescimento para concentrações típicas no caldo hidrolítico (Delgenes *et al.*, 1996, Palmqvist *et al.*, 1999, Almeida *et al.*, 2009). Porém, Taherzadeh *et al.* (1999)

e Almeida *et al.* (2009) observaram também, para *Saccharomyces cerevisiae,* que na presença de furfural e HMF, embora o rendimento de biomassa sobre substrato seja menor, o rendimento de etanol é maior. Modig *et al.* (2002) mostraram o efeito de furfural e HMF sobre algumas importantes enzimas das vias metabólicas de glicólise e produção de etanol: álcool desidrogenase (ADH), aldeído desidrogenase (ALDH), piruvato desidrogenase (PDH). Com a utilização destas enzimas e aldo-ceto-redutase e metilglioxal-redutase (GRE) (Liu 2011), a *Saccharomyces cerevisiae* e outras cepas de levedura têm a capacidade de reduzir vários aldeídos em seu álcool correspondente, porém necessitando o cofator NADH ou NADPH. Por outro lado, o aldeído furfural tem um grande efeito inibidor na atividade de ALDH e PDH, enquanto ADH é menos afetado. Assim, furfural é reduzido em álcool furfurílico, que tem propriedades inibidoras muito menores do que o furfural (Liu, 2011). Porém, a taxa desta redução é limitada. A reação provoca extensão da fase lag no crescimento (Oliva *et al.*, 2006), e a dependência de cofatores NAD(P)H desvia este importante carregador energético intercelular de outros processos bioquímicos (Palmqvist *et al.*, 1999a, Almeida *et al.*, 2009). Segundo Oliva *et al.* (2003), a *Kluyveromyces marxianus* não mostra nenhuma produção de etanol até que todo furfural no meio seja reduzido. Isto é benéfico para a viabilidade da levedura a longo prazo, sabendo que no processo redutivo de aldeídos há uma presença temporária de radicais (Larsson *et al.*, 2000) e elevada 'Reactive Oxygen Species' (ROS) (Almeida *et al.*, 2009), ambos causando danos ao DNA enquanto o furfural ainda não é reduzido, especificamente em ambiente (semi) aeróbico. A reprodução da levedura antes ou durante o reparo de material genético é obviamente problemática. Existe também um mecanismo de excreção de compostos inibidores como furfural com complexo enzimático ABC, que necessita ATP, outro carregador energético (Liu 2011).

Aldeídos - HMF

O hidroxi-metil-furfural (HMF) tem um efeito inibidor semelhante ao furfural, embora menos severo (Modig *et al.*, 2002). Por outro lado, a conversão de HMF em 2,5-bis-hidroximetilfuran (HMF álcool) ocorre em uma taxa menor do que a redução de furfural (Taherzadeh *et al.*, 1999, Almeida *et al.*, 2007). Na via metabólica, uma diminuição

da atividade de ADH, causada pela presença de HMF, foi observada, semelhante à diminuição por furfural, enquanto que as atividades de AIDH e PDH diminuem menos na presença de HMF do que na presença de furfural (Modig *et al.*, 2002).

Aldeídos - geral

A posição dos substituintes de aldeídos pode influenciar a inibição da molécula mais do que o fato de ser um grupo metil ou hidróxido (Klinke *et al.*, 2003). Por exemplo, orto-baunilha tem um efeito inibidor muito maior que baunilha ou iso-baunilha (Larsson *et al.*, 2000). Um grupo adicional pode até reduzir a toxicidade do aldeído: seringaldeído é menos tóxico do que hidroxibenzaldeído (Delgenes *et al.*, 1996). O efeito tóxico se manifesta principalmente na taxa de crescimento e, apenas em segundo lugar, no rendimento etanólico (Larsson *et al.*, 2000). Como furfural e HMF, outros aldeídos também são convertidos em seus respectivos alcoóis: baunilha em álcool baunílico, coniferil-aldeído em coniferil álcool e dihidro-coniferil álcool, etc. (Klinke *et al.*, 2004, Larsson *et al.*, 2000). A taxa desta redução nem sempre corresponde à toxicidade do componente: em concentrações pequenas o bastante para ainda permitir crescimento, a redução de orto-baunilha é comparável à redução de baunilha e isobaunilha, apesar de orto-baunilha ser muito mais tóxica (Larsson *et al.*, 2000).

Cetonas e fenóis

Conhecimento de inibição por cetonas ainda é muito limitado, mas seu efeito e mecanismo são, provavelmente, muito semelhantes a compostos fenólicos em geral, que diminuem o crescimento, produtividade e rendimento da fermentação alcoólica da levedura (Palmqvist *et al.*, 1999b). A inibição destes compostos é devida à alta lipossolubilidade, principalmente através dos anéis aromáticos, que permite sua incorporação na parede celular da levedura. Isto danifica a integridade da estrutura da membrana, prejudicando sua função de barreira seletiva, podendo diminuir o gradiente eletroquímico sobre a mesma (Almeida *et al.*, 2007). Talvez, por isto, seja frequentemente observado que um menor número de substituintes hidroxi ou metoxi aumenta a toxicidade (Larsson *et al.*, 2000):

estes grupos são polares e diminuem a lipossulibilidade da molécula. A presença de ligações duplas em substituintes é outro fator importante para sua toxicidade, independente da posição. O conhecimento da capacidade de *Saccharomyces cerevisiae*, ou outras leveduras, de metabolizar ou neutralizar estes compostos fenólicos é limitado a relatos de saturação de duplas ligações (Larsson *et al.*, 2000, 2001a): a conversão de compostos fenólicos em radicais com laccase, gerando produtos de polimerização com alto peso molecular (Almeida *et al.*, 2007). Esta conversão com laccase, também foi observada com o coniferíl aldeído (Larsson *et al.*, 2001a), necessitando um agente oxidante como o oxigênio.

Ácidos orgânicos

Ácidos orgânicos, como ácido acético, diminuem principalmente a taxa de crescimento e rendimento de biomassa. Este efeito ocorre em consequência de sobrecarga de cadeias de transporte de prótons que procuram evitar a queda de pH intracelular, devido à difusão de ácido na forma não-dissociada pela parede celular da levedura (Pampulha *et al.*, 2000) e acúmulo de ânions (Almeida *et al.*, 2007). Em geral, ácidos menores e com maior hidrofobicidade são mais lipossolúveis, o que facilita a difusão pela membrana, em baixos valores de pH (Palmqvist *et al.*, 1999b), principalmente na presença de etanol (Casal *et al.*, 1998). O influxo de prótons é neutralizado por excreção ativa dos mesmos, um processo que necessita ATP. Consequentemente, acumulam ânions na célula e há menos ATP disponível para processos de crescimento e manutenção, prejudicando a viabilidade e produção etanólica da levedura.

Os efeitos de ácidos orgânicos variam muito: ácido ferúlico inibe a produtividade alcoólica severamente, enquanto ácido siríngico não mostra nenhuma inibição (Klinke *et al.*, 2004). A Tabela 8 mostra valores de fatores que podem explicar a toxicidade de ácido ferúlico, ácido acético, ácido fórmico, ácido levulínico e ácido seríngico. O menor valor de pK_a prediz uma toxicidade maior: no mesmo pH e concentração intracelular, um ácido com baixo pK_a, como ácido fórmico, é mais dissociado e por isto mais tóxico pelo acúmulo de ânions e provocação de excreção de prótons, dispendioso em ATP. TPSA (Topological Polar Surface Area, calculada) é uma indicação da superfície polar da molécula. Quanto

menor esta superfície, tanto maior a lipossubilidade e, teoricamente, a toxicidade. O valor log P_{ow}, logaritmo de coeficiente de partição do ácido sobre octanol e água, é outra indicação da lipossubilidade (maior log P_{ow} significa um maior lipossubilidade). Um tamanho pequeno, medido por volume ou massa molecular, facilita influxo do ácido e então aumenta a toxicidade. Comparando estes fatores para todos estes ácidos, pode se concluir que nenhum fator sozinho pode indicar uma previsão definitiva das suas toxicidades relativas.

O rendimento de etanol depende da concentração: em pequenas concentrações de ácido acético, ácido levulínico ou ácido fórmico, produção de ATP é estimulado, elevando o rendimento etanólico, mas para maiores concentrações o rendimento é prejudicado pela acidificação e acúmulo de ânions na célula (Almeida *et al.*, 2007). Por outro lado, Larsson *et al.* (2000) não observaram mudanças de rendimento de etanol causado por ácido ferúlico.

Como mecanismo de desintoxicação pela levedura, a descarboxilação de ácidos via fenil-acrílico ácido decarboxilase (PAD) foi observada para vários ácidos orgânicos, produzindo derivados vinílicos (Larsson *et al.*, 2001b).

Tabela 8: Ácidos orgânicos em ordem de toxicidade e fatores que podem explicar o grau de toxicidade de cada ácido: pK_a (logaritmo da constante de acidez), TPSA (topological polar surface área), logP_{ow} (logaritmo de coeficiente de partição octanol/água), fato de haver uma dupla ligação na estrutura molecular, volume e PM (peso molecular), Nenhuma constante é consistente com a toxicidade. (Almeida et al., 2007,Klinke et al., 2004, Erdemgil et al., 2007, Pow: http://logkow.cisti.nrc.ca/logkow/, volume, TPSA: http://www.molinspiration.com/cgi-bin/properties)

ácido	toxicidade	pK_a	TPSA	log P_{ow}	dupla ligação	volume ($Å^3$)	PM (g/mol)	TPSA/V
ácido ferúlico	++++	4.56	66.8	1.25	sim	172.0	194.18	0.39
ácido fórmico	+++	3.74	37.3	-0.54	não	39.6	46.03	0.94
ácido levulínico	++	4.66	54.4	-0.49	não	108.8	116.11	0.50
ácido acético	+	4.76	37.3	-0.17	não	56.2	60.05	0.66
ácido seríngico	0	4.20	76.0	1.20	não	170.2	198.17	0.45

Observação geral

Observa-se que, ao comparar concentrações e demais estruturas moleculares, a inibição por aldeídos e cetonas é mais forte do que por fenóis, a por fenóis é mais forte do que a por ácidos orgânicos (com exceção de ácido ferúlico), e a por ácidos é muito mais

fortes do que aquelas por aldeídos reduzidos a álcoois (Larsson *et al.*, 2000, Klinke *et al.*, 2003). Porém, suas concentrações em caldo hidrolítico são muito diferentes e variam dependendo da fonte de lignocelulose e do método de hidrólise aplicado. Além disso, ácidos orgânicos, aldeídos, cetonas e fenóis mostram efeitos sinérgicos em relação à produtividade e ao rendimento de etanol e biomassa (Oliva *et al.*, 2006), tanto que a inibição total de todos os componentes presentes no meio não pode ser avaliado como se fosse a soma das inibições de cada um dos componentes individuais (Palmqvist *et al.*, 1999b, Oliva *et al.*, 2006). Somente para ácidos orgânicos diferentes entre si, tal efeito sinérgico não foi observado (Almeida *et al.*, 2007).

Sistema biológico complexo

Na última década, com o avanço de tecnologias de microbiologia molecular, surgiram mais esclarecimentos sobre a tolerância e degradação destes componentes inibidores pela levedura, que junto formam um sistema complexo, empregando diversos recursos bioquímicos de indução e repressão genética. Uma revisão de Liu (2011) resume as seguintes principais respostas da levedura aos inibidores:

- incremento de sistemas de defesa que evitam o influxo de inibidores no citoplasma e organelas vitais;
- providenciar e utilizar mais os mecanismos de reparo de membranas;
- mudanças na via glicolítica para administrar estrategicamente os balanços energéticos e promover maior produção de NADP e NADPH;
- maior produção e atividade de aldeído-desidrogenases múltiplas e outras enzimas para detoxificar inibidores aldeídicos, utilizando NADP e NADPH produzidos;
- diminuição da toxicidade pela agregação de proteínas danificadas ou proteínas mal enoveladas;
- produzindo e empregando mais chaperonas para re-enovelamento de enzimas;
- ubiquitinas, acionando a degradação destas proteínas;
- herança de código genético às gerações seguintes para a expressão induzida ou reprimida de genes envolvidos na detoxificação, incluindo a regeneração de NAD(P)H.

A Figura 17 ilustra os principais mecanismos discutidos acima, efeitos e desdobramentos através de aldeídos, cetonas, fenóis e ácidos orgânicos, no contexto das vias metabólicas de *Saccharomyces cerevisiae*. É interessante observar que a repressão e indução de várias enzimas chaves resulta na utilização da via das pentoses fosfato para maximizar a produção de NADH e ATP, para os mecanismos de detoxificação e manutenção do sistema celular.

Apesar de todo este sistema biológico, é evidente que as concentrações de inibidores no caldo hidrolítico (Martinez *et al.*, 2001, Martin *et al.*, 2007, Larsson *et al.*, 2000) são de tal magnitude que o desempenho do processo fermentativo é prejudicado em termos de produtividade, viabilidade celular e rendimento. Embora seja possível elaborar um sistema de fermentação batelada-alimentada ou contínua com reciclo de células, onde a taxa de alimentação e a massa celular são sempre mantidas de forma que o sistema mantenha um desempenho estável de produção e detoxificação pela própria levedura, (Nillson *et al.*, 2002, Almeida *et al.*, 2007), sempre ocorrerá prejuízo na produtividade e tempo prolongado de fermentação, mesmo empregando um micro-organismo geneticamente modificado (Hodge *et al.*, 2009). Isto torna interessante empregar um método de remoção de inibidores do caldo hidrolisado.

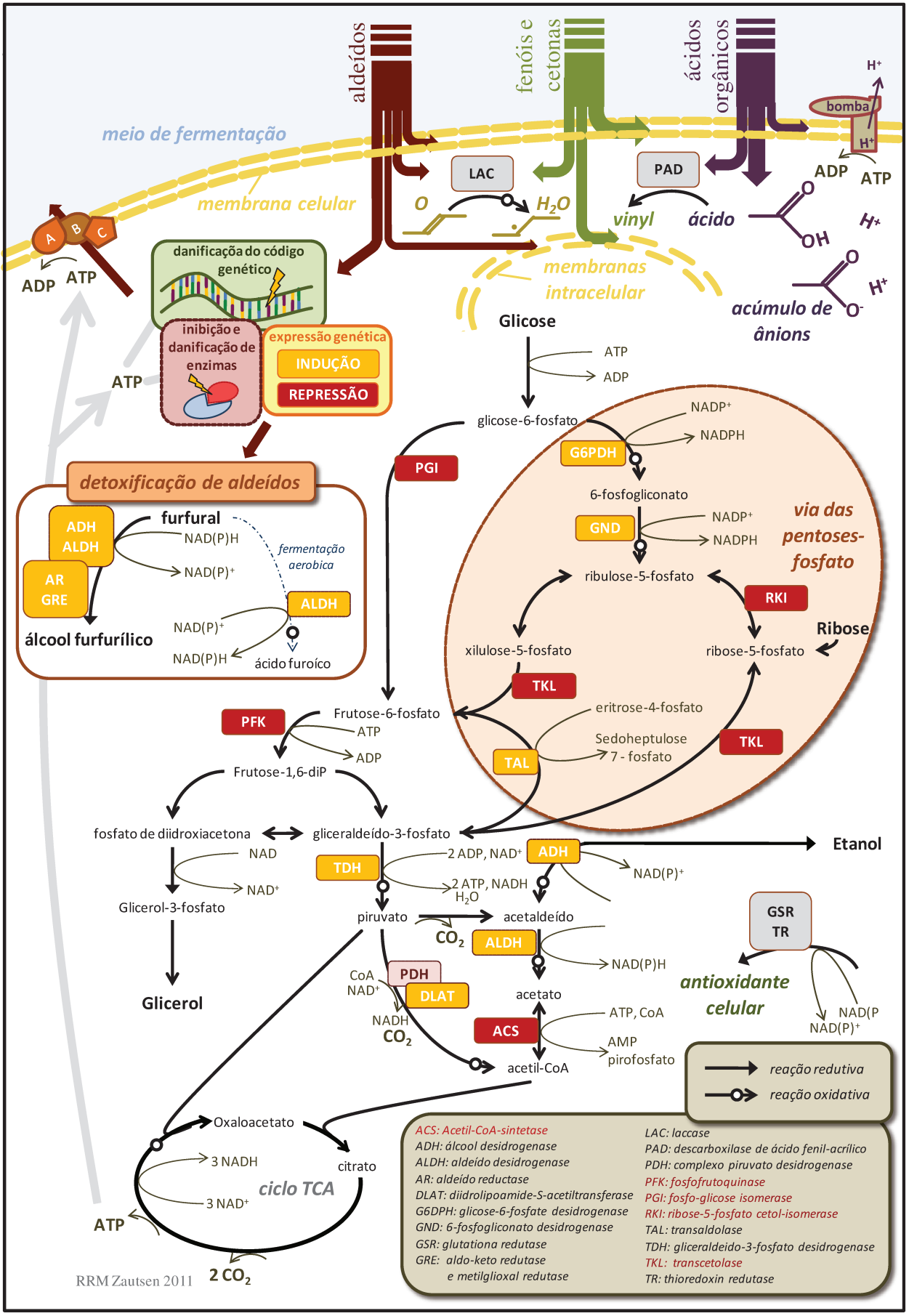


Figura 17: Mecanismos de resposta da levedura diante de inibidores: múltiplos complexos envolvendo expressão de genes (repressão e indução), danos e reparos de membrana, enzimas e código genético, equilibrio do pH interno. Repressão e indução estimulam a utilização de via das pentoses fosfato e o ciclo TCA, aumentando a produção de NAD(P)H e ATP necessários para reduzir aldeídos e para o funcionamento de enzimas de proteção contra estresse oxidativo como GSR e TR. (fontes: Liu 2011, Almeida 2007, 2008, 2009, Modig et al., 2002, Horváth et al., 2003, Larsson et al., 2001)

2.4.g Métodos de detoxificação de hidrolisados

Pesquisas recentes vêm sendo conduzidas para desenvolver meios de remoção de componentes inibidores do caldo hidrolítico, antes da introdução do mesmo no estágio de fermentação. A Tabela 9 resume alguns tratamentos reportados na literatura.

Tabela 9: Métodos de detoxificação de caldos hidrolíticos

método	anotação	referências
adsorção por carvão ativo	p.ex. carvão ativado por vapor, 60°C. De preferência pH baixo para compostos fenólicos e ácidos orgânicos (p. ex. pH 2).	Chandel *et al*., 2007, Hodge *et al*., 2009, Sainio *et al*., 2011, Mussatto *et al*., 2004
adsorção mineral (troca de iônica, zeólita, argila)	resina de troca iônica ou catiônica, p.ex. de polistirene-divenil-benzeno a pH 10, ou resina do tipo gel sem porosidade permanente	Chandel et al., 2007, Larsson *et a*l., 1999, Sainio *et al*., 2011
adsorção com material orgânico (lignina, quitina, amido)	residuo de lignina previamente lavada, pH 2, baixa temperatura (p.ex. 2°C)	Björklund *et al*., 2002,Soto *et al*., 2011
adsorção com material de origem microbiológica (vinhaça, lodos ativados)		Soto *et al*., 2011
adsorção com resinas poliméricas sintéticas	de preferência ésteres poli-aromático	Sainio *et al*., 2011, Soto *et al*., 2011
caleação, tratamento alcalino	precipitação e desestabilização de inibidores com $Ca(OH)_2$ (0,4 g/g) ou NaOH (10%), pH 9-10, 25-60°C	Martinez *et al*., 2001, Larsson *et al*., 1999, Hodge *et al*., 2009
caleação combinatório	>0,1% sulfito de sódio, pH 10, eventual evaporação de inibidores voláteis a 90°C	Larsson *et al*., 1999, Palmqvist *et al*., 2000
detoxificação microbiológica	*Trichoderma reesei*, *Issatchenkia occidentalis, IIssatchenkia orientalis*	Larsson *et al*., 1999, Hou-Rui *et al*., 2009, Palmqvist *et al*., 2000
detoxificação microbiológica in-situ	*Saccharomyces cerevisiae* imobilizada	Talebnia e Taherzadeh 2006
eletrodiálise		Cardona *et al*., 2010
evaporação e ressuspensão	baixa pressão, alta temperatura	Palmqvist *et al*., 2000
extração líquido-líquido	éter di-etílico, acetato de etila, polímeros separados por efeito térmico (25°C)	Cruz *et al*., 1999, Hasmann *et al*., 2008
reação de Fenton	oxidação química utilizando H_2O_2 (p.ex. 50 mM) como agente oxidante e Fe(II) como catalizador	Oliva *et al*., 2005
tratamento enzimático	peroxidase, laccase (pH 5,5; 0,4 g/g, 100U, 4h, 30°C)	Chandel *et al*., 2007, Larsson *et al*., 1999, Palmqvist *et al*., 2000

Um método convencionalmente aplicado é o tratamento alcalino do caldo, por exemplo a caleação: aumento de pH a 9-10 com hidróxido de cálcio. Além da neutralização do caldo e sedimentação de sais com ácido sulfúrico na forma de gipsita, compostos como furfural e HMF são quimicamente alterados, diminuindo a toxicidade destes inibidores (Martinez *et al.*, 2001). Porém, o método tem a desvantagem de reduzir a qualidade e a quantidade de açúcar.

Para tratamentos baseados em adsorção de inibidores como a adsorção por carvão ativo ou troca iônica, uma excelente adsorção dos compostos pode dificultar a regeneração do carvão ou resina, tornando sua utilização inviável (Sainio *et al.*, 2011).

A aplicação de todos estes métodos acima resulta em efeitos positivos na fermentação, mas a instalação e uso de um estágio separado de tratamento do mosto antes da fermentação encarece o processo, exceto para tratamentos *in-situ*, como a detoxificação microbiológica *in-situ* ou extração líquido-líquido utilizando um solvente biocompatível.

O mais simples tratamento microbiológico *in-situ* emprega a própria levedura do fermento, eventualmente em elevada concentração, para diminuir a toxicidade do meio como descrito no parágrafo anterior, ou pela imobilização (Talebnia e Taherzadeh, 2006), reciclo de células ou alimentação controlada na fermentação batelada-alimentada. A desvantagem destes métodos é a limitada taxa de detoxificação pela levedura, prejudicando o crescimento e produtividade e aumentando o tempo do processo fermentativo, além do acúmulo de inibidores que não podem ser convertidos, como vários ânions de ácidos orgânicos (Almeida *et al.*, 2007).

A extração líquido-líquido com um solvente biocompatível é interessante (Hasmann *et al.*, 2008) e proposta nesta tese em combinação com a remoção simultânea do produto.

2.5 Processos de fermentação

O processo biológico para conversão de substratos hexoses em produto envolve a utilização de uma levedura como *Saccharomyces cerevisiae* ou *Kluyveromyces marxianus* (Oliva *et al.*, 2004), ou bactéria como *Zymomonas mobilis* com alto rendimento, atingindo até 97% do máximo possível (Sanchez e Cardona, 2008). O micro-organismo, com capacidade de converter sacarose em glicose e frutose com auxílio da enzima invertase, consome estes monômeros como fontes de carbono e converte-os em biomassa, etanol e glicerol. Pentoses, como xilose, podem ser utilizadas através da levedura *Pichia stipitis* ou cepas heterólogas da bactéria *Escherichia coli*. Há importantes avanços em construções de bactérias e leveduras geneticamente modificadas com objetivo de melhorar o desempenho no consumo de pentoses por processos fermentativos. Exemplos disto são modificações na via de pentoses fosfato de *S. cerevisiae* (Chu e Lee, 2007) ou misturas imobilizadas de *E. coli* para converter tanto hexoses como pentoses em produto (Unrean *et al.*, 2010).

O pH do meio deve se manter entre 4,5 e 5,5 para um bom desempenho da fermentação. A fermentação pode ser executada em condições anaeróbicas ou empregando uma leve aeração sob condições de baixa concentração de açúcar. Oxigênio promove o crescimento pela produção de síntese de ácidos graxos e esteróis (Sanchez e Cardona, 2008) e oxidação de compostos inibidores (Larsson *et al.*, 2001a). Porém, com concentrações de substrato menores do que 50 $mg \cdot L^{-1}$ (Jones e Kompala., 1999) a via metabólica oxidativa é a via principal, com elevada utilização do ciclo TCA para equilibrar o balanço de ATP e conseqüentemente promover elevado rendimento de biomassa em detrimento do etanol. Para maiores concentrações de glicose, a sobrecarga desta via oxidativa provoca a produção de etanol na presença de oxigênio, conhecido como o efeito 'Crabtree' (Walker 2000 p.219).

O produto se torna um fator inibidor a partir de certas concentrações como no caso de etanol para *Saccharomyces cerevisiae* em torno de 80 $g \cdot L^{-1}$, dependendo da cepa (Rivera *et al.*, 2006). Em muitos casos, o produto perturba a estrutura da membrana celular,

promovendo a fluidez da membrana (Heipieper e de Bont, 1994) e o vazamento de prótons através desta membrana. Isto provoca aumento de uso de ATP para neutralizar este efeito, fato que diminui a quantidade de ATP que normalmente seria utilizado para o crescimento do micro-organismo. Este efeito tóxico do produto, seja ele etanol, butanol, acetona ou ácido acético, torna-se o fator limitante para a concentração final do produto no meio, independente de gargalos no sistema de vias metabólicas (Sriyudthsak *et al.*, 2010). Uma maior produção do subproduto glicerol promove resistência da levedura diante do etanol e altas concentrações de substrato (Teixeira *et al.*, 2009), mas por outro lado prejudica o rendimento. Ou seja, somente a remoção do produto durante a fermentação permite aumentar a produtividade final e diminuir o tempo da fermentação através de modificações nesta via metabólica.

O processo é exotérmico e o calor gerado deve ser retirado do meio para manter uma temperatura constante. A produção etanólica é limitada para baixas temperaturas, enquanto que para temperaturas maiores do que 37°C, tanto a produtividade como a taxa de crescimento diminuem e aumenta o risco de ocorrer baixa viabilidade e até morte da levedura. A tolerância para etanol é maior para temperaturas menores (Atala *et al*., 2001, Rivera et al, 2006). A Figura 18 mostra a relação entre temperatura e taxa de crescimento específico, sugerindo uma temperatura ótima para crescimento em torno de 37,5 °C.

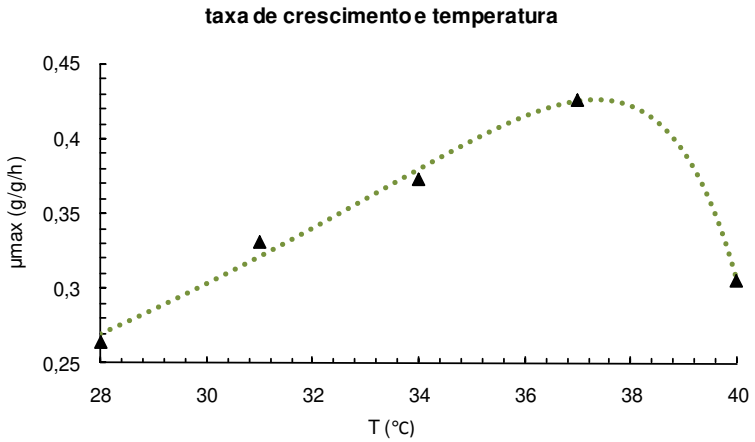


Figura 18: Relação entre temperatura e taxa de crescimento de Saccharomyces cerevisiae (Atala et al., 2001,Rivera et al., 2006)

2.5.a Regimes de fermentação

Existem vários modos possíveis para execução de processos fermentativos, como fermentação batelada, batelada alimentada ou continua. A Tabela 10 mostra uma comparação entre estes e alguns outros regimes de fermentação alcoólica. Nota-se, que as descrições do desempenho através de dados como produtividade e rendimento são meramente indicativas para comparação dos regimes e dependem muito da cepa e das condições de fermentação.

*Tabela 10: Comparação de regimes de fermentação alcoólica (Sanchez e Cardona, 2008, *Atala 2004,** Daugulis et al., 1994)*

Regime	Configuração	etanol no meio (g L^{-1})	produtividade (g $L^{-1}h^{-1}$)	rendimento (% máximo teórico)
batelada	reutilização de levedura de bateladas prévias; separação de levedura por centrifugação	80-100	1-3	85-90
bateladas repetidas	tanque agitado; levedura floculante; até 47 bateladas estáveis	89,3-92	2,7-5,3	79,5-81,7
batelada-alimentada	tanque agitado com taxa de alimentação variável	53,7-98,1	9-31	73,2-89
contínua, CSTR	recuperação e reciclo de células; eventual levedura floculante ou imobilizada; leve aeração	70-80	7,5 (*)	94.5
contínua, Biostill	tempo de residência 3-6 horas; reciclo de células por centrifugação; reciclo de vinhaça	30-70	5-20	
extração EtOH a vácuo	remoção de etanol a vácuo; reciclo de células	40-60 (*)	10 (*)	93,9 (*)
extração EtOH líquido-líquido	remoção de etanol com solvente biocompatível; reciclo de células	60-68(**)	26-32(**)	90-95(**)

No regime batelada, onde todo substrato é parte do meio a partir do início da fermentação, a levedura sofre inibição por estresse osmótico. Por outro lado, iniciar a fermentação com maior teor de açúcar apresenta vantagens de menor utilização de água no processo, maior produtividade volumétrica, obtenção de maiores concentrações finais de produto e menores custos de destilação (Mussatto *et al.*, 2010).

Bateladas repetidas são executadas utilizando a levedura produzida na fermentação prévia sem remover a mesma da dorna. Após o final de cada fermentação, a levedura floculante ou imobilizada é decantada e o vinho clarificado é removido da dorna. A levedura, ainda na dorna, funciona como inóculo para a fermentação seguinte, gerando uma massa celular cada vez maior, que por sua vez resulta em maior produtividade volumétrica a cada batelada.

No regime de batelada alimentada, a fermentação começa com um volume de meio limitado, mas com concentração do substrato adequada para impedir o efeito inibidor do mesmo. Ao longo da fermentação, o substrato é alimentado até completar o volume da dorna, momento que coincide com a conversão total do substrato. A taxa de alimentação é controlada de tal modo, que a concentração de substrato no meio se mantém bastante baixa para evitar o efeito inibidor do substrato, mas ainda bastante alta para alcançar alta produtividade, assim melhorando o desempenho e produtividade da fermentação em comparação com o regime de batelada ou bateladas repetidas.

Na fermentação contínua, o sistema é mantido em condições estáveis, possibilitando uma fermentação por tempo prolongado em condições otimizadas em termos de concentração de substrato, biomassa e produto, sendo que uma fração do meio é continuamente retirada da dorna. Este regime também tem outras vantagens inerentes, como menor custo de operação devido à inexistência, na prática, de intervalos para o enchimento e lavagem do tanque, menores custos de instalação, manutenção, resfriamento, controle do processo, além de promover a adaptação da levedura a inibidores provenientes de caldo hidrolítico (Brethauer e Wyman, 2010). Por outro lado, um sistema continuo é mais susceptível a infecções bacterianas, por exemplo por cepas de Lactobacilos, prejudicando dessa maneira o rendimento da fermentação e a viabilidade da levedura. O processo pode ser otimizado para promover maior produtividade específica ou volumétrica ou para maior rendimento, através de controle da taxa de alimentação e da concentração de substrato na mesma. Introdução de floculação controlada facilita a separação da levedura do meio para o reciclo do mesmo, ou possibilita até a retenção da levedura na dorna, que pode resultar em redução de 16% de custos totais do processo segundo Andrietta *et al.* (2008).

O regime de fermentação extrativa contínua a vácuo foi estudado por Atala (2004) para remover o etanol do meio durante a fermentação com um evaporador a vácuo do tipo flash. O sistema, atualmente sendo estudado em escala de planta piloto, diminui a concentração de etanol no meio até em torno de 5 ºGL, ou seja, abaixo do nível de inibição. Em conseqüência, a fermentação, alimentada com fluxo com alto teor de açúcar (até em

torno de 500g/L), mostrou menor inibição pelo etanol produzido, alta viabilidade e produtividade volumétrica. Além disso, a concentração de etanol no fluxo de vapor proveniente do evaporador é favorável para reduzir custos na recuperação do produto (Junqueira *et al.*, 2009). A alta concentração de substrato resulta em menor quantidade de água utilizada no processo integral de produção de etanol, implicando em menor gasto energético, e flegmaça e vinhaça mais concentrados. Esta última característica resulta em menor custo de transporte de vinhaça para os campos de cana para fertilização dos mesmos. Também foi previsto um resfriamento eficiente da dorna, pelo próprio sistema a vácuo.

Neste sistema, ainda não foi estudado o uso de caldo hidrolítico como substrato, a economia do uso de membrana para reciclo de células e o evaporador flash na escala industrial em combinação com a taxa elevada do reciclo pelo sistema. O evaporador a vácuo pode provavelmente diminuir as concentrações de inibidores voláteis como furfural e ácido acético, enquanto outros inibidores como baunilha e 4-hidroxibenzaldeído podem acumular caso a massa celular não for bastante elevada para conversão efetiva dos mesmos. Também, o estresse elevado do fermento, gerado pela alta taxa de reciclo de levedura, pode influenciar negativamente na viabilidade da mesma em sistema em escala industrial, embora este efeito não tenha sido observado em escala de bancada. No entanto, é possível que a baixa concentração de etanol seja mais importante para manter altas viabilidades. Neste contexto, é importante lembrar que o fator de estresse devido à presença de inibidores no caldo hidrolítico, pode se somar ao estresse que ocorre na membrana celular através do sistema de reciclo de células. Por exemplo, os inibidores fenóis justamente perturbam a integridade da membrana celular e outros inibidores provocam desvio de ATP para reparos intracelulares, em detrimento de manutenção da integridade da membrana celular.

Contudo, destacam-se os efeitos evidentemente positivos no desempenho da fermentação na remoção de etanol durante a fermentação, um dos principais objetivos do processo, que também é estudado nesta tese.

2.5.b Fermentação extrativa líquido-líquido

Descrição do processo

A fermentação extrativa, como proposta nesta tese, envolve a utilização de um solvente biocompatível durante a fermentação alcoólica para remover o produto da fermentação e os inibidores provenientes de caldo hidrolítico do meio. Desta maneira, o desempenho do micro-organismo, em termos de taxa de crescimento, viabilidade, produtividade volumétrica e específica e rendimento, pode ser otimizado, assim como o uso de energia do processo de produção, incluindo a recuperação e retificação do produto e o reciclo do solvente. A Figura 19 mostra um diagrama simplificado do processo. Um mosto rico em açúcar (A) entra no fermentador, que contém um sistema bifásico: uma fase aquosa, na qual a fermentação é realizada, e uma fase orgânica acima da fase aquosa, parcialmente dispersa na fase aquosa, composta pelo solvente. O solvente é injetado no fundo do fermentador (C).

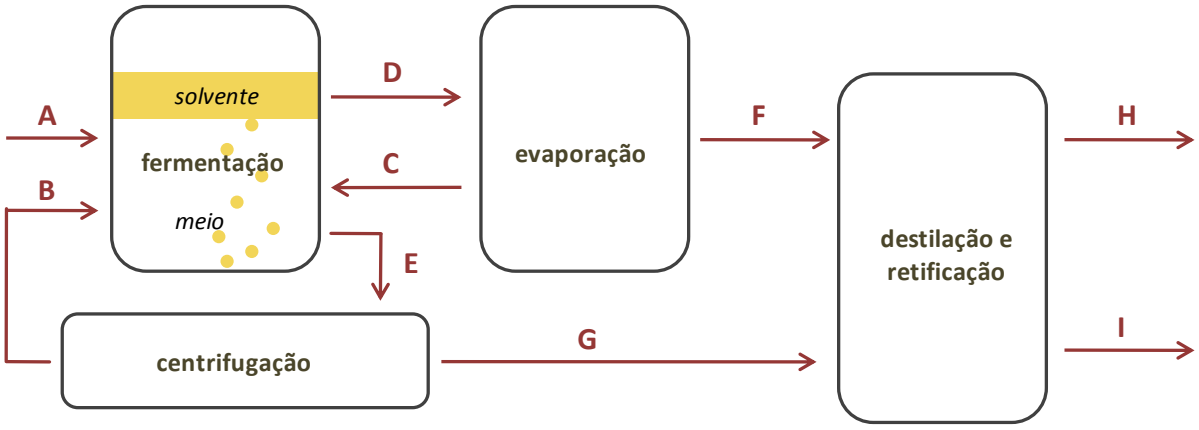


Figura 19: Princípio da fermentação extrativa líquido-líquido

O solvente é bem misturado com o meio de fermentação, mas sem provocar uma emulsão estável. A densidade do solvente é relativamente baixa, forçando escoamento do solvente para cima onde o mesmo se junta com a camada orgânica. Assim o solvente funciona como um extrativo *in-situ* do produto da fermentação, o etanol, e os inibidores do caldo. Na parte superior o solvente é mais rico em etanol e a água é removida do fermentador (D). Em seguida, o solvente é bombeado para uma unidade de evaporação. O

solvente tem uma volatilidade menor do que a água e o etanol, que então podem ser recuperados como mistura pelo aquecimento e evaporação a baixa pressão. A fase vapor, composta por etanol e água, é enviada para colunas de destilação e retificação (F). A camada aquosa é bombeada do fermentador como vinho (E) e a levedura é recuperada por centrifugação. A maior parte é re-utilizada (B), enquanto o vinho centrifugado (G) é bombeado para as colunas de destilação e retificação. As colunas de destilação e retificação geram etanol hidratado como produto (H) e vinhaça, flegmaça e óleo fúsel como subprodutos (I). A Figura 20 mostra ainda o diagrama de fluxo deste processo.

Desta forma, a remoção *in-situ* de produto e demais inibidores do meio de fermentação pelo solvente biocompatível, concentra de fato o produto, elimina uma unidade extra de tratamento do meio e pode diminuir o custo total de produção de etanol, tanto em termos energéticos quanto em termos de água utilizada.

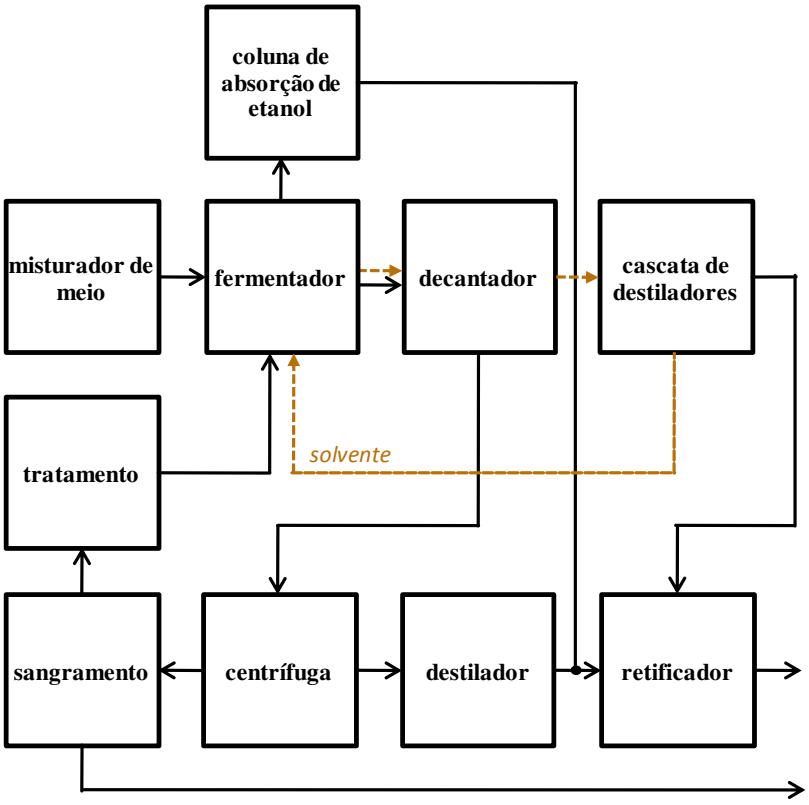


Figura 20: Diagrama de fluxo de fermentação extrativa líquido-líquido

Exemplos na literatura

Há alguns exemplos na literatura onde o solvente empregado não é biocompatível, necessitando a separação da levedura do meio ou vinho antes da extração ou utilização de uma membrana para evitar contato direto entre o fermento e o solvente, por exemplo, decanol (Eckert e Schügerl, 1987, Kapucu *et al.*, 1999), iso-octano, octano, gasolina, querosene e diesel (Rahman *et al.*, 2007), fluidos supercríticos como gases comprimidos CO_2, etano e propano (Bothun *et al.*, 2003) para remoção de etanol, ou éter etílico ou acetato de etila (Cruz *et al.*, 1999) e 2-metil-2-butanol ou 2-etil-1-hexanol (Cabezas *et al.*, 1988) para remoção de inibidores no hidrolisado.

Por outro lado, também existem exemplos de utilização de um solvente biocompatível. Kollerup e Daugulis (1986) fizeram uma evaluação teórica de 1361 possíveis solventes para extração de etanol para propriedades como coeficientes de partição, biocompatibilidade, disponibilidade e custo. Para a biocompatibilidade foi observado que solventes com uma cadeia de carbono com número de carbonos menor do que 12 têm efeitos tóxicos ou inibidores no crescimento da levedura (Minier e Goma, 1982). Exemplos destes e outros solventes na literatura são uso de óleo oléico (Daugulis *et al.*, 1987, Roffler *et al.*, 1987, Weilnhammer e Blass, 1994), dodecanol (Gyamerah e Glover, 1996, Minier e Goma, 1982) e ácido oléico para remoção de etanol (Jassal *et al.*, 1994) ou para produção simultânea de biodiesel (Oliveira *et al.*, 1998, Csányi *et al.*, 2004), ácido ricinoleico ou outros ácidos graxos (Waibel *et al.*, 2010, Boudreau e Hill, 2006). Uso de biodiesel, ésteres metílicos de ácidos graxos de óleos vegetais, para remoção de etanol, butanol ou acetona também foram avaliados (Waibel *et al.*, 2010, Grobben *et al.*, 1993, Ishizaki *et al.*, 1999), mas não para remoção de inibidores provenientes do caldo hidrolítico. Utilizando álcool oléico em um processo de sacarificação e fermentação extrativa com celulase, Moritz e Duff (1996) chegaram perto de realizar a extração simultânea de produto e inibidores do caldo hidrolítico, mas sua literatura não mostra esta compreensão.

Outros estudos mostram resultados positivos utilizando polímeros para extração de etanol (Seiler *et al.*, 2003, Offeman *et al.*, 2008) e, para extração de compostos fenólicos do caldo hidrolítico, polímeros cuja separação do meio é induzido por um aumento da temperatura (Hasmann *et al.*, 2008). Utilização de variações de polietilenoglicol foi revisado por Banik *et al.* (2003). Remoção de etanol por adsorventes, em combinação com levedura imobilizada foi estudada por Cartón *et al.* (1998).

Maiorella *et al.* (1984) estudaram a concentração de inibidores provenientes de vinhaça reciclado como cálcio no meio de fermentação por causa da baixa seletividade do solvente para este tipo de inibidores ou extração a vácuo, e concluiram não misturar mais que um terço de vinhaça reciclado no meio.

Somente para a remoção *in-situ* de etanol (então sem remoção de demais inibidores), com solvente biocompatível, o processo foi modelado e executado na escala laboratorial, mostrando retornos de custos de investimento para usinas existentes e novos usinas (Daugulis *et al.*, 1991, 1994). O processo propiciou a utilização de elevadas concentrações de açúcar, menor inibição de etanol durante a fermentação e menor requisito de energia. O renovado interesse neste processo é demonstrado pelo pedido de patente recente de Waibel *et al.* (2010).

Demais vantagens de fermentação extrativa líquido-líquido

A fermentação é um processo exotérmico, ou seja, calor está sendo gerado durante a conversão do substrato. Este calor pode ser efetivamente retirado do meio de fermentação utilizando o próprio fluxo de solvente em sistema contínuo, sendo que o solvente pode ser introduzido na dorna com baixa energia interna após resfriamento prévio do mesmo. Desta forma, a temperatura da fermentação pode ser mantida em torno da ótima, com menores gradientes de temperatura na dorna, elevando a eficiência em geral, tanto em termos de custos energéticos, como em termos de custos de equipamento e manutenção.

Com a remoção de etanol do meio de fermentação, o solvente também incorpora uma quantidade de água. Dependendo do tipo solvente empregado e das condições termodinâmicas, como temperatura, concentração de etanol e sais no meio de fermentação, a proporção de etanol versus água no solvente pode ser até 60-70% de etanol (Daugulis *et al.*, 1991). Porém, uma concentração menor de em torno 50% já pode ser muito interessante para a transformação de etanol em hidrogênio para a produção de energia elétrica (Lopes, 2009, Fatsikostas *et al.*, 2002). A equação 1 mostra que a estequiometria desta reação é tal que cada molécula de etanol precisa de três moléculas de água para formação de 6 moléculas de hidrogênio. Em porcentagem mássica isto se traduz em 46% de etanol e 54% de água.

$$C_2H_5OH + 3H_2O \rightarrow 6H_2 + 2CO_2 \quad - \quad [1]$$

Contudo, não há exemplos de extração simultânea de produto e inibidores provenientes de caldo hidrolítico, tema desta tese. A extração líquido-líquido pode tornar a fermentação etanólica um processo mais eficiente, provocando menos estresse à levedura e resultando em menor custo energético. Adicionalmente, o uso de um solvente para o resfriamento da dorna durante a fermentação extrativa líquido-líquido nunca foi reportado.

2.6 Escolha de Solvente

O solvente ideal para fermentação extrativa líquido-líquido pode ser idealizado a partir dos seguintes critérios (Offeman *et al.*, 2008, Wielen e Luyben, 1992, Kollerup e Daugulis, 1985, Grobben *et al.*, 1993, Maiorella *et al.*, 1984, Bruce e Daugulis, 1991, Banik *et al.*, 2003, Wang e Achenie, 2002 e elaboração própria):

- elevados coeficientes de partição para os componentes a ser extraídos (etanol e os inibidores);

- seletividade alta para os componentes de interesse (etanol e os inibidores) em comparação com outros componentes (substrato, sais e minerais, mas não necessariamente água);
- baixa solubilidade do solvente na fase aquosa;
- baixa toxicidade do solvente, tanto para o organismo empregado na fermentação (biocompatibilidade) como para operários e meio-ambiente. Por exemplo, a plantação de cana pode receber o solvente através de vinhaça usada na fertilização;
- taxa de transporte elevada dos componentes a serem extraídos;
- permitir a recuperação do produto e a regeneração do solvente;
- não formar emulsões (estáveis) durante a fermentação e/ou processos downstream;
- densidade diferente do meio, de preferência menor, para separação facilitada de fases orgânica e aquosa;
- estabilidade química do solvente, independente da utilização de altas temperaturas nos processos de recuperação ou da presença de componentes reativos, como os ácidos, no meio fermentativo;
- não degradar o produto ou causar sua degradação, ou ainda reagir com o produto para formar outro, salvo se isto for desejado, como no caso de Csányi *et al.* (2004) na formação de biodiesel a partir do solvente e etanol;
- baixos ponto de fusão e viscosidade na faixa de temperatura utilizada e ponto de ebulição acima da faixa da temperatura utilizada;
- opção de re-utilização do solvente por outros fins após ser usado várias vezes no processo fermentativo, como por exemplo como combustível no caso de ácido graxo (m)etilizado;
- alta disponibilidade no mercado e de preferência no mercado local;
- preço do solvente economicamente viável.

2.6.a Coeficientes de partição

Em um sistema bifásico, o soluto é distribuído em ambas as fases, mas em cada fase em concentrações diferentes. O coeficiente de partição do soluto descreve esta distribuição

como a razão da concentração mássica do componente na fase orgânica e na fase aquosa (equação 2, onde: m_p é o coeficiente de partição (sem unidade), w_{org}: a fração mássica do componente na fase orgânica (-), w_{aq}: a fração mássica do componente na fase aquosa).

Este coeficiente depende da temperatura, pH e concentrações de outros componentes

$$m_p = \frac{w_{aq}}{w_{org}} \quad - \quad [2]$$

em ambas as fases, que influenciam a polaridade e outras propriedades termodinâmicas do sistema. Efeitos eletroquímicos, hidrofóbicos, tamanho das moléculas envolvidas e suas conformações e outros fatores são determinantes para estabilizar corretamente o valor de coeficientes de partição (Banik *et al.*, 2003). Estes efeitos tendem a contribuir positivamente, pois na maioria dos casos, os coeficientes de partição são maiores para o meio de fermentação do que para água destilada, por causa de presença de sais, substrato, levedura e outros componentes no meio de fermentação (Kollerup e Daugulis, 1986).

Quanto maior o coeficiente de partição, maior a quantidade de produto e inibidores que podem ser removidos do meio com determinada quantidade de solvente, em certo período de tempo. Porém, para atingir uma remoção completa destes compostos seria necessário, em tese, um fluxo infinito de solvente, ou um sistema de extração com um fluxo contracorrente. Na prática, é desejável introduzir apenas um baixo fluxo de solvente no biorreator. Desta maneira evita-se a formação de emulsões e ocorre uma remoção parcial dos componentes. Assim, as concentrações de produto e inibidores no meio de fermentação (fase aquosa) podem ser mantidas suficientemente baixas para manter a produtividade do processo. Neste contexto, é interessante ressaltar que, como explicado no item 2.4.f, existe uma contribuição considerável do próprio micro-organismo na remoção de inibidores do meio, pela conversão bioquímica destes inibidores em compostos menos tóxicos, processo que pode resultar em rendimento elevado, desde que as concentrações sejam moderadas (Palmqvist *et al.*, 1999b, Almeida *et al.*, 2009). A dinâmica desta forma de remoção em relação à temperatura e a outras condições do meio de cultura também é tema de estudo neste trabalho. Embora a importância dos coeficientes de partição para a remoção dos componentes de interesse seja grande, ela não é decisiva.

2.6.b Seletividade e remoção de água

A seletividade descreve a preferência de extração de um componente em comparação a água (Bruce e Daugulis, 1991) ou outros componentes presentes no meio de fermentação (Banik *et al.*, 2003). No último caso, a seletividade pode ser anotada da seguinte forma, onde $m_p a$ é o coeficiente de partição de um componente 'a' e $m_p b$ o coeficiente de partição de componente 'b':

$$S = \frac{m_p a}{m_p b} \quad - \quad [3]$$

Jassal *et al.* (1994) verificaram, para ácido oléico como solvente, que a alta seletividade por etanol sobre a água, em um processo com fermentação extrativa líquido-líquido, seguido por evaporação flash do etanol, resultaria em custos de destilação menores do que os custos convencionais de destilação de vinho proveniente diretamente do fermentador. Daugulis *et al.* (1991) chegaram a uma conclusão semelhante para álcool oléico. Porém, estes autores deixaram de observar dois fatos.

O primeiro, como mencionado no item 2.5.b, relaciona-se com a seletividade para etanol, que quanto menor pode resultar em uma concentração de +/- 50% de etanol em água, no fluxo obtido na evaporação destes componentes do solvente após da fermentação, o que é vantajoso num processo de transformação de etanol em hidrogênio (Lopes 2009).

O segundo relaciona-se à remoção de água da dorna, que resulta em melhor utilização do substrato em um sistema contínuo, levando a rendimentos mais elevados. Em sistemas contínuos, a produtividade do micro-organismo é, entre outros fatores, determinada pela concentração de açúcar. Por exemplo, existe uma concentração de substrato ótima para produção específica de etanol. Em sistemas contínuos, no entanto, é importante manter a concentração de substrato baixa, uma vez que a concentração na saída do sistema determina a perda do substrato e, assim, diminuindo o rendimento do processo. Para sublinhar a

importância de evitar esta perda, basta lembrar que muitas vezes dornas são instaladas em série, com o único fim de converter ao máximo o substrato, mesmo que isso signifique perda de produtividade. No entanto, o uso de solvente também extrai certa quantidade de água do meio, o que concentra o substrato. Este aumento da concentração pode ser aproveitado pelo micro-organismo e a concentração final, na saída da última dorna, sendo igual ao sistema convenional (da fermentação sem solvente), resulta em um melhor aproveitamento do substrato, significando aumento de rendimento. A Figura 21 explica este princípio graficamente.

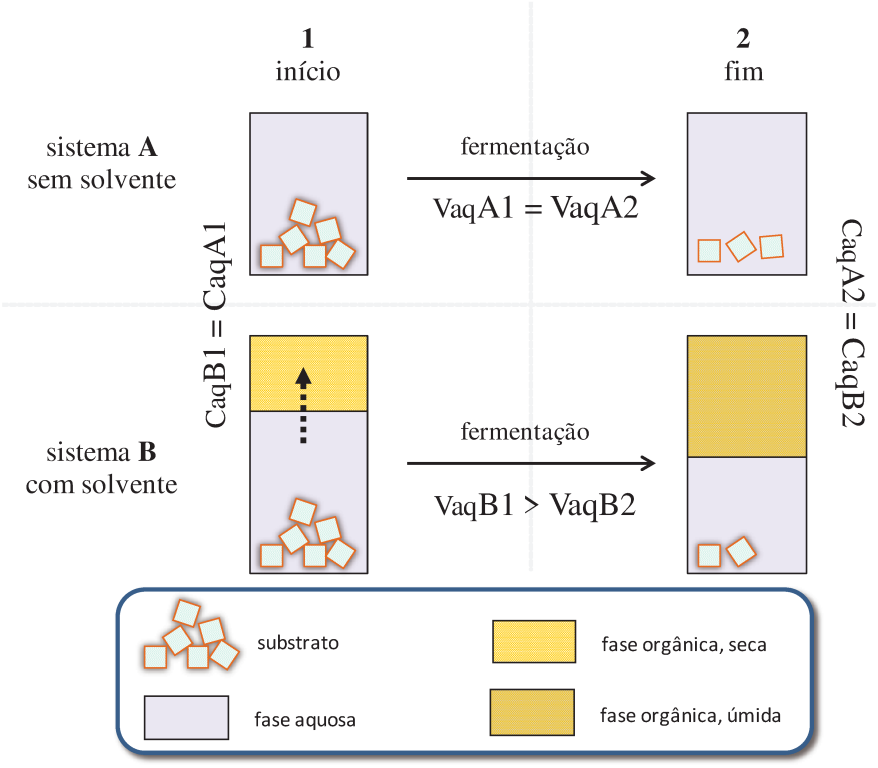


Figura 21: Aumento de conversão total de substrato no sistema com solvente. Com a extração seletiva de água da fase aquosa para a fase orgância, o volume aquoso diminui para sistema B. Porém, as concentrações finais de substrato são iguais para sistema A e B.

2.6.c Biocompatibilidade

Caso a viabilidade e a produtividade do micro-organismo diminuam devido ao uso do solvente, a produtividade específica e o rendimento da fermentação são prejudicados.

Portanto, não é viável escolher um solvente que não seja completamente biocompatível. Um fator menos crítico é o desempenho do solvente como extrator de inibidores, pois o objetivo principal do uso de qualquer solvente orgânico durante a fermentação é reduzir a concentração dos inibidores (inclusive o produto) o suficiente para otimizar o desempenho do processo, e não necessariamente remover completamente os mesmos. Com redução suficiente de suas concentrações, o micro-organismo encontra um ambiente menos estressante, com efeitos inibidores minimizados, promovendo um aumento de atividade e permitindo o melhor crescimento microbiano e produção do produto.

Evidentemente, um solvente orgânico que é biocompatível, mas que não funcione como extrator, não atuaria em termos de rendimento ou produtividade, apenas incrementaria os custos operacionais e energéticos do processo. Embora esses custos sejam relativamente baixos quando comparados aos custos de matéria-prima, pode-se entender que a escolha do solvente é um compromisso entre os custos e os benefícios das propriedades extrativas no rendimento, produtividade específica e volumétrica.

2.6.d Valor log P_{ow}

O valor log P_{ow}, logaritmo do coeficiente de partição do componente sobre a água e o octanol, é freqüentemente utilizado para a seleção de solventes orgânicos em termos de biocompatibilidade (Bruce e Daugulis, 1991): solventes com um valor log P_{ow} maior do que 4 são geralmente considerados biocompatíveis. Por definição, o coeficiente de partição para cada inibidor é também relacionado ao valor log P_{ow} do solvente, sendo o valor log P_{ow} uma função da polaridade do mesmo. Para prever a viabilidade e desempenho extrativo do solvente, pode-se, por exemplo, verificar se existe uma relação entre o valor log P_{ow} do solvente, a biocompatibilidade do mesmo e os coeficientes de partição dos componentes.

2.6.e Ponto de ebulição

Para facilitar a remoção do produto do solvente extrativo após a fermentação, os pontos de ebulição do solvente e do produto têm que ser suficientemente diferentes. Um

solvente com ponto de ebulição menor que o do produto e dos inibidores não é viável do ponto de vista biológico. Tais solventes costumam ser pequenos e/ou apolares, o que facilitaria sua difusão pela parede celular da levedura prejudicando a biocompatibilidade do solvente. Entretanto, solventes com cadeia longa como álcoois C_{12+}-H_n-OH ou ácidos graxos têm um ponto de ebulição alto e mostram maior biocompatibilidade (Minier e Goma, 1982). Um exemplo é álcool oléico, usado nas pesquisas de Daugulis e Kollerup (1985, 1994).

2.6.f Estrutura molecular

A Figura 22 mostra a estrutura química do álcool oléico: uma cadeia longa de átomos de carbono. A ligação dupla na posição 9 aumenta o tamanho tridimensional da molécula, resultando em menor densidade e menor força de Van der Waals entre as moléculas, o que é importante pois resulta em menor viscosidade.

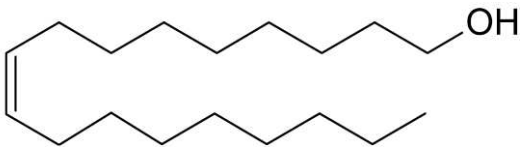


Figura 22: Álcool oléico (cis-9-octadecen-1-ol)

Porém, o grupo hidróxido é o único grupo polar. Uma razão maior entre o número de grupos polares e o tamanho total de cadeia de carbonos, ou maior hidrofilicidade do solvente em geral favoreceria coeficientes de partição maiores para etanol por causa da hidrofilicidade deste produto. Por outro lado, a maioria dos inibidores tem propriedades hidrofóbicas, requerendo justamente um solvente menos polar para maximizar sua remoção do meio de fermentação.

2.6.g Óleo de mamona e biodiesel

As características necessárias a um solvente adequado também podem ser encontradas em óleo de mamona ou no biodiesel obtido a partir de óleo de mamona. O óleo de mamona consiste por de 85-90% em ésteres de ácido ricinoléico (Conceição *et al.*, 2007, Vaisman *et al.*, 2008), uma molécula de cadeia longa que tem um grupo hidróxido na posição C-12 e uma carbonila na posição C-1. Com este último grupo o ácido ricinoléico (ácido 12-hidroxi-9-cis-octadecenóico) tem um grupo polar a mais em comparação com o álcool oléico. O biodiesel, um éster metílico ou etílico deste ácido ricinoléico, deve ter uma polaridade ainda maior, proveniente do éster com alta disponibilidade espacial. Como no caso do álcool oléico, a dupla ligação na posição C-9 favorece uma menor viscosidade.

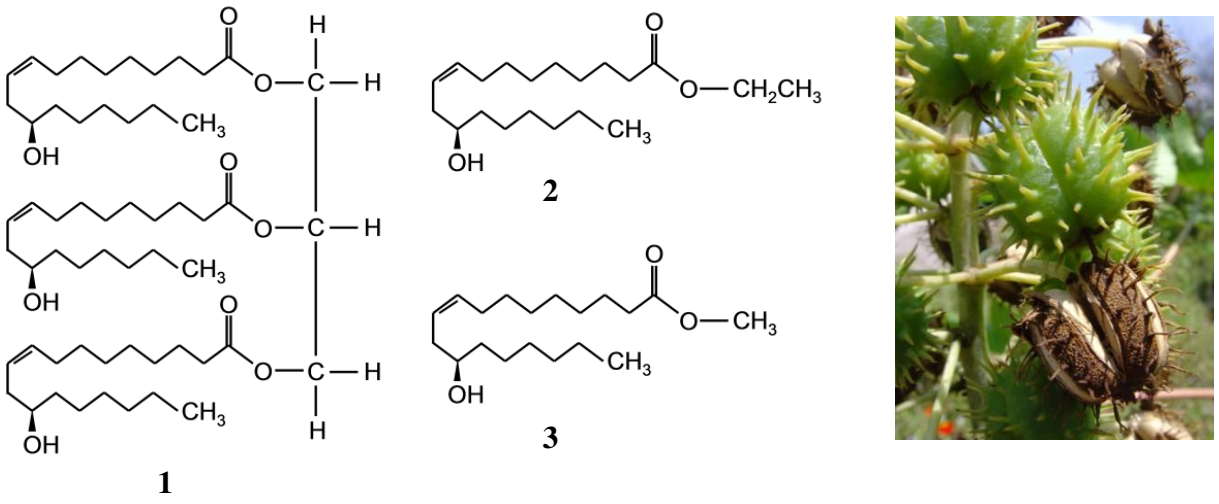


Figura 23: (1) Triglicéride de ácido ricinoléico ou óleo de mamona, (2) éster etílico de ácido ricinoléico e (3) éster metílico de ácido ricinoléico. Foto ao lado direito: mamona (fonte: imagem própria, Paraíba 2008)

Com a previsão de aumento de produção de biodiesel, e a disponibilidade de óleo de mamona e biodiesel a partir deste óleo, a utilização destes produtos como extrator *in-situ* de inibidores e produtos de fermentação pode ser economicamente interessante. Sobretudo, depois de vários ciclos de recuperação, o óleo de mamona ou o biodiesel, poderiam ser re-utilizados como combustível para a frota de transporte da própria planta como observado também por Grobben *et al.*, 1993.

2.7 Referências

Adsul M.G., Ghule J.E., Shaikh H., Singh R., Bastawde K.B., Gokhale D.V., Varma A.J. Enzymatic hydrolysis of delignified bagasse polysaccharides. Carbohydrate Polymers 62 (2005) 6-10

Aguilar R., Ramírez J.A., Garrote G., Vázquez M. Kinetic study of the acid hydrolysis of sugar cane bagasse. Journal of Food Engineering 55 (2002) 309-318

Almeida J.R.M., Modig T., Petersson A., Hahn-Hägerdal B., Lidén G., Gorwa-Grauslund M.F. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae. Journal of Chemical Technology and Biotechnology. 82 (2007) 340-349

Almeida J.R.M., Röder A., Modig T., Laadan B., Lidén G., Gorwa-Grauslund M.F. NADH-vs NADPH-coupled reduction of 5-hydroxymethyl furfural (HMF) and its implications on product distribution in *Saccharomyces cerevisiae*. Applied Microbial Biotechnology 78 (2008) 939-945

Almeida J.R.M., Bertilsson M., Gorwa-Grauslund M.F., Gorsich S., Lidén G. Metabolic effects of furaldehydes and impacts on biotechnological processes. Applied Microbial Biotechnology 82 (2009) 625-638

Andrietta S.R., Study of flocculent yeast performance in tower reactors for bioethanol production in a continuous fermentation process with no cell recycling. Bioresource Technology 99 (2008) 3002-3008.

Atala D.I.P., Costa A.C., Maciel R., Maugeri-Filho F. Kinetics of ethanol fermentation with high biomass concentration considering the effect of temperature. Applied Biochemistry and Biotechnology (2001) 91-93:353-365

Atala D.I.P. Montagem, instrumentação controle e desenvolvimento experimental de um processo fermentativo extrativo de produção de etanol. Tese de doutorado, FEA/UNICAMP 2004

Balabin R.M., Syunyaev R.Z., Karpov S.A. Molar enthalpy of vaporization of ethanol-gasoline mixtures and their colloid state. Fuel 86 (2007) 323-327

Balat M., Balat H. Recent trends in global production and utilization of bio-ethanol fuel. Applied Energy 86 (2009) 2273-2282

Banik R.M., Santhiagu A., Kanari B., Sabarinath C., Upadhyay S.N. Technological aspects of extractive fermentation using aquous two-phase systems. World Journal of Microbiology and Biotechnology 19 (2003) 337-348

Belyea R.L., Rausch K.D., Tumbleson M.E. Composition of corn and distillers dried grains with soluble from dry grind ethanol processing. Bioresource Technology 94 (2004) 293-298

Björklund L., Larsson S., Jönsson L.J., Reimann A., Nilvebrant N-O. Treatment with lignin residue; a novel method for detoxification of lignocellulose hydrolysates. Applied biochemistry and Biotechnology. 98-100 (2002) 563-575

Bothun G.D., Knutson B.L., Strobel H.J., Nokes S.E., Brignole E.A., Díaz S. Compressed solvents for the extraction of fermentation products within a hollow fiber membrane contactor. Journal of Supercritical fluids 25 (2003) 119-134

Boudreau T.M., Hill A.H. Improved ethanol-water separation using fatty acids. Process Biochemistry 41 (2006) 980-983

Brethauer S., Wyman C.E. Review: continuous hydrolysis and fermentation for cellulosic ethanol production. Bioresource Technology 101 (2010) 4862-4874

Bruce L.J., Daugulis A.J. Solvent selection strategies for extractive Biocatalysis. Biotechnology Progress 7 (1991) 116-124

Cabezas J.L., Bárcena L.a. Extraction of furfural from aqueous solutions using alcohols. Journal of Chemical Engineering Data 33 (1988) 435-437

Cândida Rabelo, S. Avaliação e otimização de pré-tratamentos e hidrólise enzimática do bagaço de cana-de-açúcar para a produção de etanol de segunda geração. Doctor Thesis (2010). State University of Campinas, Brazil. 000770600

Cartón A., González Benito G., Rey J.A., Fuente M. Selection of adsorbents to be used in an ethanol fermentation process. Adsorption isotherms and kinetics. Bioresource Technology 66 (1998) 75-78

Cardona C.A., Quintero J.A., Paz I.C. Production of bioethanol from sugarcane bagasse: status and perspectives. Bioresource Technology 101 (2010) 4754-4766

Casal M., Cardoso H., Leão C. Effects of ethanol and other alkanols on transporto f acetic acid in *Saccharomyces cereviseae*. Applied and Environmental Microbiology 64 (1998) 665-668

Chandel A.K., Kapoor R.K., Singh A., Chander-Kuhad R. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501. Bioresource Technology 98 (2007) 1947-1950

Chauvel B., Guillemin J.P., Colbach N., Gasquez, J. Evaluation of cropping systems for management of herbicide-resistant populations of blackgrass (*Alopecurus myosuroides* Huds). Crop Protection 20 (2001) 127-137

Chen W.H., Tu Y.J., Sheen H.K. Disruption of sugarcane bagasse lignocellulosic structure by means of dilute sulfuric acid pretreatment with microwave-assisted heating. Applied Energy 88 (2011) 2726-2734

Chu B.C.H., Lee H., Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation. Biotechnology Advances 25 (2007) 425-441

Conceição M.M., Candeia R.A., Silva F.C., Bezerra A.F., Fernandes V.J., Souza A.G. Thermoanalytical characterization of castor oil biodiesel. Renewable and Sustainable Energy Reviews 11 (2007) 964-975

Cordova J., Nemmaoui M., Ismaili-Alaoui M., Morin A., Roussos S., Raimbault M., Benjilaili B. Lipase production by solid state fermentation of olive cake and sugar cane bagasse. Journal of Molecular Catalysis B: Enzymatic 5 (1998) 75-78

Corradini E., Marconcini J.M., Anelli J.A.M., Mattoso L.H.C. Thermoplastic blends of corn gluten meal/starch (CGM/Starch) and corn gluten meal/polyvinyl alcohol and corn gluten meal/poly (hydroxybutyrate-co-hydroxyvalerate) (CGM/PHB-V). Carbohydrate polymers 83 (2011) 959-965

Cruz J.M., Domínguez J.M., Domínguez H., Parajó J.C. Solvent extraction of hemicellulosic Wood hydrolysates: a procedure useful for obtaining detoxified fermentation media and polyphenols with antioxidant activity. Food Chemistry 67 (1999) 147-153

Csányi E., Bélafi-Bakó K., Nemestóthy N., Gubicza L. Study on ethanol fermentation integrated with simultaneous solvent extraction and enzymatic reaction. Acta Alimentaria 33 (2004) 63-70

Daugulis A.J., Swaine D.E., Kollerup F., Groom C.A. Extractive fermentation – integrated reaction and product recovery. Biotechnology Letters 9 (1987) 425-430

Daugulis A.J., The economics of ethanol production by extractive fermentation. The Canadian Journal of Chemical Engineering 69 (1991) 488-497

Daugulis A.J., Axford D.B., Ciszek B., Malinowski JJ. Continuous fermentation of high-strength glucose feeds to ethanol. Biotechnology Letters 16 (1994) 637-642

Delgenes J.P., Moletta R., Navarro J.M. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stiptis* and *Candida shehatae*. Enzyme and Microbial Technology 19 (1996) 220-225

Doherty W.O.S., Mousavioun P., Fellows C.M. Value-adding to cellulosic ethanol: lignin polymers. Industrial crops and products 33 (2011) 259-276

Domínguez J.M.O. Efecto de los productos de degradación originados em La explosión por vapor de biomasa de chopo sobre *Kluyveromyces marxianus*. Tese de doutorado, Madrid (2003). ISBN: 84-669-1709-8

Eckert G., Schügerl K. Continuous acetone-butanol production with direct product removal. Applied Microbiology and Biotechnology 27 (1987) 221-228

EIA – Energy Information Administration. Federal Financial Interventions and subsidies in energy markets 2007. (2008) p114 (diponível em: http://www.eia.doe.gov/oiaf/servicerpt/subsidy2)

Erdemgil F.Z., Şanli S., Şanli N., Özkan G., Barbosa J., Guiteras J., Beltrán J.L. Determination of pKa values of some hydroxylated benzoic acids in methanol-water binary mixtures by LC methodology and potentiometery. Talanta 72 (2007) 489-496.

Ezeji T., Qureshi N., Blaschek P. Butanol production from agricultural residues: Impact of degradation products on *Clostridium beijerinckii* growth and butanol fermenatation. Biotechnology and Bioengineering 97 (2007) 1460-1469

Fatsikostas A. N., Kondarides D.I., Verykios X.E. Production of hydrogen for fuel cells by reformation of biomass-derived ethanol. Catalysis Today 75 (2002) 145-155.

Fenske J.J., Griffin D.A., Penner M.H. Comparison of aromatic monomers in lignocellulosic biomass prehydrolysates. Journal of Industrial Microbiology and Biotechnology. 20 (1998) 364-368

Finguerut J. I Workshop tecnológico sobre produção de etanol. Lorena EEL/USP 2006 (disponível via: http://www.apta.sp.gov.br/cana)

García-Cubero M.T. González-Benito G., Indacoechea I., Coca M., Bolado S. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresource Technology 100 (2009) 1608-1613

Giamalva M.J., Clarke S.J., Stein J.M. Sugarcane hybrids of biomass. Biomass 6 (1984) 61-68.

Gírio F.M., Fonseca C., Carvalheiro F., Duarte L.C., Marques L.C., Bogel-Łukasik R. Hemicelluloses for fuel ethanol: a review. Bioresource Technology 101 (2010) 4775-4800

Grobben N.G., Eggink G., Cuperus F.P., Huizing H.J. Production of acetone, butanol and ethanol (ABE) from potato wastes: fermentation with integrated membrane extraction. Applied Microbiol Biotechnolgy 39 (1993) 494-498

Gyamerah M., Glover J. Production of ethanol by continuous fermentation and liquid-liquid extraction. Journal of Chemical Biotechnology 66 (1996) 145-152

Hasmann F.A., Santos V.C., Gurpilhares D.B., Pessoa-Junior A., Roberto I.C. Aqueous two-phase extraction using thermoseparating copolymer: a new system for phenolic compounds removal from hemicelullosic hydrolysate. Journal of Chemical Technology and Biotechnology 83 (2008) 167-173

Heipieper H.J., de Bont J.A.M. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. Applied and Environmental Microbiology 60 (1994) 4440-4444

Herrera A., Téllez-Luis S.J., González-Cabriales J.J., Ramírez J.A., Vázquez M. Effect of the hydrochloric acid concentration on the hydrolysis of sorghum straw at atmospheric pressure. Journal of food engineering. 63 (2004) 103-109

Hodge D.B., Andersson C., Berglund K.A., Rova U. Detoxification requirements for bioconversion of softwood dilute acid hydrolyzates to succinic acid. Enzyme and Microbial Technology 44 (2009) 309-316

Holtzapple M.T., Jun J.H., Ashok G., Patibandla S.L., Dale B.E. The ammonia freeze explosion (AFEX) Process. Applied Biochemistry and Biotechnology 28-29 (1991) 59-74

Hou-Rui Z., Xiang-Xiang Q., Silva S.S., Sarrouh B.F., Ai-Hua C., Yu-Heng Z., Ke J., Qui X. Novel isolates for biological detoxification of lignocellulosic hydrolysate. Applied Biochemistry and Biotechnology. 152 (2009) 199-212

Iranmahboob J., Nadim F., Monemi S. Optimizing acid-hydrolysis: a critical step for production of ethanol from mixed Wood chips. Biomass and Bioenergy 22 (2002) 401-404

Ishizaki A.,, Michiwaki S., Crabbé E., Kobayashi G., Sonomoto K., Yoshino S. Extractive acetone-butanol-ethanol fermentation using methylated palm oil as extractant in batch culture of *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). Journal of Bioscience and Bioengineering 87 (1999) 352-556

Jassal DS, Zhang Z, Hill GA. In-situ extraction and purification of ethanol using commercial oleic acid. Can J Chem Eng 1994;72:822–827.

Jones K.D., Kompala D.S. Cybernetic model of the growth dynamics of Saccharomyces cerevisiae in batch and continuous cultures. Journal of Biotechnology 71 (1999) 105-131

Junqueira T.L., Dias M.O.S, Maciel M.R.W., Maciel-Filho R., Rossell C.E.V., Atala D.I.P. Simulation and optimization of the continuous vacuum extractive fermentation for bioethanol production and evaluation of the influence on distillation process. Computer Aided Chemical Engineering 26 (2009) 827-832

Kapucu H., Mehmetoglu U. The effects of bioprocess parameters on the yield in extractive ethanol fermentation. Reviews in Chemical Engineering 15 (1999) 307-318

Kent J.A., Riegel E.R. Handbook of industrial chemistry and biotechnology. 11th edition, volume 1 (2007) p1485. New York, Springer. ISBN: 978-0-387-27842-1

Kline K.L., Oladosu G.A., Wolfe W.K., Perlack R.D., Dale V.H. Biofuel feedstock assessment for selected countries. Oak Ridge National Laboratory, 2007. U.S. department of energy, relatório público ORNL/TM-2007/224 (disponível via: http://www.osti.gov/bridge)

Klinke H.B., Ahring B.K., Schmidt A.S., Thomsen A.B. Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresource Technology 82 (2002) 15-26

Klinke H.B., Olsson L., Thomson A.B., Ahring B.K. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: wet oxidation and fermentation by yeast. Biotechnology and Bioengineering 81 (2003) 738-747

Klinke H.B., Thomson A.B., Ahrling B.K. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Applied Microbial Biotechnology 66 (2004) 10-26

Kollerup F., Daugulis A.J. Screening and identification of extractive fermentation solvents using a database. The Canadian Journal of Chemical Engineering 63 (1985) 919-927

Kollerup F., Daugulis A.J. Ethanol production by extractive fermentation – solven identification and prototype development. The Canadian Journal of Chemical Engineering. 64 (1986) 598 - 606

Koo B.W., Kim H.Y., Park N., Lee S.M., Yeo H., Ghoi I.G. Organosolv pretreatment of Liriodendron tulipifera and simultaneous saccharification and fermentation for bioethanol production. Biomass and Bioenergy 35 (2011) 1833-1840

Kroumov A.D., Módenes A.N., Araujo-Tait M.C. Development of new unstructured model for simultaneous saccharification and fermentation of starch to ethanol by recombinant strain. Biochemical Engineering Journal 28 (2006) 243-255

Kelley K.W., Long J.H., Todd T.C. Long-term crop rotations affect soybeen yield, seed weight and soil chemical properties. Field Crops Research 83 (2003) 41-50.

Laane, C., Boeren, S., Vos, K., Veeger, C. Rules for optimization of biocatalysis in organic solvents. Biotechnology and Bioengineering, 30 (1987) 81-87

Larsson S., Palmqvist E., Hahn-Hägerdal B., Tengborg C., Stenberg K., Zacchi G., Nilvebrant N.O. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme and Microbial Technology 24 (1999) 151-159

Larsson S., Reimann A., Nilvebrant N-O., Jönsson L.J. Comparison of different methods for the detoxification of lignocellulosic hydrolyzates of spruce. Applied Biochemistry and Biotechnology 77-79 (1999) 91-103

Larsson S., Quintana-Sáinz A., Reimann A., Nilvebrant N.O., Jönsson L.J. Influence of Lignocellulosic-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*. Appliec Biochemistry and Biotechnology 84 (2000) 617-632.

Larsson S., Cassland P., Jönsson L.J. Development of a Saccharomyces cerevisiae strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. Applied and Environmental Microbiology. 67 (2001) 1163-1170

Larsson S., Nilvebrant N.O., Jönsson L.J. Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. Applied Microbial Biotechnology. 57 (2001) 167-174

Lee D., Owens V.N., Boe A., Jeranyama P. Composition of Herbaceous Biomass Feedstocks. South Dakota State University Report 2007 (disponível via: http://ncsungrant1.sdstate.org/uploads/publications/SGINC1-07.pdf)

Lee W.L., Jeffries T.W. Efficiencies of acid catalysts in the hydrolysis of lignocellulosic biomass over a range of combined severity factors. Bioresource Technology 102 (2011) 5884-5890

Legendre B.L., Burner D.M. Biomass production of sugarcane cultivars and early-generation hybrids. Biomass and Bioenergy 8 (1995) 55-61

Leibbrandt N.H., Knoetze J.H., Görgens J.F. Comparing biological and thermochemical processing of sugarcane bagasse: an energy balance perspective. Biomass and Bioenergy 35 (2011) 2117-2126

Lin C.W., Wu C.H., Tran D.T., Shih M.C., Li W.H., Wu C.F. Mixed culture fermentation from lignocellulosic materials using thermofillic lignocellulose-degrading anaerobes. Process Biochemistry 46 (2011) 489-493

Liu D.L., Christians N.E. Bioactivity of pentapeptide isolated from corn gluten hydrolysate on *Lolium perenne L.* Journal of Plant Growth Regulation 15 (1996) 13-17

Liu Z.L., Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Applied Microbial Biotechnology 90 (2011) 809-825

Lopes D.G. Análise técnica e econômica da inserção da tecnologia de produção de hidrogênio a partir da reforma de etanol para geração de energia elétrica com células a combustível. Tese de Doutorado (2009) FEM/UNICAMP

Lynd L.R., Zyl W.H., McBride J.E., Laser M. Consolidated bioprocessing of cellulosic biomass: an update. Current Opinion in Biotechnology 16 (2005) 577-583

Maiorella B.L., Blanch H.W., Wilke C.R. Feed component inhibition in ethanolic fermentation by *Saccharomyces cerevisiae*. Biotechnology and Bioengineering 26 (1984) 1155-1166

Mantelatto P.E. Estudo do processo de cristalização de soluções impuras de sacarose de cana-de-açúcar por resfriamento. Dissertação de Mestrado UFSCAR, São Carlos, 2005

Martin C, Marcet M, Almazán O, Jönssen LJ. Adaptation of a recombinant xylose-utilizing Saccharomyces cerevisiae strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresource Technology 98 (2007) 1767-1773

Martin M.A. First generation biofuels compete. New Biotechnology 27 (2010) 597-607

Martinez A. Rodriguez M.E., Wells M.L., York S.W., Presont J.F., Ingram L.O. Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnology Progress 17 (2001) 287-293

Martínez Á.T., Speranza M., Ruiz-Dueñas F.J., Ferreira P., Camerero S., Guillén F., Martínez M.J., Gutiérrez A., Río J.C. Biodegradation of lignocellulosics: microbial, chemical and enzymatic aspects of the fungal attack of lignin. International Microbiology 8 (2005) 195-204

Méjean A., Hope C., Modelling the costs of energy crops: A case study of US corn and Brazilian sugar cane. Energy Policy 38 (2010) 547-561

Minier M., Goma G. Ethanol production by extractive fermentation. Biotechnology and Bioengineering. 24 (1982) 1565-1579

Modig T., Lidén G., Taherzadeh M.J. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochemistry Journal 363 (2002) 769-776

Moritz J.W., Duff S.J. Simultaneous saccharification and extractive fermentation of cellulosic substates. Biotechnology and Bioengineering 49 (1996) 504-511

Mosier N., Wyman C., Dale B., Elander R., Lee Y.Y., Holtzapple M., Ladisch M. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96 (2005) 673-686

Mussatto S.I., Roberto I.C. Alternatives for detoxificationof diluted-acid lignocellulosic hydrolyzates for use in fermentative process: a review. Bioresource Technology 93 (2004) 1-10

Mussatto S.I., Dragone G., Guimarães P.M.R., Silva J.P.A., Carneiro L.M., Roberto I.C., Vicente A., Domingues L., Teixeira J.A. Technological trends, global market and challenges of bio-ethanol production. Biotechnology Advances 28 (2010) 817-830

Nilsson A., Taherzadeh M.J., Lidén G. On-line estimation of sugar concentration for control of fed-batch fermentation of lignocellulosic hydrolyzates by *Saccharomyces cerevisiae*. Bioprocess Biosystems Engineering 25 (2002) 183-191

Nolasco-Junior J. Desenvolvimento de processo térmico otimizado para mosto de caldo de cana na fermentação alcoólica. Dissertação de Mestrado UNCAMP, Campinas 2005

Offeman R.D., Stephenson S.K., Franqui D., Cline J.L., Robertson G.H., Orts W.J. Extraction of ethanol with higher alcohol solvents and their toxicity to yeast. Separation and Purification Technology 63 (2008) 444-451

Ogeda T.L., Petri D.F.S. Hidrólise enzimática de biomassa. Química Nova 33 (2010) 1549-1558

Oliva J.M., Sáez F., Ballesteros I., González A., Negro M.J., Manzanares P., Ballesteros M. Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. Applied Biochemistry and Biotechnology 105 (2003) 141-153

Oliva J.M., Ballesteros I., Negro M.J., Manzanares P., Cabañas A, Ballesteros M. Effect of Binary Combinations of selected toxic compounds on growth and fermentation of *Kluyveromyces marxianus*. Biotechnology Progress 20 (2004) 715-720

Oliva J.M., Manzanares P., Ballesteros I., Negro M.J., González A., Ballesteros M. Application of Fenton´s reaction to steam explosion prehydrolysates from poplar biomass. Applied Biochemistry and Biotechnology 121-124 (2005) 886-899

Oliva J.M., Negro M.J., Sáez F., Ballesteros I., Manzanares P., González A., Ballesteros M. Effects of acetic acid, furfural and catechol combinations on ethanol fermentation of *Kluyveromyces marxianus*. Process Biochemistry 41 (2006) 1223-1228

Oliveira A.C., Rosa M.F., Cabral J.M.S., Aires-Barros M.R. Improvement of alcoholic fermentations by simultaneous extraction and enzymatic esterification of ethanol. Journal of Molecular Catalysis B: Enzymatic 5 (1998) 29-33

Olivério J.L., Hilst, A.G.P. Revolutionary process for producing alcohol from sugar cane bagasse. Guatemala, 2005, XXV ISSCT Congress

Olivério J.L., Augosto-Soares P. Produção de álcool a partir de bagaço: o processo DHR – Dedini Hidrólise Rápida. Novas Tecnologias para Bioenergia, Piracicaba 2007 (Desponível via: http://www.iea.usp.br/)

Olsson L., Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme and microbial technology 18 (1996) 312-331

Palmqvist E., Almeida J.S., Hahn-Hägerdal B. Influence of furfural on anaerobic glycolitic kinetics of *Saccharomyces cerevisia*e in batch culture. Biotechnology and Bioengineering 62 (1999a) 447-454

Palmqvist E., Grage H., Meinander N.Q., Hahn-Hägerdal B. Main and interaction effects of acetic acid, furfural and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnology and Bioengineering 83 (1999b) 47-55

Palmqvist E., Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification 74 (2000) 17-24

Pampulha M.E., Loureio-Dias M.C. Energetics of the effect of acetic acido on growth of *Saccharomyces cerevisiae*. FEMS Microbiology Letters 184 (2000) 69-72

Rahman M.A., Asadullah M., Rahman M.S., Nabi M.N., Azad M.A.K. Extractioon of gasohol grade ethanol from aqueous solution using gasoline as solvent. Bangladesh Journal of Scientific and Industrial Research 42 (2007) 287-298

Raminez E.C., Johnston D.B., McAloon A.J., Yee W., Singh V. Engineering process and cost model for a conventional corn wet milling facility. Industrial crops and products 27 (2008) 91-97

Ramos L.P. The Chemistry involved in the steam treatment of lignocelulosic materials. Química Nova 26 (2003) 863-871

Rivera E.C., Costa A.C., Atala D.I.P, Maugeri-Filho F., Wolf-Maciel M.R., Maciel-Filho R. Evaluation of optimization techniques for parameter estimation: Application to ethanol fermentation considering the effect of temperature. Process Biochemistry 41 (2006) 1682-1687

Roffler S.R., Blanch H.W., Wilke C.R. In Situ Extractive Fermentation of Acetone and Butanol. Biotechnology and Bioengineering 31 (1987)135-143

Rossell, C.E.V., Qualidade da matéria-prima. I Workshop tecnológico sobre produção de etanol. Lorena EEL/USP 2006 (disponível via: http://www.apta.sp.gov.br/cana)

Rubin E.M. Genomics of cellulosic biofuels. Nature 451 (2008) 841-845

Sánchez O.J., Cardona C.A. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresource Technology 99 (2008) 5270-5295

Sacks W.J., Kucharik C.J. Crop management and phenology trends in the U.S. Corn Belt: impacts on yields evapotranspiration and energy balance. Agricultural and forest meteorology (2011). (Artigo na imprensa)

Sainio T., Turku I., Heinonen J. Adsorptive removal of fermentation inhibitors from concentrated hydrolyzates of lignocellulosic biomass. Bioresource Technology 102 (2011) 6048-6057

Sánchez Ó.J. Cardona C.A. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresource Technology 99 (2008) 5270-5295

Seiler M., Köhler D., Arlt W. Hyperbranched polymers: new selective solvents for extractive distillation and solvent extraction. Separation and Purification Technology 30 (2003) 179-197

Soto M.L., Moure A., Domínguez H., Parajó J.C. Recovery, concentration and purification of phenolic compounds by adsorption: A review. Journal of Food Engineering 105 (2011) 1-27

Spiridon I., Popa V.I. 13: Hemicelluloses: major sources, properties and applications. Monomers, Polymers and Composities from Renewable Resources ISBN: 978-0-08-045316-3 (2008) p289

Sriyudthsak K., Shiraishi F. Investigation of the performance of fermentation process using a mathematical model including effects of metabolic bottleneck and toxic product on cells. Mathematical Biosciences 228 (2010) 1-9

Sun Y., Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83 (2002) 1-11

Szijárto N., Siika-aho M., Tenkanen M., Alapuranen M., Vehmaanperä J., Réczey K., Viikari L. Hydrolysis of amorphous and crystalline cellulose by heterologously produced cellulases of *Melanocarpus albomyces*. Journal of Biotechnology 136 (2008) 140-147

Tadeu J. Produção de Alimentos vezes produção de biocombustíveis. Apresentação SBPC 2008 (desponível em: www.apta.sp.gov.br/cana/anexos/)

Taherzadeh M.J., Niklasson C., Lidén G. Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation. Bioresource Technology 69 (1999) 59-66

Talebnia F., Taherzadeh M.,J. In situ detoxification and continuous cultivation of dilute-acid hydrolyzate to ethanol by encapsulated S. cerevisiae. Journal of Biotechnology 125 (2006) 377-384

Teixeira M.C., Raposo L.R., Mira N.P., Lourenço A.B., Sá-Correia I. Genome-wide identification of *Saccharomyces cerevisiae* genes required for maximal tolerance to ethanol. Appied and Environmental Microbiology 75 (2009) 5761-5772

Timilsina G.R., Shrestha A. The growth of transport sector CO_2 emissions and underlying factors in latin America and the Carribian. World-Bank – Policy research working paper 4734 (2008)

Unrean P., Srienc F. Continuous production of ethanol from hexoses and pentoses using immobilized mixed cultures of *Escherichia coli* strains. Journal of Biotechnology 150 (2010) 215-223

Vaisman, B., Shikanov, A., Domb, A.J., "The isolation of ricinoleic acid from castor oil by salt-solubility-based Fractionation for the biopharmaceutical applications" Journal of the American Oil Chemists' Society 85 (2008) 169-184

Vasilakoglou I., Dhima K., Karagiannidis N., Gatsis T., Sweet sorghum productivity for biofuels under increased soil salinity and reduced irrigation. Field crops research 120 (2011) 38-46

Waibel B.J., Square K., Krukonis V.J. Energy Efficient Separation of ethanol from aqueous solution. US Patent 2010 0069686 A1

Walker G.M. Yeast physiology and biotechnology. 2000. Wiley and Sons, West Sussex, England.

Wang Y., Achenie E.K. Computer aided solvent design for extractive fermentation. Fluid Phase Equilibria 201 (2002) 1-18

Weilnhammer C., Blass E. Continuous fermentation with product recovery by in-situ extraction. Chemical Engineering and Technology 17 (1994) 365-373

Wielen L.A.M, Luyben KC. Integrated product formation and recovery in fermentation. Current Opinion in Biotechnology 3 (1992) 130–138.

World Energy Council. Survey of Energy Resources 2010. p371-373. (disponível via: http://www.worldenergy.org/publications)

Yeoman C.J., Han Y., Dodd D., Schroeder C.M., Mackie R.I., Cann I.K.O. Thermostable enzymes as biocatalists in the biofuel industrie. Advances in Applied Microbiology 70 (2010) 1-55

Yu H., Guo G., Zhang X., Yan K., Xu C. The effect of biological pretreatment with the selective whit-rot fungus *Echinodontium taxodii* on enzymatic hydrolysis of softwoods and hardwoods. Bioresource Technology 100 (2009) 5170-5175

Zautsen R.R.M. Influence of aeration on fermentation of hydrolitic liquor from lignocellulosic biomass. CTC relatório interno, Piracicaba 2004.

Capítulo 3

Liquid-liquid extraction of fermentation inhibiting compounds in lignocellulose hydrolyzate

3.1 Abstract

Several compounds that are formed or released during hydrolysis of lignocellulosic biomass inhibit the fermentation of the hydrolysate. The use of a liquid extractive agent is suggested as a method for removal of these fermentation inhibitors. The method can be applied before or during the fermentation. For a series of alkanes and alcohols, partition coefficients were measured at low concentrations of the inhibiting compounds furfural, hydroxymethyl furfural, vanillin, syringaldehyde, coniferyl aldehyde, acetic acid, as well as for ethanol as the fermentation product. Carbon dioxide production was measured during fermentation in the presence of each organic solvent to indicate its biocompatibility. The feasibility of extractive fermentation of hydrolysate was investigated by ethanolic glucose fermentation in synthetic medium containing several concentrations of furfural and vanillin and in the presence of decanol, oleyl alcohol and oleic acid. Volumetric ethanol productivity with 6 g/L vanillin in the medium increased twofold with 30% volume oleyl alcohol. Decanol showed interesting extractive properties for most fermentation inhibiting compounds, but it is not suitable for *in situ* application due to its poor biocompatibility.

Referência da publicação:

Zautsen RRM, Maugeri-Filho F, Vaz-Rossell CE, Straathof AJJ, Wielen LAM, Bont JAM. 2008. Biotechnology and Bioengineering 102:1354-1360

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Liquid–Liquid Extraction of Fermentation Inhibiting Compounds in Lignocellulose Hydrolysate

R.R.M. Zautsen,[1] F. Maugeri-Filho,[1] C.E. Vaz-Rossell,[2] A.J.J. Straathof,[3] L.A.M. van der Wielen,[3] J.A.M. de Bont[3]

[1]Department of Food Engineering, FEA, State University of Campinas, UNICAMP, R. Monteiro Lobato 80, CEP: 13081-970, Cidade Universitária, Campinas, SP, Brazil; telephone: +55 19 3521 4034; fax: 55 19 3521 4027; e-mail: maugeri@fea.unicamp.br

[2]Department of Chemical Processes, FEQ, State University of Campinas, UNICAMP, Av. Albert Einstein 500, Campinas, SP, Brazil

[3]Department of Biotechnology, Delft University of Technology, Delft, The Netherlands

Received 7 July 2008; revision received 14 October 2008; accepted 27 October 2008

Published online 3 November 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22189

3.2 Introduction

During hydrolysis of lignocellulosic biomass, such as sugar cane bagasse or wheat straw, hexoses and pentoses are produced that can be fermented into ethanol or other organic products. However, furans and carboxylic acids are often formed as by-products of the hydrolysis process, and phenolic compounds are released from complex lignin structures. These substances are known to inhibit the fermentation process (Larsson *et al.*, 1999). To improve fermentation of hydrolysed lignocellulosic biomass, inhibiting compounds should be removed from the fermentation medium, for example by an extractive solvent. Such an extraction process could take place *in situ* during fermentation or outside the fermenter as a parallel or pretreatment process, each option leading to different thermodynamical extraction environments and a distinct organic solvent and product recovery process. *In situ* removal during fermentation has the advantage of being a one-step process, potentially reducing investment and operating costs. Extraction of inhibiting components could be performed simultaneously with *in situ* product recovery e.g. in the case of ethanol, reducing product inhibition (Daugulis *et al.*, 1987). Disadvantage is the necessity for the organic solvent to be highly biocompatible. In this study, several ethanol fermentation inhibitors were subjected to partitioning over a series of alkanes, higher alcohols and other organic solvents. Biocompatibility of these organic solvents was measured and feasibility of *in situ* fermentation was indicated by conducting fermentations in defined medium.

Generally three groups are distinguished as inhibiting components that result from the hydrolysis of biomass: furans, phenolic compounds and carboxylic acids (Klinke *et al.*, 2004). Furfural and hydroxymethyl furfural are the furans that are most often mentioned for their inhibitive effect. Furfural, formed by acidic pentose degradation, inhibits specific growth rate and ethanol productivity. It is typically present in bagasse hydrolysate but can be slowly converted into the less toxic furfuryl alcohol by *Saccharomyces cerevisiae*. Hydroxymethyl furfural, formed by acidic hexose degradation, is generally present in lower concentrations than furfural and is less inhibiting than furfural, but is converted into

hydroxymethyl-furfuryl alcohol at a four times lower rate, overall resulting in important inhibiting effects (Taherzadeh, 1999).

Vanillin, syringaldehyde and coniferylaldehyde have been identified as the most important inhibiting phenols (Delgenes *et al.*, 1996, Larsson *et al.*, 2000). These aromatic compounds are released from lignin structures during hydrolysis and tend to integrate in the yeast cell membrane. This uncontrolled modification of the membrane leads to loss of integrety and selectivity of the membrane as a barrier and eventually decreased ethanol yield (Palmqvist *et al.*, 2000).

Acetic acid, produced during hydrolysis and during fermentation by yeast, is an important inhibiting carboxylic acid, as are lactic, formic and levulinic acid. Undissociated acids diffuse through the cell membrane and subsequently dissociate in the yeast cytosol and acidify the pH of the cytosol, decreasing the proton motive force over the membrane (Palmqvist *et al.*, 2000). This effect becomes more important as the ethanol concentration increases, since ethanol inhibits the efflux of acetate and thus causes anion accumulation (Casal *et al.*, 1997). Also decreased xylose reductase activities have been reported as a result of acetic acid (Lima *et al.*, 2004). Finally, the fermentation product ethanol reduces the specific growth rate of the yeast, depending on its concentration. Figure 1 shows the chemical structures of several studied inhibitors.

As extractive agents, alkanes were chosen as a first approach *i.e.* hexane, octane, decane, dodecane, tetradecane and hexadecane. Alkanes lack any functional polar group, in contrast to all studied fermentation inhibiting solutes, thus elevated partition coefficients could be expected if alcohols were to be used as solvent instead of alkanes. However, to invest a consistent range of organic solvents on biocompatibility and partition coefficients, it is a disadvantage that the melting points of dodecanol and higher alcohols are relatively high, ruling out these solvents. Partition coefficients for hexanol, octanol and decanol were also measured, as were partition coefficients for oleyl alcohol and oleic acid because these latter solvents have been indicated by literature as interesting for *in situ* (ethanol) extraction due to their complete biocompatibility (Daugulis *et al.*, 1994, Jassal *et al.*, 1994).

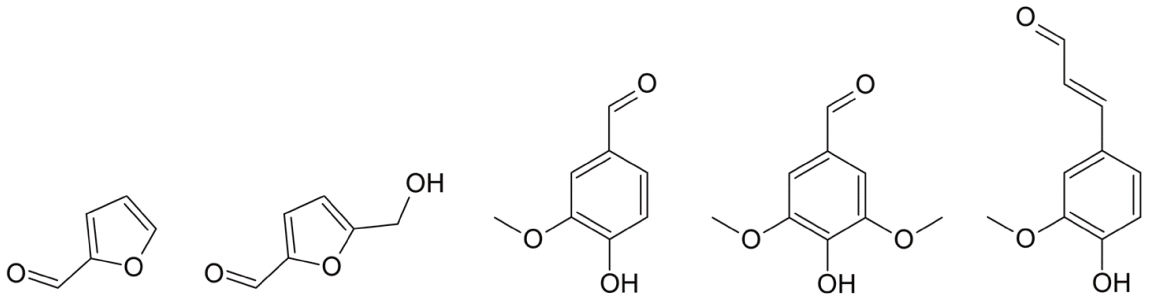


Figure 1: Chemical structure of investigated furans and phenolic compounds

In general, partition coefficients depend on the concentration of the solute. However, except for ethanol, the concentrations of the studied inhibitors were 0.1% w/w in the initial aqueous phase, which is relatively low and representative for the concentrations of the solutes in the actual hydrolytic liquor. In this order of magnitude, changes in concentration during fermentation due to metabolic processes can be assumed to be too small to influence the partition coefficient. Ethanol is produced and its concentration generally increases from 0 % w/w to up to 15 % w/w in long batch fermentations, whereas furfural is converted by the yeast cells in furfuryl alcohol, thus its concentration typically decreases. Also it was decided to determine all partition coefficients both at the typical pH of hydrolysed lignocellulose (pH 2.3) and at the pH of a typical fermentation (pH 4.5). Partition coefficients for ethanol were only measured at pH 4.5, since ethanol is only present during fermentation.

The log P_{ow} value, the logarithmic of the partition coefficient of a compound over water and octanol, is traditionally used as a rule of thumb for selection of organic solvents in terms of biocompatibility (Laane *et al.*, 1987). Solvents with log P_{ow} values higher than 4 are mostly found to be biocompatible, thus not negatively influencing the viability or the stability of the yeast cell. To predict the viability of the suggested extractive process, it is interesting to obtain practical insight in the relation between the solvent log P_{ow} value, partition coefficients of the solutes, and biocompatibility of the organic solvent. As the log P value of the solvent is a function of the polarity of the solvent, the biocompatibility and

the partition coefficient for different solutes are probably related to the log P_{ow} value of the organic solvent.

The goal of this investigation was to obtain a fundamental understanding of the extractive removal of inhibitors. In future work, this knowledge can be used for optimizing extraction procedures at industrially relevant conditions.

3.3 Material and methods

3.3.a Partition coefficients

HPLC was used for analysis of furfural, HMF, vanillin, syringaldehyde and coniferyl aldehyde (Waters 486 UV detector, Waters auto injector 717 and pump 510, and a SpeedROD Chromolith column RP 18-e no. UM2112/006, 50-4-6mm, connected to a Phenomenex C18 4mm*3mm as precolumn), using furfuryl alcohol, furfural or vanillin as internal standard (average R^2 0.999). HPLC was also used to measure acetic acid concentrations (Shimadzu SPD10A UV, Phenomenex, Rezex ROA Organic Acid Column 00H-0138-K0, 300*7.80mm, 8μm; precolumn Phenomenex Carbo-Ca-2+ 4mmL * 3mm, Chrompack column heater at 60 °C, Perkin Elmer 200 injector and a Waters 590 pump). For furfural, vanillin, syringaldehyde and coniferyl aldehyde, an 18% acetonitrile solution with 0.002 M H_3PO_4 was used as eluent (1 mL/min). For HMF, a 2.5% acetonitrile solution with 0.002 M H_3PO_4 was used (1 mL/min). For acetic acid, a 0.005 N H_2SO_4 solution was used (6.5 mL/min). UV spectra were made from each compound to decide for the best UV absorbance compromise for each solute and external standard.

Concentrations of furans and phenolic compounds were measured according to the following protocol. A solute solution in milli-Q water was prepared and adjusted to the desired pH by addition of a sulphuric acid or potassium hydroxide solution to reach both the desired pH and a solute concentration of 0.1 % w/w. Approximately 5 g of the solution were added to a 10 ml bottle, determining the exact weight of added solution with an analytical balance (Sartorius CP224S analytical balance), as was done for 5 g of organic solvent. The bottle was shaken heavily and stirred for 4 h with a magnetic stirrer in a water

bath at 34.0 ºC. In at least triplo, of both the organic and the aqueous phase, 100 µL were removed and added to 9 mL of a stock external standard solution of milli-Q water containing 0.0015 % w/w external standard. Sample and stock external standard solution were both quantified by weight. The solutions were shaken heavily. The bottles containing the organic phase samples were left for at least 2 h at 34.0 ºC to ascertain reversed extraction of the solute from the organic phase sample into the stock external standard solution. From each sample solution, 10 µL aqueous phase were injected into the HPLC system to determine the mass fraction of the solute. An initial mass fraction of the solute in the original organic phase was estimated by a mass balance calculation. Subsequently, an initial partition coefficient was calculated as the quotient of the mass fractions of the solute in the organic and the water phase. The concentration of solute present in the relatively small organic phase in the reversed extracted sample system was then determined iteratively, using the measured concentration of the solute in the aqueous phase of both the original two-phase system and the reversed extracted sample system and adapting the partition coefficient to close the total mass balance over the two systems. From each triple sample set the resulting partition coefficients were averaged.

Partition of ethanol was measured at least in triplicate by gas chromatography (Varian Star 3400CX, CP WAX column 52CB 50m*0.53mm, N_2 carrier gas), using butanol as external standard ($R^2 > 0.999$). Both phases were diluted hundred times in stock Methyl Tert-Butyl Ether (MTBE) containing butanol as external standard. These solutions were then dried with magnesium sulphate to prevent injection of water into the gas chromatograph and to force the ethanol to migrate completely into the stock MTBE external standard solution. From each triple sample set the results were again averaged.

The following chemicals were used: furfural (Acros Organics, 99%), hydryxymethyl furfural (Acros Organics, 98%), vanillin (Fluka, 98%), syringaldehyde (Acros Organics, 98%), coniferyl aldehyde (Aldrich, 98%), acetic acid (Acros Organics, 99.5%), hexane (Acros Organics, 99.85%), octane (Acros Organics, 99%), decane (Acros Organics, 99%), dodecane (Acros Organics, 99%), tetradecane (Acros Organics, 99%), hexadecane (Acros Organics, 99%), hexanol (Acros Organics, 98%), octanol (Acros Organics, 99%), decanol

(Acros Organics, 99%), oleyl alcohol (Acros organics, 70%), oleic acid (Fisher Scientific, 70%), D-Glucose, (Acros Organics, anhydrous), Yeast extract powder (Acros Organics), Peptone (Brunschwig Chemie, bacto, enzymatic protein digest).

3.3.b Biocompatibility

Biocompatibility was derived from a change in CO_2 production during fermentation after addition of 5% organic solvent to the medium. Standard Yeast Peptone Dextrose (YPD) fermentation medium was prepared with 22 g/L glucose, 20 g/L peptone and 10 g/L yeast extract. To 100 ml bottles with septum covers, 200 mg bakers yeast was added and flushed with nitrogen to reduce the initial carbon dioxide concentration in the bottle to zero and create an anaerobic gas phase. Ten mL of YPD medium was chosen empirically to establish a constant CO_2 production rate during 40 min in organic solvent free fermentation medium. Fermentations took place at 34 ºC and uncontrolled pH while gently stirring the flasks. CO_2 samples of the gas phase were taken every 3 min and analysed with a Gas Chromatograph (Hewlett Packard 5890 series II, Carboplot P7 column, 0.53 mm, 25 m, He carrier gas) equipped with a TCD detector, allowing two periods of six measurements during the constant CO_2 production phase. Organic solvent was added after 30 min of fermentation except to blanc fermentations. The slopes of the first and the last 20 min of constant CO_2 production were compared and their quotient, expressed in percentage, was used to indicate biocompatibility. Thus with both slopes equal, the quotient was 100%, indicating 100% biocompatibility as shown in Figure 2.

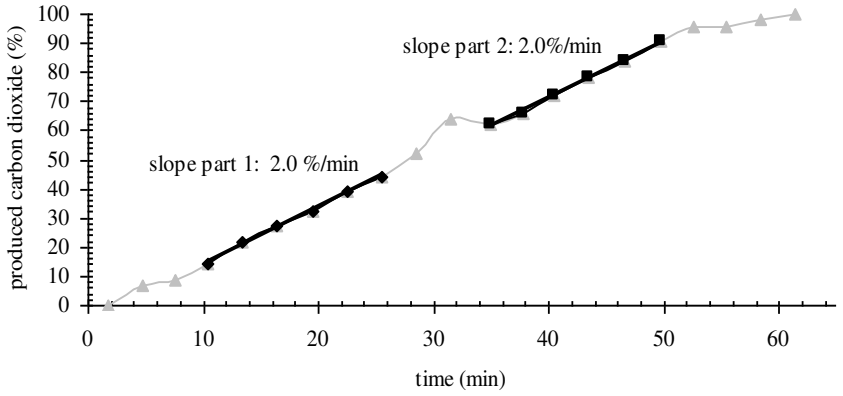
On the other hand, with a hypothetical horizontal slope of the second 20 min indicating no further CO_2 production after addition of the organic solvent, the quotient would be zero, thus demonstrating 0% biocompatibility. The measured CO_2 concentration was not calibrated, but expressed in percentage of concentration measured after 60 min of fermentation. To compensate for inconsistencies in the 5 µL gas samples injection volumes, the CO_2 concentration was measured relative to the sum of detected carbon dioxide and

nitrogen, corrected for the difference in thermal conductivity relative to the carrying gas helium, according to equation 1.

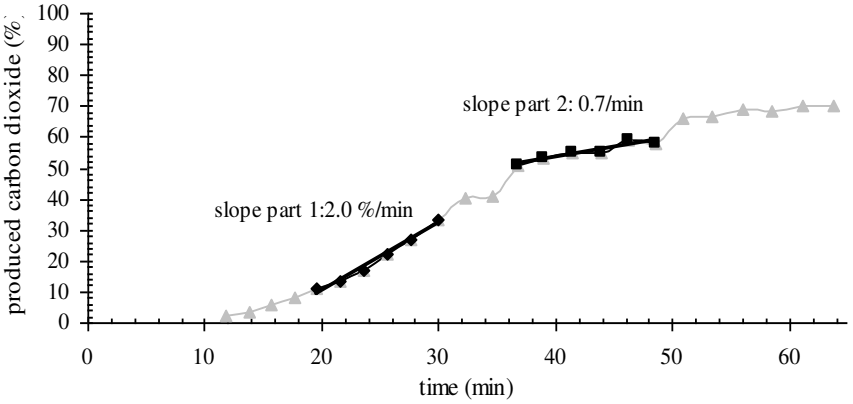
$$\left[\frac{Area_{CO_2}}{Area_{N_2}+Area_{CO_2}\cdot b}\right]_t \cdot \left[\frac{Area_{CO_2}}{Area_{N_2}+Area_{CO_2}\cdot b}\right]_{t_{end}}^{-1} \cdot 100 \qquad \textit{CO}_2\textit{ produced (\% of total)} \qquad [1]$$

$Area_X$: the peak area of the gas fraction in the gas chromatogram, tc_{N1}: thermal conductivity of Nitrogen, 18.7 mW/cm/K, tc_{CO2}: thermal conductivity of carbon dioxide, 9.6 mW/cm/K, tc_{He}: thermal conductivity of helium, 119.3 mW/cm/K, values from CRC Press with b a correction factor for the difference of thermal conductivity of CO_2 and N_2 relative to the thermal conductivity of He:

$$b = 1 + \frac{tc_{N_2} - tc_{CO_2}}{tc_{He}} \qquad - \qquad [2]$$



A



B

Figure 2: A. Blank experiment; the quotient of the slope of second part and the first part equals 1. The slope does not increase during the first 60 min, thus no growth of the yeast takes place. B. Addition of hexane after 30 minutes

3.3.c Feasibility of the approach

The feasibility of the extractive fermentation of lignocellulosic hydrolysate was demonstrated by a series of defined medium fermentations in 30 ml flasks. Each flask contained 18 ml YPD medium with 80 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 3 g/L dried bakers yeast, Milli-Q water and 0 to 6 g/L furfural or vanillin. Each flask further contained either no organic solvent, or 30% in volume decanol, oleyl alcohol or oleic acid. The flasks were stirred heavily to maximize contact between organic solvent and yeast. After 1 h fermenting at 34ºC and at uncontrolled pH, 1 ml sample was taken every hour during 5 h. The samples were centrifuged and 300 µL of the aqueous phase was added to

400 µL 2% citric acid (external standard) in water solution and 300 µL of the organic phase was added to 1 ml 1% hexanol (external standard) in decanol solution, weighing both samples and external standard solutions with an analytical balance.

Glucose and ethanol were measured in the water phase with HPLC (waters 590 pump, Phenomenex, Rezex ROA Organic Acid Column 00H-0138-K0, 300*7.80mm 8µm; precolumn Phenomenex Carbo-Ca-2+ 4mmL * 3mm, 60 °C Chrompack column heater, Perkin Elmer 200 injector, equipped with a RI detector (Shimadzu RID10A), using 0.0025 M H_2SO_4 as eluent at 6.5 mL/min). Ethanol was measured in the organic phase with a gas chromatograph (Shimadzu GC2014, CPSil5CB 50m*0.53mm, equipped with a Shimadzu AOC-20I auto injector).

3.4 Results

3.4.a Partition coefficients

Table 1 shows the partition coefficients of the solutes as measured for each organic solvent, at pH 2.3 and pH 4.5. As can be seen in Table , the partition coefficients are pH independent except for acetic acid. For both the series of alkanes and alkanols, partition coefficients of all solutes decrease as the log P_{ow} of the organic solvent increases. However, partition coefficients of the solutes into alkanols are higher than into alkanes, even for comparable log P_{ow} values. Partition coefficients for ethanol are elevated at higher ethanol concentrations.

3.4.b Biocompatibility

Experiments without addition of organic solvent showed no change in CO_2 production rate (Figure 2A). However, Figure 2b shows that the CO_2 concentration decreased after addition of hexane at 30 min of fermentation. 100% CO_2 corresponds to the

CO_2 concentration measured without addition of an organic solvent at 60 min. Results from all shaked flask fermentations in the presence of alkanes, alkanols and oleic acid as solvents are summarised in Figure 3, where the biocompatibility of each tested organic solvent is expressed as a function of the log P_{ow} value of that organic solvent.

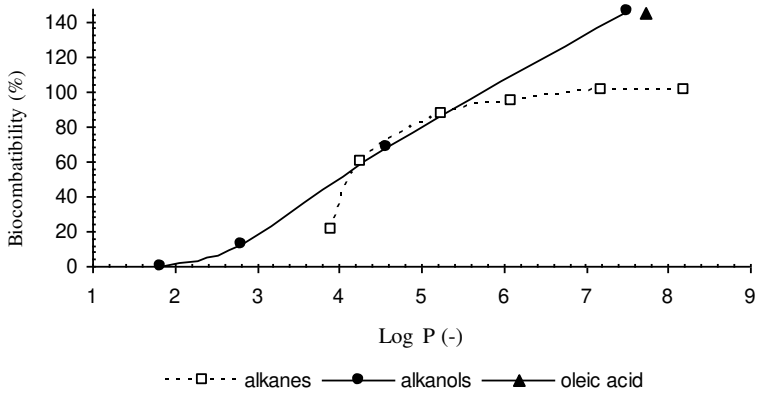


Figure 3: Biocompatibility during 1 h fermentation of alkanes, alkanols and oleic acid according to Log P value

As could be expected, the biocompatibility increases with increasing log P_{ow} value according to the shaked flask fermentations. However, only for tetradecane and hexadecane (log P_{ow} 7.2 and 8.2 respectively), 100% biocompatibility was observed. Dodecane (log P_{ow} 6.1) is only 95% biocompatible, and decane (log P_{ow} 5.25), octane (log P_{ow} 4.27) and hexane (log P_{ow} 3.9) are respectively 85%, 60% and 25% biocompatible. (log P_{ow} values retrieved from Hansch, 1995). Further it is interesting to see that for oleyl alcohol and oleic acid, (log P_{ow} respectively 7.5 and 7.7), the observed CO_2 production exceeds the blank production rate.

Table 1: Partition coefficients of fermentation inhibiting solutes (g solute in organic phase per g solute in aqueous phase)

solute	pH	hexane	octane	decane	dodecane	tetra-decane	hexa-decane	hexanol	octanol	decanol	oleyl alcohol	oleic acid
furfural	2,3	0,699	0,581	0,522	0,484	0,438	0,423	3,85	2,82	2,14	1,49	1,44
	4,5	0,686	0,575	0,523	0,478	0,442	0,419	3,84	2,86	2,15	1,50	1,44
HMF	2,3	0,0017	0,0015	0,0014	0,0012	0,0012	0,0011	1,28	0,82	0,55	0,26	0,059
	4,5	0,0017	0,0015	0,0014	0,0013	0,0012	0,0011	1,26	0,81	0,55	0,24	0,062
Vanillin	2,3	0,266	0,252	0,239	0,222	0,211	0,206	31,5	19,6	13,3	7,14	2,49
	4,5	0,265	0,250	0,237	0,223	0,209	0,204	26,2	16,9	12,2	5,56	2,56
Syringaldehyde	2,3	0,094	0,090	0,085	0,078	0,076	0,074	19,4	12,4	8,09	3,22	1,39
	4,5	0,093	0,087	0,084	0,081	0,077	0,075	16,2	11,1	7,61	3,67	1,66
Coniferyl aldehyde	2,3	0,238	0,228	0,226	0,218	0,205	0,200	104	48,2	29,5	13,1	4,89
	4,5	0,239	0,229	0,223	0,210	0,201	0,200	91,2	44,6	26,9	9,10	5,02
Acetic acid	2,3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0,89	0,65	0,45	0,21	0,15
	4,5	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0,39	0,29	0,23	0,15	0,084
Ethanol 1%	4,5							0,73	0,40	0,22	0,20	0,12
Ethanol 10%	4,5							0,97	0,73	0,54	0,30	0,21
log Pow		3,9	4,27	5,25	6,1	7,2	8,2	1,82	2,81	4,57	7,5	7,73

3.4.c Feasibility

Figure 4 shows the volumetric ethanol production rate in the aqueous phase for different concentrations of furfural without an organic solvent phase and with 33% volume of decanol, oleyl alcohol or oleic acid. Fermentations performed in the presence of decanol showed virtually no ethanol production, whereas during fermentation in the presence of oleic acid, the ethanol production was observed to be virtually equal to ethanol production during fermentation without the presence of organic solvent. For fermentation with oleyl alcohol, increased ethanol production rates were observed compared to fermentation without organic solvent for furfural concentrations higher than 0 g/L.

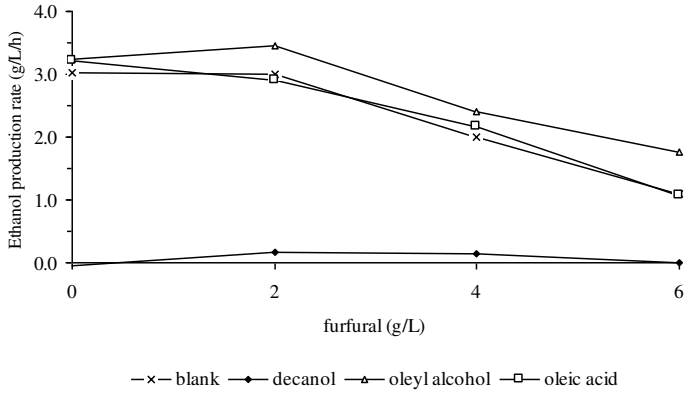


Figure 4: Volumetric ethanol production rate after 4.5 h of fermentation in the presence or absence of organic solvents in the media, for different furfural concentrations

Figure 5 shows the ethanol concentration during fermentations with 6 g/L vanillin. At the end of the fermentation, the ethanol concentration was more than 7 times higher when the fermentation was performed in the presence of oleyl alcohol than without the presence of this organic solvent. Figure 6 shows the volumetric ethanol production rate in the aqueous phase for different concentrations of vanillin without an organic solvent phase and with 33 % volume of decanol, oleyl alcohol or oleic acid. Fermentations performed in the presence of decanol again showed virtually no ethanol production, whereas for fermentation in the presence of oleic acid, the ethanol production rate was observed to be higher as for fermentation without the presence of organic solvent. For fermentation in the presence of oleyl alcohol, ethanol production rates further increased.

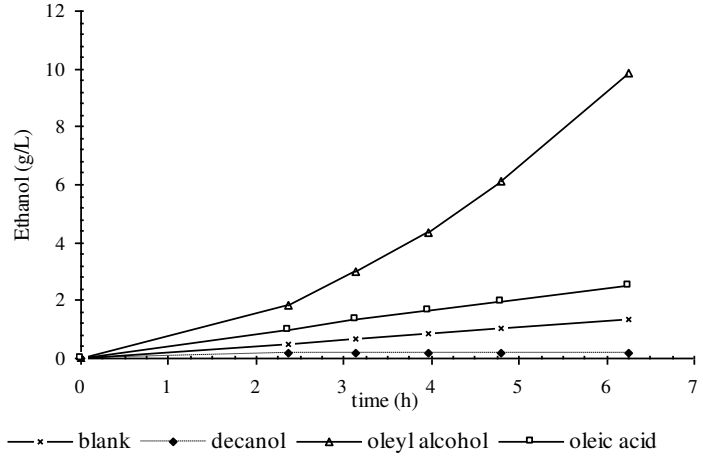


Figure 5: Ethanol concentration during fermentation with 6 g/L vanillin in the media, in the presence or absence of organic solvents

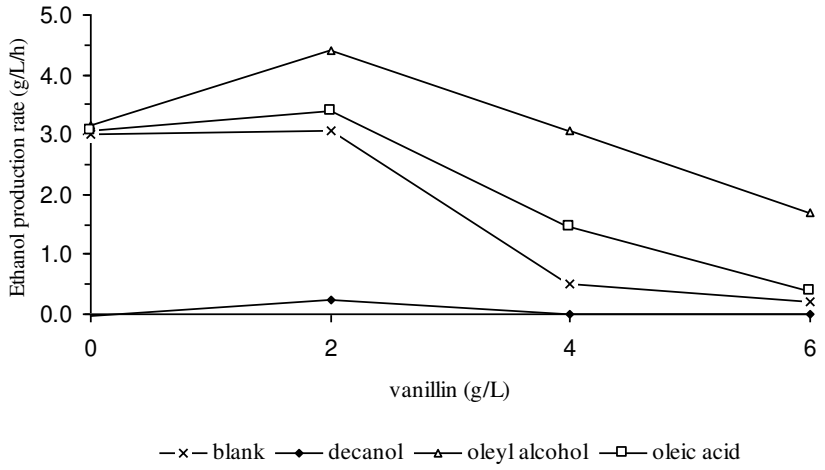


Figure 6: Volumetric ethanol production rate after 4.5 h of fermentation in the presence or absence of organic solvents in the media, for different vanillin concentrations

3.5 Discussion

3.5.a Partition coefficients

In water, an aldehyde group reversibly reacts with water, forming a hydrate, two hydroxyl groups that promote dissolution of the compound in an aqueous phase rather than into an alkane organic phase. This is the case for all referred aldehyde solutes in alkanes as the partition coefficients are below 1. The differences in partition coefficients between the solutes can be explained as follows. In contrast to furfural, hydroxymethyl furfural possesses a hydroxymethyl group. Apparently, the presence of this particular group increases the favoring for the aqueous phase by 400 fold in comparison to furfural if alkanes are used as organic solvent. For higher alcohols and oleyl alcohol, this increase is merely 4 fold. For oleic acid however, HMF has 25 times less affinity than furfural. Compared to HMF, syringaldehyde possesses a 6 carbon aromatic ring rather than a furan, which slightly increases the apolar surface of the molecule. However, apart from an aldehyde and a hyxdroxyl group, it possesses two methoxy groups, from which the oxygen atom can form hydrogen bonds with water. Thus both the aldehyde, hydroxyl group and the

two methoxy groups promote dissolution of the syringaldehyde molecule in water rather than in an alkane organic phase and explain the lower partition coefficient relative to furfural and HMF for alkanes. For the other, more polar organic solvents, the partition coefficient for syringaldehyde is consequently higher than for furfural.

Vanillin, compared to syringaldehyde, lacks one methyl ester, decreasing the polarity of the molecule relative to syringaldehyde and thus increasing its partition coefficient for an alkane organic phase and increasing its partition coefficient for an alcoholic organic phase.

The aldehyde group of coniferyl aldehyde is not directly coupled to the aromatic ring as is the case for vanillin, but is present as an allylic aldehyde. Apparently, this increases the polarity of coniferyl aldehyde compared to vanillin; hence the slightly lower partition coefficients for this compound in alkanes and higher partition coefficient in the higher alcohols.

Acetic acid showed different partition coefficients at different pH of the water phase. At pH 2.3, the partition coefficients tend to be twice as high as at pH 4.5, which can be explained by realizing that acetic acid, with a pKa value of 4.76, is only 65% protonated at pH 4.5 and thus more hydrophilic than at pH 2.3 where acetic acid is 99.7% protonated, and thus less polar.

The elevated partition coefficients for ethanol at a higher ethanol concentration as shown in Table are a result of the reduced polarity of the aqueous phase due to the presence of ethanol in the aqueous phase. This reduced polarity in fact decreases the polarity difference between the aqueous and organic phase, encouraging a more equal distribution of the ethanol over the two phases and hence elevating the partition coefficients.

In general it can be concluded, that the higher the $\log P_{ow}$ value of the organic solvent, the lower the partition coefficient of the tested solutes for that organic solvent, thus the least suitable the organic solvent is in terms of extractive properties.

3.5.b Biocompatibility

Alkanes as well as alcohols show a wide range of biocompatibility. Again a relation can be seen with the log P_{ow} value of the organic solvent. The higher the log P_{ow} value of the organic solvent, the higher the biocompatibility and thus the more suitable the organic solvent is in terms of biocompatibility.

The observed elevated apparent biocompatibility relative to no addition of organic solvent for oleic acid and oleyl alcohol is not consistent with the observation in the feasibility experiment without addition of inhibiting component, though the increment in CO_2 production directly after addition of oleic acid or oleyl alcohol is consistent and repeatedly observed.

3.5.c Feasibility

From the results of the feasibility experiments it can be concluded that the presence of a high fraction of decanol in a heavily stirred medium, disturbs the fermentation process in contrast to a low fraction and gently stirred decanol as during the biocompatibility experiments. Decanol can therefore be disregarded as a solvent for being non-biocompatible. From oleyl alcohol and oleic acid, the former shows most elevated volumetric ethanol production rates.

Calculated specific production rates (data not shown) did not show significant differences, indicating that the observed increase in production rate can be accounted for by the increased biomass rather than higher ethanol production of individual yeast cells. With higher biomass yields, the overall ethanol yields might thus in effect be lower which decreases the attractiveness of the extractive fermentation method. On the other hand, observed higher biomass indicates an elevated viability, which may be desirable in higher stress fermentations as is often the case in industrial production.

Using a series of alkanes as solvent, it could be concluded that with increasing $\log P_{ow}$ of the organic solvent, the partition coefficient for all fermentation inhibiting components decreased while the biocompatibility of the organic solvent for yeast cells increased. The choice of a solvent therefore is a trade-off between partition coefficient and biocompatibility. No optimum $\log P_{ow}$ value exists for which the organic solvent is both satisfyingly biocompatible and has high enough partition coefficients for the inhibiting solutes in case of *in situ* application of the organic solvent, because the relation between $\log P_{ow}$ value and both biocompatibility and partition coefficients is not the same for alkanes as it is for higher alcohols. In industrial processes, key performance indicators for an optimal organic solvent are product yield and specific productivity. In this respect, it is not viable to opt for a solvent that is not completely biocompatible, since both product yield and specific productivity would be negatively affected if viability and productivity decrease due to the use of the organic solvent. Less vital in this respect is the performance of the organic solvent as an extractor of an inhibitor. The main objective of using an organic solvent during fermentation is to reduce the concentration of as many inhibitors as possible, rather than complete removal or recovery of these inhibitors. With sufficient reduction of concentration, the inhibiting effects can be avoided, giving rise to higher growth and ethanol production rates. As a consequence, yeast cells suffer less stress, which leads to increased yeast viability. Obviously, an organic solvent that is biocompatible but which has no extractive property, would be neutral to yield or productivity, but adds to operational and energetic costs of the production plant. Although these costs are minor compared to the costs of raw material, it can be understood that the choice of a solvent is a trade-off between these costs and the benefits that its extractive properties have towards product yield and specific productivity.

Within the series of tested alkanes, tetradecane and hexadecane are completely biocompatible. However, their extractive properties are poor. Higher alcohols clearly show more interesting partition coefficients. Amongst these alcohols, only oleyl alcohol is fully biocompatible, also according to the feasibility experiment. Oleic acid is biocompatible, but its extractive properties are poorer than the extractive properties of oleyl alcohol. It can be concluded that oleyl alcohol is the best candidate for *in situ* application. It might be

interesting to determine if its extractive properties are sufficient for a cost-effective application during fermentation of lignocellulosic substrate.

3.6 Acknowledgement

This project is financially supported by the Netherlands Ministry of Economic Affairs and the B-Basic partner organizations (www.b-basic.nl) through B-Basic, a public-private NWO-ACTS programme (ACTS = Advanced Chemical Technologies for Sustainability).

3.7 References

Casal M, Cardoso H, Leão C, 1998. Effects of Ethanol and other alkanols on transport of acetic acid in *Saccharomyces cerevisiae*. Appl Environ Microbiol 64:665-668

Daugulis AJ, Axford DB, Ciszek B, Malinowski JJ. 1994. Continuous fermentation of high-strength glucose feeds to ethanol Biotechnol Lett 16:637-642

Daugulis AJ, Swaine DE, Kollerup F, Groom CA. 1987. Extractive fermentation - integrated reaction and product recovery Biotechnol Lett 9:425-430

Delgenes JP, Moletta R, Navarro JM. 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. Enzym Microb Tech 19:220-225

Hansch C, Leo A, Hoekman D. 1995. Exploring QSAR. Hydrophobic, Electronic, and Steric Constants. Professional Reference Book. Washington:ACS Publications 1223 p.

Klinke HB, Thomsen AB, Ahring BK. 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol and Biotechnol 66:10-26

Laane C, Boeren S, Vos K, Veeger C. 1987. Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng 30:81-87

Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant N-O. 1999. The generation of fermentation inhibitors during dilute acid hydrolyis of softwood. Enzym Microb Tech 24:151-159

Larsson S, Quintana-Sáinz A, Reimann A, Nilvebrant N, Jönsson LJ. 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation *by Saccharomyces cerevisiae*. Appl Biochem Biotechnol 84:617-632

Lima LHA, Graças de Almeida Felipe M, Vitolo M, Araripe Gonçalves Torres F, 2004. Effect of acetic acid present in bagasse hydrolysate on the activities of xylose reductase and xylitol dehydrogenase in *Candida guilliermondii*. Appl Microbiol Biotechnol 65:734-738

Palmqvist E, Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol 74:25-33

Taherzadeh MJ. 1999. Ethanol from lignocellulose: Physiological effects of inhibitors and fermentation strategies. Dissertation. Sweden:Chalmers University of Technology. ISBN 91-7197-780-5

Capítulo 4

Comparison of vegetable oil and vegetable oil based biodiesel as organic solvent for *in-situ* extraction of fermentation inhibitors in hydrolysed bagasse

4.1 Abstract

Upon acidic hydrolysis of lignocellulosic feedstocks as sugarcane bagasse, phenolic compounds are released and carboxylic acids and furans are generated, which inhibit subsequent fermentation of the liquor. During fermentation, products as ethanol or butanol inhibit the bioprocess at certain concentrations. Both inhibitive phenomena, by lignocellulose-based inhibitors and by fermentation products, can be overcome by selective liquid-liquid extraction. In this work, castor oil and an ethyl ester of ricinoleic acid (biodiesel) are compared for their extractive properties and biocompatibility. Both solvents are predicted to be relatively cheaply available in the near future due to the increase in biodiesel production, and could eventually be reutilized as fuel or fuel feedstock. Final results show that biodiesel has most interesting properties with respect to the partition coefficients of inhibitors in hydrolyzed liquor, the partition coefficients of ethanol and fermentation by-products, water absorption and low viscosity. Both biodiesel and castor oil allow yeast to be completely recovered by centrifugation of the emulsified fermentation two-phase system with biodiesel having the lowest density, and both also show a high biocompatibility and low partition coefficients of fermentation substrates. Actual batch fermentation with inhibitors and biodiesel proves the advantages of the use of the extractive solvent in terms of reduced total fermentation time, improved ethanol production rates and ethanol yields.

4.2 Introduction

With an increasing footprint on environment and natural resources, the urge to move to a bio-based society is ever more seriously being considered. Agricultural non-food by-products as lignocelluloses can play an important role in a sustainable industry, for example as substrate for fermentations that yield fuels, fine chemicals or polymer building blocks. To ferment such substrate, it must first be hydrolyzed to generate fermentable sugars. In the case of lignocellulosic biomass, such as sugarcane bagasse, hydrolysis can be performed rapidly, for example by organosolv acidic hydrolysis (Rossell *et al.*, 2005). However, several fermentation inhibiting compounds are generated or liberated during this process, such as furfural, 5-hydroxyl-methyl furfural (HMF), acetic acid and vanillin (Larsson *et al.*, 1999). Also pretreatment of lignocelluloses for enzymatic hydrolysis causes generation of these substances (Oliva *et al.*, 2006), albeit in lower concentrations. The inhibitive effects have been found for ethanol production with *Saccharomyces cerevisiae* and *Zymomonas mobilis*, *Pichia stipitis* and *Candida shehatae* (Delgenes *et al.*, 1996) and *Kluyveromyces marxianus* (Oliva *et al.*, 2006), as well as for ethanol, butanol and acetone production with *Clostridium beijerinckii* (Ezeji *et al.*, 2007).

Furfural is generated from xylose during hydrolysis and has a negative effect on specific growth rate, cell mass yield on ATP, volumetric and specific ethanol productivities. It is reduced to furfuryl alcohol at a certain rate by *Saccharomyces cerevisiae* and other yeast strains, which has less inhibitory properties under anaerobic conditions (Palmqvist *et al.*, 1999a) than furfural. Oliva *et al.* (2003) found that *Kluyveromyces marxianus* even does not grow at all until all furfural in the media is reduced. 5-Hydroxy-methyl-furfural is generated from hexoses and plays a similar inhibitory role as furfural, though less severe (Modig *et al.*, 2002). 5-Hydroxy-methyl-furfural is converted into 5-hydroxy-methyl-furfuryl alcohol at a slower rate than the reduction of furfural (Taherzadeh, 1999). Vanillin is generated by the breakdown of complex lignin structures during bagasse hydrolysis and negatively affects the fermentation ethanol yield (Palmqvist *et al.*, 2000). It is reduced to vanillic alcohol during fermentation

(Liu 2011). Acetic acid, formed during hydrolyis of hemiceluloses, diminishes growth rate and biomass yield by overcharging proton motive force pumps to overcome an intracellular drop in pH when diffusing undissociated into the yeast cell (Pampulha *et al.*, 2000).

Several methods to remove these inhibitors from the fermentation broth have been suggested to enhance the fermentation performance, like degradation and precipitation by addition of calcium or sodium hydroxide (overliming) (Palmqvist *et al.*, 2000), treatment with activated charcoal, ion exchange resins, enzymatic detoxification (Chandel, 2007), growth stimulation with acetaldehyde (Barber *et al.*, 2000), microbial treatment, evaporation (Palmqvist *et al.*, 2000), treatment with lignin residue (Björklund *et al.*, 2002) or liquid-liquid extraction using diethyl ether (Palmqvist *et al.*, 2000) or thermo-separating copolymers (Hasmann, 2008). With in situ liquid-liquid extraction techniques, the fermentation product can be removed to prevent it from reaching inhibiting levels as in the case of ethanol (Daugulis *et al.*, 1987, Atala *et al.*, 2001, Weilnhammer 1994, Junqueira 2009). To facilitate a subsequent removal of the fermentation product from the extractive solvent, the volatility of the extractive solvent must differentiate substantially from the volatility of the product. An extractant with a higher volatility than the product and inhibitors is not feasible, since volatile substances tend to be small and easily diffuse through or integrate into the cell wall, leading to low biocompatibility in terms of growth distortion and consequent losses in biomass and product yield. Low volatile extractive solvents however are more feasible, like long chain alcohols or organic acids (Zautsen *et al.*, 2008). Several long-chain alkanes or alcohols have been evaluated only for *in-situ* product removal, as oleyl alcohol (Daugulis *et al.*, 1987), decanol (Kapucu *et al.*, 1999), dodecanol (Minier and Goma, 1982), and long chain organic acids as oleic acid (Csanyi *et al.*, 2003). With the projected growth in biodiesel production, vegetable oils and organic acids will become available for relative low prices. Castor oil is particularly interesting, since it contains for 85-90% esthers of ricinoleic acid (cis-12-hydroxyoctadeca-9-enoic acid) (Vaisman *et al.*, 2008), a C-18 molecule with a hydroxyl group at its C-12 position as shown in Figure 1. This hydroxyl group decreases the hydrophobicity of the molecule, favoring partition coefficients of inhibitors and fermentation products with polar characteristics. A methyl or ethyl-esther further decreases hydrophobicity. Since castor oil

and castor oil based biodiesel, a methyl or ethyl ester of ricinoleic acid, are projected to become commodities in Brazil, utilization of these products for *in-situ* inhibitor and product extraction can be interesting. After several recovery cycles, the castor oil or biodiesel could even be used as on-site fuel or fuel source for the truck float of the production plant (Grobben *et al*., 1993).

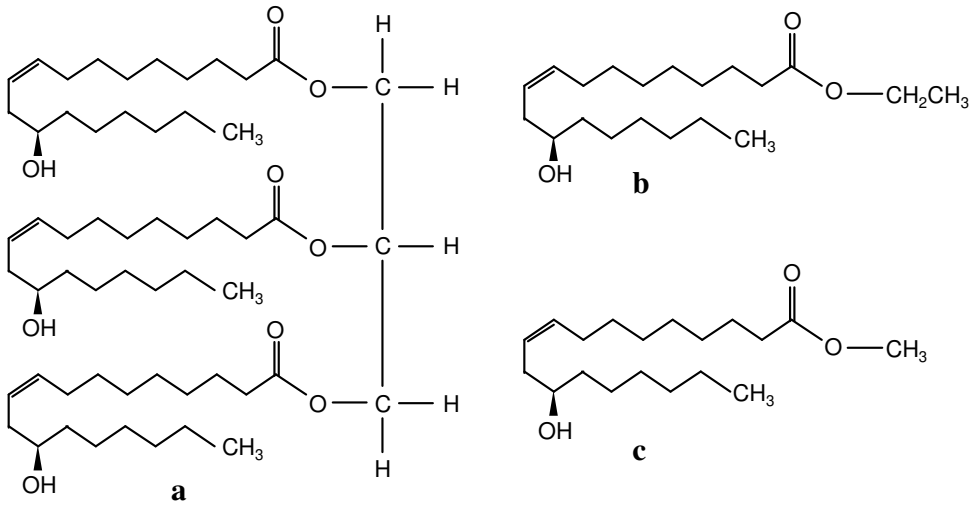


Figure 1: Castor oil, triglyceride of ricinoleic acid(a), ethyl ester of ricinoleic acid (b) and methyl ester of ricinoleic acid (c)

In the case of a continuous fermentation, depending on the extractive characteristics and saturation due to a chosen dilution rate of the organic solvent, extraction of inhibitors and fermentation product can be only partial. The before mentioned biochemical reduction of several other inhibitors to less toxic components, allows even an incomplete extraction to substantially improve the fermentation performance by mere reduction of inhibitor concentrations to less severe levels. Moreover, complete removal of, for example, furfural from the fermentation broth is not necessarily beneficial, as Palmqvist *et al.* (1999b) found that a low intracellular concentration of furfural (2 mmol l^{-1}) results in a lower glycerol production and consequently a higher ethanol yield. This effect is probably caused by offering the yeast an alternative NADH consuming processes (aldehydes reduction with aldehyde dehydrogenase) which compete with NADH dependent glycerol production and thus pushing the metabolic flux to glyceraldehyde-3P and eventually biomass and ethanol as exposed for hydroxymethylfurfural by Almeida *et al.* (2009).

In this work, castor oil and castor oil based ethyl-esther, henceforth indicated as 'biodiesel', are compared in terms of biocompatibility, partition coefficients for various inhibitors, substrates and ethanol, viscosity and water absorption. Water absorption can be of significance by concentrating the substrate in the aqueous phase, on one hand, but diluting the fermentation product in the organic phase, on the other hand. Fermentations of synthetic hydrolytic liquor are also performed to verify the expected performance enhancement of extractive solvent-mediated ethanol fermentation.

4.3 Material and methods

4.3.a Biocompatibility

Biocompatibility of biodiesel was measured using four 125 mL Erlenmeyers equipped with a swan-neck gas outlet that was filled with sulphuric acid as gas drying seal. Fermentation medium, consisting of 10 g/L peptone, 10 g/L yeast extract, 2.5 g/L K_2HPO_4 and 60 g/L glucose was autoclaved at 121 ˚C for 15 minutes and inoculated with an industrial *Saccharomyces cerevisiae* strain (*Santa Adélia Sugar Mill*), that was grown overnight in medium with the same composition. Each Erlenmeyer was filled with 50 mL of the inoculated medium. To two Erlenmeyers, 10 mL biodiesel was added and the weight of the Erlenmeyers was measured every 10 minutes during 10 hours on an analytical balance (Scientech SA210), indicating the loss of produced carbon dioxide in grams per litre of fermentation medium. The weight profiles of flasks with and without biodiesel were compared to indicate the biocompatibility of biodiesel. The same experiment was repeated for castor oil.

4.3.b Water and ethanol absorption

The absorption of water and ethanol into biodiesel and castor oil was determined for several ethanol concentrations in the range of 0 to 200 g/L. For each concentration of

ethanol and for each of the two organic solvents, the following procedure was followed. First, a two-phase system was made out of weighed 5 mL milli-Q water, 5 mL organic solvent and a varying amount of ethanol. The two-phase system was left unshaken for 48 hours in a water bath (Tecnal TE092) at 34 ˚C. Then a sample was withdrawn from the organic phase and put into a previously weighed glass bulb. The glass bulb was weighed again and kept under a low pressure of 0.5 bars at 35 ˚C with a rotary evaporator (Marconi MA120). At intervals of about 5 minutes, the weight was monitored with the analytical balance and upon stabilizing, the amount of ethanol and water previously present in the organic phase was concluded from the loss of weight during the evaporation in the rotary evaporator. From the aqueous phase another sample was taken, filtered with a 0.22 µm sample filter (Millipore), and analyzed with HPLC, equipped with an auto sampler (Varian 9095), a UV detector (Varian 9095), a refractive index detector (Varian RI-4), a binary pump (Varian 9010) and using an Aminex HPX-87H ion-exchange column (Bio-Rad, 7.8*300 mm), with a Biorad micro-guard Cation-H 125-0129 precolumn, at 25 ˚C, and 5 mM H_2SO_4 as a mobile phase at 0.7 ml/min. From the mass balance over the two-phase system, the now known concentration of ethanol in the aqueous phase and the total mass of water and ethanol present in the organic phase, the concentrations of ethanol and water in the organic phase were calculated.

4.3.c Partition coefficients

Partition coefficients were measured for the hydrolysis by-products furfural, 5-hydroxymethyl furfural, vanillin, syringaldehyde, coniferylaldehyde and acetic acid. Coefficients were also measured for lactic acid, which is often produced on industrial sites due to bacterial contaminations. For the fermentation substrates glucose, sucrose, fructose and xylose, as well as for fermentation (by-) products glycerol and ethanol, partition coefficients were measured as well. All partition coefficients were measured for six different concentrations of ethanol in the aqueous phase between 0 g/L and 200 g/L. For each of these components and for each ethanol concentration, a two-phase system was made in an Eppendorf tube, containing 1 g of a 1 g/L solution of the component, except for

ethanol, in milli-Q water as the aqueous phase and 1 g biodiesel or castor oil as the organic phase. The two-phase system was left closed and unshaken for 48 hours in a water bath at 34 ˚C. After centrifuging for 5 minutes at 2000 rpm, a 500 mg sample of the organic phase was added to 700 mg milli-Q water, thus forming a secondary two-phase system for back-extraction of the component. From the aqueous phase of the original two-phase system, 200 mg was added to a HPLC sample vial filled with 1 g water and the concentration of the component in the aqueous phase was measured with HPLC. The secondary two-phase system was left unshaken for another 48 hours at 34˚C. Then, from the aqueous phase of this secondary two-phase system, a 200 mg sample was diluted five times and analyzed with HPLC. The exact weight of each sample, water and organic phase transferred into an eppendorf tube or HPLC vial was measured with an analytical balance to avoid errors due to density differences or viscosity issues. From the mass balance over the original and the secondary two-phase system, the partition coefficient was determined for each component and each ethanol concentration. HPLC measurements for glucose, xylose, glycerol and ethanol were done using an Aminex HPX-87H ion-exchange column (Bio-Rad, 7.8*300 mm), at 25 ˚C and 5 mM H_2SO_4 as a mobile phase at 0.7 ml/min; detection was done by a refractive index detector (Varian RI-4). Acetic acid and lactic acid were measured on the same column but with a UV detector (Varian 9095) at 210 nm. Furfural, 5-hydroxymethyl furfural, vanillin, syringaldehyde e coniferylaldehyde, were retained using a C-18 column (μBondapak, 10 μm, 3.9*300 mm) and quantified with UV detection (Varian 9095) at wavelengths that were most appropriate for each individual compound. A 10% acetonitrile solution was used as eluent (1 ml/min). Eluents were prepared with Milli-Q water, filtered with 0.45 μm filter (Millipore). All measurements were performed in at least in duplicate.

4.3.d Viscosity

For both biodiesel and castor oil, viscosity measurements were carried out using a Physica MCR301 (Anton Paar GMbH, Graz, Austria) rheometer, equipped with a stainless steel parallel plate (75 mm diameter, 0.5mm gap). Flow curve measurements were performed in triplicate with shear rate $\dot{\gamma}$ varying between 0 and 300 s^{-1}. An up-down-up

steps program was carried out in order to evaluate thixotropy of evaluated products. Flow behavior of biodiesel and castor oil was modeled in order to obtain rheological parameters (yield stress σ_0, consistency index K and flow behavior index n), according to the Herschel-Bulkley model (Equation 1), from which subsequently the viscosity was determined. The effect of temperature on the viscosity of biodiesel and castor oil was studied within 5 to 40°C, using a temperature interval of 5°C. The results of this effect on viscosity were evaluated according to the Arrhenius equation (Equation 2).

$$\sigma = \sigma_0 + K \cdot \dot{\gamma}^{\,n} \quad [1]$$

$$\ln(\eta) = \ln(\eta_0) + \left(\frac{E_a}{R}\right)\frac{1}{T} \quad [2]$$

Where η: viscosity (Pa·s), η_0: viscosity at infinite temperature (Pa·s), E_a: activation energy for viscous flow (J mol^{-1}), R: gas constant (J mol^{-1} K^{-1}), T: temperature (K).

4.3.e Fermentations

Furfural, acetic acid and vanillin were chosen as inhibitors to represent furans, carboxylic acids and phenolic compounds as the main inhibitor groups. Inhibitors were added to inoculums slants and fermentation medium in the ratio as furfural:acetic acid:vanillin compares to 1:2.2:0.6, which was found representative for hydrolytic liquor hydrolyzed during weak acids treatment as deduced from Martin *et al.* (2007), although much depends on the source, pretreatment and hydrolysis of the material. The actual inhibitor concentrations were established by gradual increased exposure of the industrial strain (*Saccharomyces cerevisiae,* from Santa Adélia Sugar Mill) to the inhibitors in YPD agar medium. Subsequently, the yeast was transferred daily to a fresh agar slant with the highest viable inhibitor concentration found, to have a constant active stock of recently exposed yeast available. The fermentation inoculum was prepared from this stock yeast by growing for 48 hours in shaken flasks, in 100mL medium without inhibitors, upon which

the whole culture was transferred to 100 mL medium with double this highest viable inhibitor concentration for another 48 hours or fermentation. The medium for both these incubation steps was prepared with 150 g/L glucose, 10 g/L peptone, 10 g/L yeast extract and 2.5 g/L K_2HPO_4, autoclaving glucose separate from the other ingredients-solution at 121°C for 15 minutes. The incubations took place in a shaker (Tecnallab TE420) at 34°C and 150 rpm and the optical cell density was monitored every 12 hours using a spectrophotometer (Beckman Coulter DU640) at 600 nm. Calibration of optical density was done by measuring dry weight by volume in triple Eppendorf tubes on an analytical balance (Scientech SA210), discounting the weight of the empty tubes with 0.1 mg accuracy. Herefore, the biomass sample was washed twice with distilled water, sedimenting the yeast after each wash with a microcentrifuge (Hettich Mikro200) at 5000 rpm for 5 minutes and drying afterwards for more than 24 hours at 50°C in a oven (Fanem Ltda. 320-SE). The pH, initially adjusted to 5 with a 4M H_2SO_4 solution before autoclaving, dropped on average during incubation 4.05.

The prepared inoculum was used for three fermentations, which were carried out upon choosing biodiesel, an ethyl esther of natural castor oil based ricinoleic acid, as the best organic solvent for *in-situ* extractive fermentation, to demonstrate the advantage of the use of this organic solvent for fermenting in the presence of inhibitors. No inhibitors or biodiesel were applied to the first fermentation, whereas inhibitors were added to the medium of the second fermentation and both inhibitors and biodiesel were applied to the third fermentation. Prior to application, 500mL batches were bubble-washed four times with 2500mL distilled water at 25°C during two hours for each wash, using a circular stainless steel air sparger and compressed air flow at 0.2 $L \cdot min^{-1}$, measured with a digital gas flow meter (Cole-Parmer EW-32907-62). Between each wash cycle, the biodiesel was left two hours before draining and refilling the aqueous layer. A sample from the aqueous layer was analyzed (HPLC, Varian 9010, Hyperrez XP organic acids column 100mm 5µm 25°C 1$mL \cdot min^{-1}$ 2mM H_2SO_4, detection with a ChromTech RI2000 RI detector and Varian 9095 UV at 215nm) after each wash to verify complete removal of any traces of glycerol, ethanol and other impurities. After washing, the biodiesel was siphoned off the aqueous layer and dried and clarified by heating in a rotary evaporator (Marconi MA 120) at 55°C

and 0.5 bar. The drying timeframe was previously established by measuring weight loss over time under the same temperature and pressure conditions of a water-saturated sample of equal amount.

The fermentations were performed in 1L fermenters, using 1L medium for the fermentations without and with inhibitors and 800mL medium for a third fermentation with inhibitors and 400mL biodiesel. The biodiesel was added to the medium 12 hours prior to inoculation at fermentation temperature, pH and agitation conditions to assure equilibrium. The furfural:acetic-acid:vanillin ratio was 1:2.2:0.6 as in the inoculums, with 2g/L furfural, 4.4 g/L acetic acid, 1.2g/L vanillin. Both fermentation medium and biodiesel were autoclaved and glucose was autoclaved separately to prevent browning, as were furfural and acetic acid to prevent evaporation. The pH was kept constant during the fermentation to 4.5 with a 3MKOH solution. Samples of twice 2mL were taken at regular intervals during the course of the fermentations for analysis of dry weight (0.1mg accuracy) and substrate, inhibitors and product concentrations. The biomass samples were washed twice with distilled water, sedimenting the yeast after each wash with a microcentrifuge (Hettich Mikro200) at 5000 rpm for 5 minutes and drying afterwards for more than 24 hours at 50°C in a oven (Fanem Ltda. 320-SE). Samples containing biodiesel however, were washed twice with an over-saturated 20% butanol emulsion. For HPLC measurements, the supernatant of samples without biodiesel and the aqueous phase of samples with biodiesel, were diluted four times and filtered with 5.5cm^2·0.22µm syringe filters (Microgon DynaGard®). For samples with biodiesel, the organic phase was analysed using back extraction, as described in previous work (Zautsen *et al.*, 2008). HPLC measurements for glucose and ethanol were done using a HyperRez XP organic acids column (Thermo Scientific, 8µm 7,7·100mm), at 25˚C and 2 mM H_2SO_4 as a mobile phase at 1 ml/min; detection was done by a refractive index detection (ChromTech RI2000 RI) at 35˚C. Furfural, furfuryl alcohol, vanillin and vanillyl alcohol were retained using a C-18 column (Merck Chromolith C18) with a precolumn (Bischoff Prontosil 120-5-C18 5µm) and quantified with UV detection (Varian 9095) at 229nm. The mobile phase consisted of a 10% acetonitrile solution (A) and 100% Milli-Q H_20 (B), of which A was run at a proportion of 10% (1%ACN) for 1 min, then ramped up to 100% at 1.5min until 3.5min

when it was ramped down again to 10% at 4min until the end of the run at 7min. Both eluents were prepared with Milli-Q water, filtered with 0.22 μm filter (Millipore).

4.3.f Used chemicals

The following chemicals were used: peptone, bacto (Oxoid Ltd, Basingstoke Hampshire, England), yeast extract (Oxoid Ltd, Basingstoke Hampshire, England), agar (Merck kGaA, Darmstadt, Germany, extra pure), glucose (Labsynth, Diadema, SP Brazil, 98%), sucrose (Labsynth, Diadema, SP Brazil, 99.9%), fructose (Labsynth,Diadema, SP Brazil, 98%), xylose (Aldrich, Milwaukee, WI USA, 99%), sulfuric acid (Labsynth, Diadema, SP Brazil, 95-98%), K_2HPO_4 (Ecibra, São Paulo, SP Brazil, 98%), furfural (J.T.Baker, Phillipsburg, NJ USA, 98.8%), furfuryl alcohol (Acros Organics, Geel, Belgium, 99%), 5-hydroxy-methyl-furfural (Acros Organics, Geel, Belgium, 98%), vanillin (J.T. Baker, Phillipsburg, NJ USA), vanillyl alcohol (4-hydroxy-3-methoxy benzalyl alcohol, Acros Organics, Geel Belgium, 99%), syringaldehyde (Acros Organics, Geel, Belgium, 98%), coniferylaldehyde (Aldrich, Milwaukee, WI USA, 98%), acetic acid (Dinâmica, São Paulo, SP Brazil, 99.7%), lactic acid (Vetec, Rio de Janeiro, RJ Brazil, 85-90%), glycerol (Aldrich, Milwaukee WI USA, 99.5%), ethanol (Ecibra, São Paulo, SP Brazil, 99.5%), Acetonitrile (J.T. Baker, Phillipsburg, NJ USA, 99.9%), Castor oil (Amaral Ltda. Farmax, medical grade), mineral water (Bonafonte, Danone Ltda., Jacutinga MG Brasil), Biodiesel (brandless, industrial grade ethyl esther of natural castor oil based ricinoleic acid, kindly provided by the Microbial Laboratory of Biotechnology at the Chemistry Institute, Federal University of Rio de Janeiro, UFRJ.

4.4 Results and discussion

4.4.a Biocompatibility

Both addition of castor oil and castor oil based biodiesel to fermentation media showed no difference in the weight profile of the Erlenmeyer flasks in the biocompatibility experiments compared to fermentation without addition of these solvents. At the end of the fermentation, all flasks with extractive solvent had lost 3% less carbon dioxide per litre than flasks without solvent, but consistent retardation of the weight profile of flasks with organic solvent, indicated that this was due to accumulation of carbon dioxide in the organic layer rather than lack of biocompatibility. Figure 2 shows the weight profile for fermentation with biodiesel as extractive solvent.

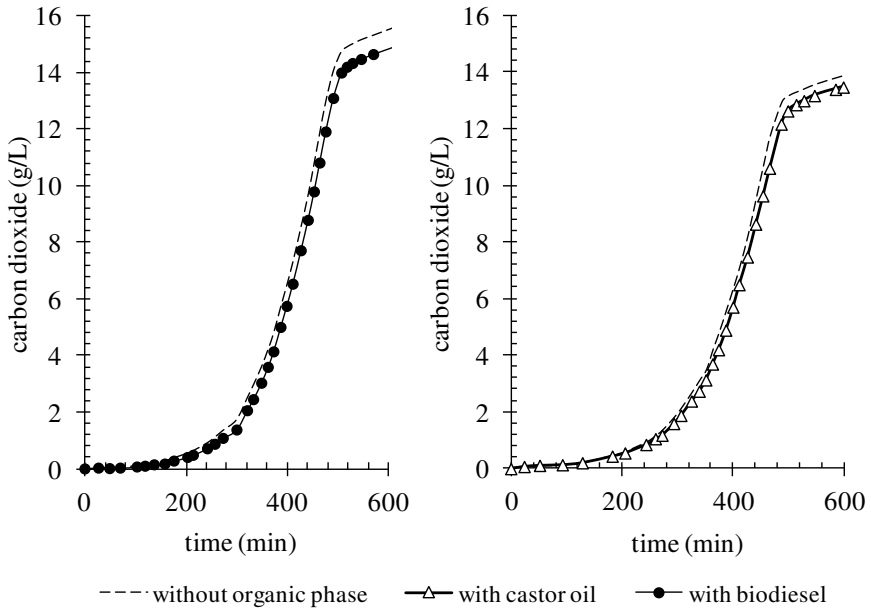


Figure 2: Biocompatibility of castor oil and biodiesel demonstrated by carbon dioxide production during fermentation without organic phase (dotted line), with castor oil (white triangle) and with biodiesel (black circle)

4.4.b Water and ethanol absorption

Biodiesel absorbed 2.4 to 6.8 times more water then castor oil, while ethanol absorption was roughly the same for both organic solvents. As shown in Figure 3, with

increasing ethanol concentration in the aqueous phase, both organic solvents absorbed more ethanol and water. There is no constant ratio of absorbed water and absorbed ethanol, as suggested by Malinowski *et al.* (1993) for oleyl alcohol. Although a clear relation with the ethanol concentration in the aqueous or organic phase could not be concluded from the experimental data, an increase in ethanol/water absorption ratio can be seen as the ethanol concentration increases, especially for biodiesel. This means that with a higher ethanol concentration in the aqueous phase, ethanol is relatively absorbed more. This is most strongly the case for biodiesel, where the water/ethanol ratio diminishes from 2.13 to 1.1 between 35 and 182 g/kg ethanol in the aqueous phase. Whereas the difference in water absorption is considerable between biodiesel and castor oil, the difference in ethanol absorption mostly lies within the standard deviation of the experiments. Overall, biodiesel has an advantage over castor oil in that it absorbs more water and can thus in effect concentrate substrate in the aqueous phase during fermentation, which leads to reduced loss of substrate in continuous fermentations or increased production rate for (fed-) batch fermentations.

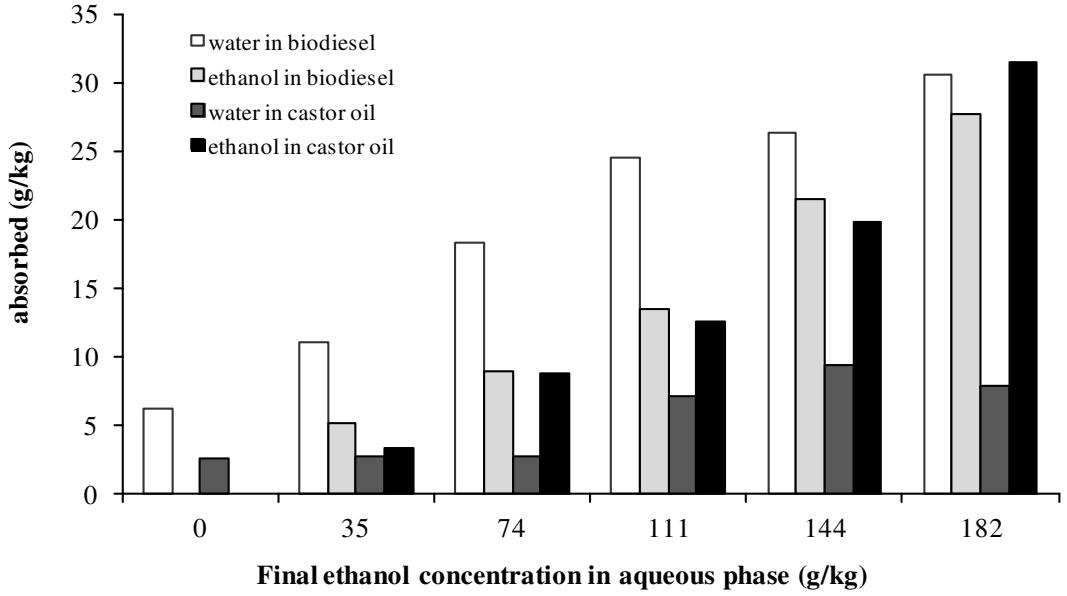


Figure 3: Water and ethanol absorption in water-biodiesel and water-castor oil two-phase systems for different final ethanol concentrations in the aqueous phase

4.4.c Partition coefficients

Measured partition coefficients are represented in Table 1. Up to concentrations of 100 g/L of ethanol in the aqueous phase, biodiesel outperforms castor oil as extractive agent for all fermentation inhibiting compounds, HMF, furfural, vanillin, syringaldehyde and coniferyl aldehyde. Apparently, all these compounds have more affinity for biodiesel than for castor oil. This affinity depends however strongly on the amount of ethanol present in the two phase system. Partition coefficients for HMF and furfural increase with increasing ethanol concentration. This is also the case for partition coefficients of vanillin, syringaldehyde and coniferylaldehyde in the case of castor oil as organic solvent, but not in the case of biodiesel for which partition coefficients tend to decrease with increasing amount of ethanol in the system. It can also be seen that for these compounds maximum partition coefficients exist for ethanol concentrations within the interval of 0 to 200 g/L. The ethanol concentrations to reach these maximum partition coefficients are however not the same for biodiesel and castor oil. For biodiesel as organic solvent, maximum partition coefficients can be found at significantly lower ethanol concentrations than for castor oil. Coincidently, for biodiesel, these maximum partition coefficients are found at ethanol concentrations below 80 g/L, at which ethanol becomes inhibitive to the fermentation at most standard fermentation temperatures (Rivera *et al.*, 2006). During ethanol fermentation, the extractive properties of biodiesel can thus be exploited maximally without suffering ethanol inhibition.

For the substrates sucrose, glucose, fructose and xylose, no significant difference can be seen between partition coefficients for biodiesel and castor oil, but in all cases the partition coefficients are relatively small, with measured values ranging between 0.04 for glucose and fructose down to $1.2 \cdot 10^{-3}$ for xylose. Moreover, partition coefficients for the studied substrates decrease at increasing amount of ethanol in the system, both for biodiesel as for castor oil. Thus, as desired, the selectivity for sugar is low both in the case of biodiesel as in the case of castor oil as organic solvent. Usage of either of these solvents during fermentation will consequently not lead to any significant decrease in sugar concentrations in the aqueous phase.

Partition coefficients for glycerol, acetic acid and lactic acid are two to seventy times higher for biodiesel than for castor oil, but still relatively low and do not exceed 0.3 g/g. Thus for extraction of these compounds, biodiesel does not perform significantly, though considerably better than castor oil. Overall, the optimum ethanol concentration in the aqueous phase for maximal exploration of the partition coefficients for glycerol, acetic acid and lactic acid can be found below 80 g/L.

Table 1: Partition coefficients of various compounds for Biodiesel (BD) and Castor Oil (CO) in the presence of different concentrations of ethanol in the aqueous phase

Ethanol (g/L)	Solvent	HMF	furfural	vanillin	syring aldehyde	coniferyl aldehyde	Sucrose	glucose	fructose	xylose	glycerol	acetic acid	lactic acid	Ethanol
0	BD	0.33	1.8	3.1	1.6	18	0.018	0.034	0.035	0.0031	0.093	0.071	0.132	-
	CO	< 0,01	0.37	1.1	0.5	2.5	0.025	0.040	0.040	0.0026	0.044	0.006	0.0019	-
37	BD	0.26	2.1	3.7	1.8	28	0.018	0.033	0.034	0.0018	0.068	0.127	0.096	0.12
	CO	< 0,01	0.45	1.0	0.4	2.6	0.0092	0.027	0.027	0.0032	0.023	0.048	0.0024	0.09
74	BD	0.17	2.3	2.8	1.4	29	0.0073	0.022	0.022	0.0012	0.084	0.258	0.070	0.12
	CO	0.012	0.85	1.5	0.6	3.8	0.011	0.024	0.024	0.0038	0.021	0.068	0.0042	0.11
110	BD	0.40	2.4	1.8	0.97	22	0.0046	0.020	0.023	0.0014	0.041	0.304	0.065	0.12
	CO	< 0,01	1.5	5.4	1.98	16	0.0061	0.019	0.019	0.0034	0.018	0.076	0.0084	0.12
149	BD	0.49	2.5	1.5	0.86	18	0.0093	0.020	0.021	0.0014	0.054	0.337	0.071	0.15
	CO	0.15	1.4	4.5	1.84	13	0.0075	0.022	0.019	0.0022	0.018	0.104	0.013	0.14
183	BD	0.61	2.5	1.4	0.81	15	0.0022	0.014	0.014	0.0016	0.038	0.346	0.068	0.15
	CO	0.19	1.4	4.1	1.72	9.7	0.0058	0.017	0.018	0.0018	0.016	0.102	0.013	0.17

4.4.d Viscosity

For both biodiesel and castor oil, shear stress demonstrated a strong linear relation to shear rate with intersection at zero on both axes and an average regression of 0.9999 for all temperatures. Hence, there is no yield stress (σ_0) and the flow behavior index (n) is equal to 1, leading to a Newtonian behavior with a consistency index (K) equal to the viscosity (η)

(equation 3) at a same temperature, where σ: shear stress (Pa), $\dot{\gamma}$: shear rate (s^{-1}), η: viscosity (Pa·s) and K: consistency index (Pa·s).

$$\sigma = K \cdot \dot{\gamma} \quad \text{or} \quad \eta = \frac{\sigma}{\dot{\gamma}} = K \qquad \textit{Newtonian viscosity} \qquad [3]$$

Figure 4 shows the effect of temperature on the viscosity of biodiesel and castor oil. Plotting the natural logarithmic of the viscosity against the inverse of the temperature in Kelvin reveals a clearly linear relation for both solvents with regressions of 0.999. For both biodiesel and castor oil, increasing temperatures resulted in lower viscosities. Biodiesel however, consistently had a lower viscosity than castor oil which was expected since biodiesel was originally invented as a fuel oil replacement with lower viscosity. For biodiesel and castor oil, η_0 is found to be $3.126 \cdot 10^{-8}$ and $5.751 \cdot 10^{-11}$ respectively; η_0 of biodiesel is therefore higher than of castor oil, but both are relatively small. E_a was found 33556 for biodiesel and 57431 for castor oil, which means that the activation energy needed to initiate flow is considerably lower for biodiesel, and can be understood in Figure 4, as the temperature needed for low viscosity is much higher for castor oil. With the order of magnitude of increase in activation energy for castor oil, the importance of the relatively lower η_0 is overruled which in effect explains the higher viscosity for this oil in the mathematical sense.

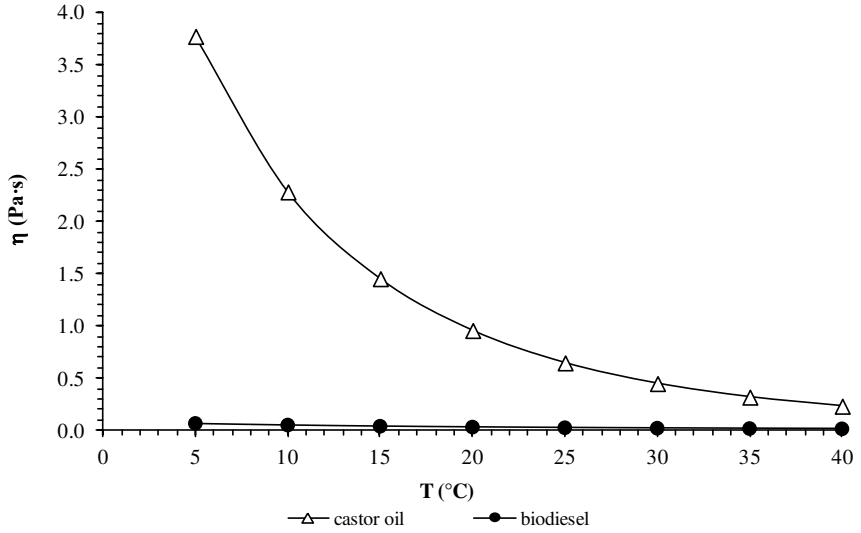


Figure 4: Viscosity comparison between castor oil and biodiesel for various temperatures

4.4.e Fermentations

Fermentation results with fitted sigmoidal curves are shown for fermentation without inhibitors in Figure 5, fermentation with inhibitors in ***Erro! Fonte de referência não encontrada.*** *and fermentation with inhibitors and biodiesel in*

Figure 7. Concentrations of furfural, furfuryl alcohol, vanillin and vanillic alcohol during fermentation without biodiesel are shown in Figure 8 and with biodiesel in Figure 9. Acetic acid concentrations are not shown, but increased slightly during the fermentations. Table 2 summarizes several fermentation parameters for each batch fermentation.

Table 2: Initial inhibitor concentrations and fermentation parameters in the following order: maximal specific growth-, furfural and vanillin reduction- and ethanol production rates, maximal volumetric ethanol production rate, ethanol, glycerol and biomass yields.

furfural	vanillin	acetic acid	biodiesel	μ_{max}	$q_{F\,max}$	$q_{V\,max}$	$q_{EtOH\,max}$	$r_{EtOH\,max}$	Y_{EtOH}	$Y_{glycerol}$	$Y_{biomass}$
$g \cdot l^{-1}$	$g \cdot l^{-1}$	$g \cdot l^{-1}$	%	$g \cdot g \cdot h^{-1}$	$g \cdot g \cdot h^{-1}$	$g \cdot g \cdot h^{-1}$	$g \cdot g \cdot h^{-1}$	$g \cdot l^{-1} \cdot h^{-1}$	$g \cdot g^{-1}$	$g \cdot g^{-1}$	$g \cdot g^{-1}$
0	0	0	0	0.47	-	-	1.7	3.4	0.46	0.040	0.043
2	1.2	4.4	0	0.09	0.05	0.03	1.2	3.0	0.41	0.014	0.018
2	1.2	4.4	33.3	0.13	0.27	0.20	1.3	4.1	0.45	>0.026	0.045

Whereas the conventional fermentation without inhibitors was complete within 24 hours, addition of the inhibitors required a total of 90 hours for the fermentation to complete. For the fermentation system with both inhibitors and biodiesel, the total time required to finish the fermentation was reduced to 24 hours. Fermentation with inhibitors remained in a stationary state until furfural and vanillin had been converted to less toxic levels, which was only the case after 60 hours. From this moment, the ethanol fermentation was no longer significantly affected by the inhibitors and the fermentation completed within a 24 hours time-span as seen for fermentation without inhibitors under the same conditions.

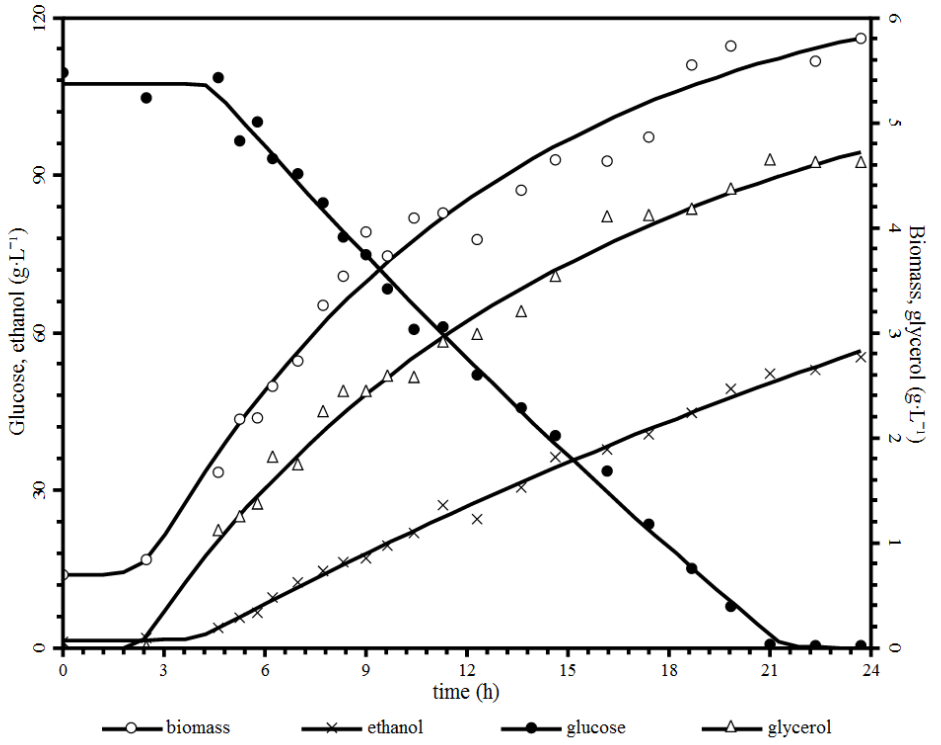


Figure 5: Fermentation without inhibitors

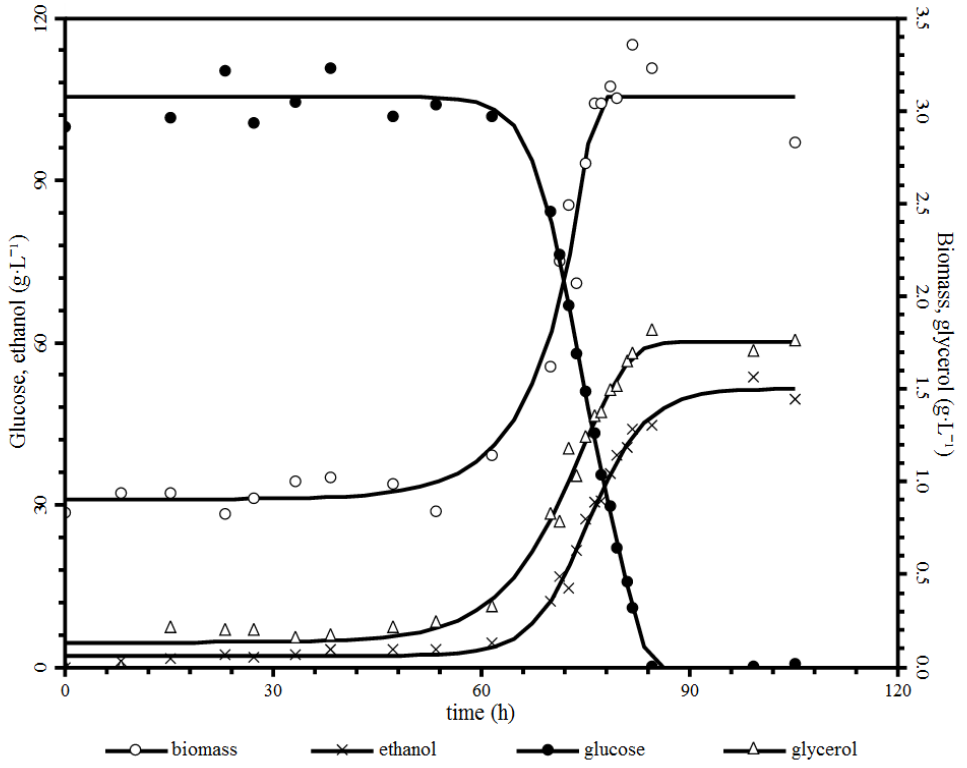


Figure 6: Fermentation with inhibitors

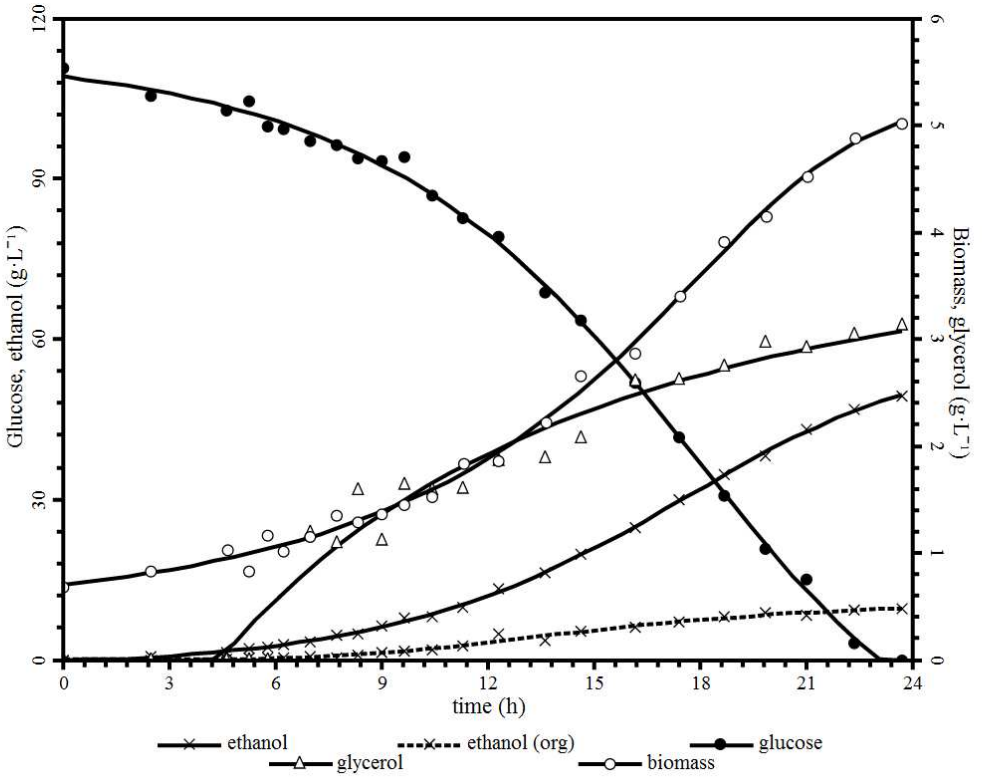


Figure 7: Fermentation with inhibitors and with biodiesel

As for furfural and vanillin, the detoxification mechanism works by reducing furfural to non-toxic furfuryl alcohol and vanillin to vanillic alcohol as shown in Figure 8. At first, furfural is reduced with a rate that decreases when the furfural concentration becomes smaller. Vanillin reduction, on the contrary, is slow or negligible initially, after which its rate increases until all vanillin has been reduced. As can be seen in Figure 9, in the presence of biodiesel the studied compounds are partitioned over the aqueous and organic phase. The same reduction pattern can be seen as in Figure 8, with the exception of two main observations. Firstly, there is a lag phase of 5 hours before the furfural starts to be reduced significantly, while the ethanol fermentation itself starts directly after the introduction of the inoculums to the medium. The yeast, which was freshly grown under equally inhibiting conditions, apparently had to induce again its metabolic capacity to reduce furfural, but was triggered later to do so and to a much less extend. The lower concentrations of furfural, vanillin and acetic acid in the aqueous phase may have reduced the need for a prompt reaction to the presence of these toxic compounds. Secondly, calculating reduction rates

from the combined organic and aqueous phase, the maximum specific reduction rate of furfural (0.27 $g \cdot g^{-1} \cdot h^{-1}$) is higher than in the case without organic phase (0.05 $g \cdot g^{-1} \cdot h^{-1}$). The maximum specific reduction rates for vanillin with and without the organic phase are 0.20 and 0.03 $g \cdot g^{-1} \cdot h^{-1}$ respectively. Apparently, the lower combined inhibition level in the presence of an organic phase allows for a more effective furfural and vanillin reduction. Thus, while in the presence of an organic phase the furfural concentration to which the yeast is exposed (0.9 $g \cdot l^{-1}$ in the aqueous phase) is lower than without the organic phase (2 $g \cdot l^{-1}$), the specific reduction rate is five to six times higher. In contrast, previous work has shown that furfural reduction rates increase with higher furfural concentrations to up to 4 $g \cdot l^{-1}$ of furfural, after which reduction rates decrease (Palmqvist *et al.*, 1999a). Probably, the synergistic toxicity effect of vanillin and acetic acid in addition to furfural decreases the furfural concentration for which furfural reduction rate is at maximum. An important implication of this observation is that for fed-batch of hydrolysed liquor, independent of the application of an organic phase, the conversion kinetics of furfural and vanillin can be more favourable than for batch fermentation by keeping the inhibitor concentrations constant but low. For a continuous fermentation however, growth inhibition can be the actual limiting factor for the maximal specific inhibitor conversion, because washout will occur at relatively low inhibitor concentrations as noted also by Horvath *et al.*, 2001. Moreover, before reaching washout conditions, low sugar conversion rates will cause loss of substrate and low ethanol production rates. Thus especially for continuous fermentations, the proposed two-phase system is a promising option for its inherent increased capacity of inhibitor reduction.

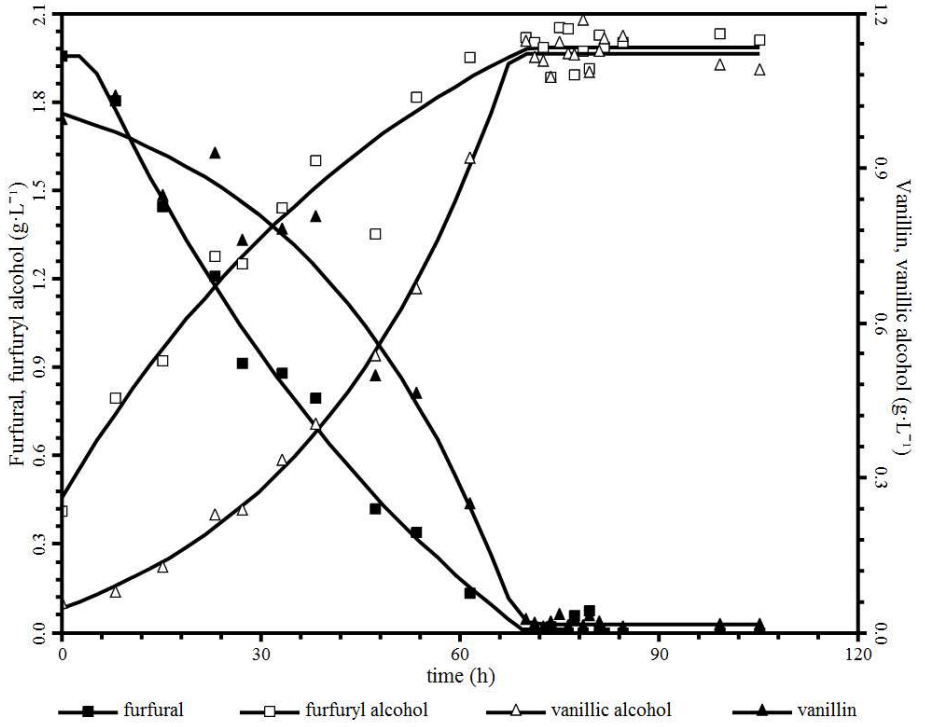


Figure 8: Concentration profiles of inhibitors during fermentation without biodiesel

For the fermentation with the organic phase (Figure 9), all furfural and vanillin was reduced within 13 hours, well before the end of the fermentation. This has implications for the ethanol yield relative to glycerol, which is slightly higher only while furfural is still present in the medium (Palmqvist *et al.*, 1999b, Almeida *et al.*, 2009). On the other hand, specific growth rate and growth coupled specific ethanol production suffer from the presence of the inhibitors (Palmqvist *et al.*, 1999a), while for acetic acid it is known that it also has a positive effect on specific ethanol production because of a higher demand for ATP (Taherzadeh, 1999). The fermentation process could thus be optimized for total yield, by reducing the amount of organic phase such that the inhibitors are present until the end of the batch fermentation in limited concentrations, with a slight increase in total fermentation time.

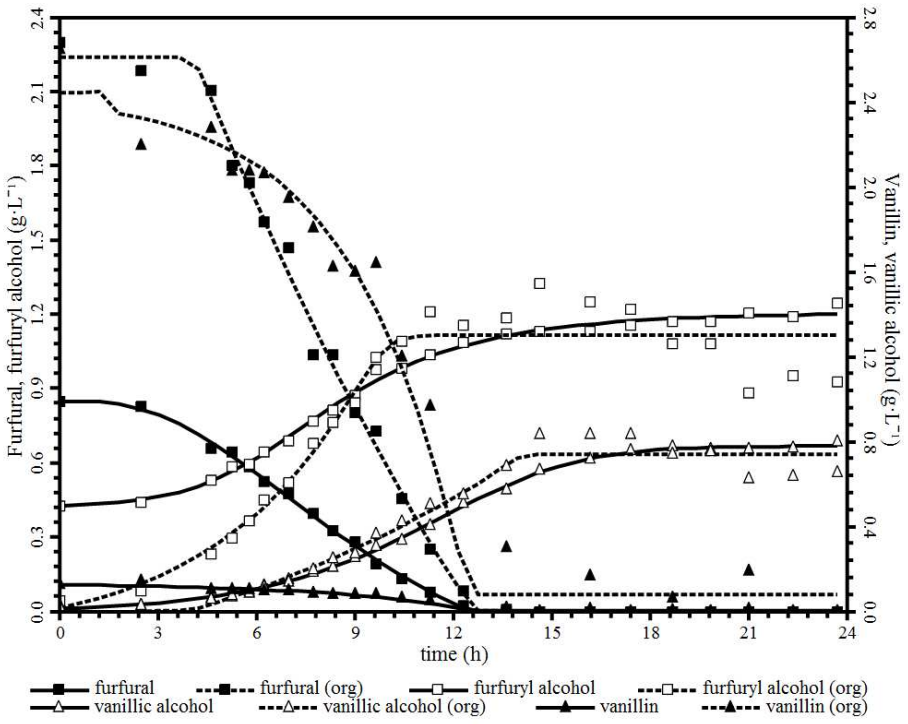


Figure 9: Concentration profiles of inhibitors during fermentation with biodiesel in the organic phase (org) and aqueous phase (no suffix).

4.4.f Further observations

For high turbulence of the aqueous two-phase system with biodiesel or castor oil, emulsification was unavoidable. The higher the turbulence, the more stable this emulsification maintained. Both biodiesel and castor oil however allowed yeast to be completely recovered by centrifugation of the emulsified fermentation broth, which then could be easily de-emulsified by heating to 50˚C. Conceição *et al.* (2007) determined the densities of both solvents at 20°C and showed that the density of biodiesel (0.925 $g \cdot cm^{-3}$) is lower than the density of castor oil (0.958 $g \cdot cm^{-3}$), favouring biodiesel for its separation characteristics. Partition coefficients in the actual two-phase fermentation system, were different from the partition coefficients as depicted in Table 1, due to the different composition of the aqueous phase mainly with respect to salts. Interestingly, these differences generally favoured the fermentation process in that inhibitors, including

ethanol, had more affinity with the organic phase than predicted by the measured partition coefficients in the pure water-biodiesel two phase system.

4.5 Conclusion

Biodiesel as an ethyl-esther of ricinoleic acid derived from castor-oil has several characteristics that make it an interesting choice as an extractive solvent for *in-situ* extractive fermentation of hydrolyzed lignocellulose biomass. It is a relatively cheap, renewable bioproduct that can be produced on-site. It is biodegradable and waste that is produced after several recovery cycles can be sold or used as biofuel for the plant's own truck float. Both biodiesel and castor oil show no toxicity and optimal biocompatibility for industrial yeast. Biodiesel has a lower density than castor oil (Conceição *et al.*, 2007) favouring its separation from fermentation broth. Biodiesel absorbs more water than castor oil, which in effect concentrates substrate in the aqueous phase and water and ethanol are absorbed more with increasing total amounts of ethanol in the system. For a batch fermentation this means that sugar is concentrated mostly at the end of the fermentation when the ethanol concentration is high, which is a desired effect since it avoids undesired substrate inhibition at the beginning of the fermentation while it promotes maximum yields and use of sugar towards the end of the fermentation. Partition coefficients for the undesired fermentation inhibitors are highest for biodiesel, resulting in the removal of these inhibitors from the fermentation broth in favour of the overall fermentation process. Ethanol production starts sooner in a batch fermentation and the production rate will be higher in a (fed-) batch process than without the extractive solvent. For optimal use of the extractive capabilities of biodiesel, the ethanol concentration in the aqueous phase should be slightly below 80 g/L, coincidently the concentration at which ethanol becomes inhibitive to the fermentation at most standard fermentation temperatures. Comparison of a conventional fermentation without inhibitors, fermentation with inhibitors and fermentation with inhibitors and biodiesel, confirm the positive effect of the use of biodiesel for batch

fermentation with furfural, vanillin and acetic acid in terms of total fermentation time, growth rates, ethanol production rates and ethanol yields.

4.6 Acknowledgement

Biodiesel was kindly provided by the Coppe institute of the Federal University of Rio de Janeiro with the intermediation of Prof. D. Freire.

4.7 References

Atala D.I.P., Costa A.C., Maciel R., Maugeri-Filho F. 2001. Kinetics of ethanol fermentation with high biomass concentration considering the effect of temperature. Applied Biochemistry and Biotechnology 91-93:353-365

Almeida JRM, Bertilsson M, Hahn-Hägerdal B, Lidén G, Gorwa-Grauslund MF. 2009. Carbon fluxes of xylose-consuming Saccharomyces cerevisiea strains are affected differently by NADH and NADPH usage in HMF reduction. Appl Microbiol Biotechnol 84:751-761

Barber AR, Hansson H, Pamment NB. 2000. Acetaldehyde stimulation of the growth of Saccharomyces cerevisiae in the presence of inhibitors found in lignocellulose-to-ethanol fermentations. J Ind Microbiol Biotechnol 25:104-108

Björklund L, Larsson S, Jönsson LJ, Reimann A, Nivebrant N-O. 2002. Treatment with lignin residue. A novel method for detoxification of lignocellulose hydrolysates. Appl Biochem Biotechnol 98:563-575

Chandel AK, Kapoor RK, Singh A, Kahud RC. 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501. Bioresour Technol 98:1947-1950

Conceição M.M., Candeia R.A., Silva F.C., Bezerra A.F., Fernandes V.J., Souza A.G. 2007. Thermoanalytical characterization of castor oil biodiesel. Renew Sust Energ Rev 11:964-975

Csányi E, Bélafi-Bakó K, Nemestóthy N, Gubicza L. 2004. Study on ethanol fermentation integrated with simultaneous solvent extraction and enzymatic reaction. Acta Aliment 33:63-70

Daugulis AJ, Swaine DE. 1987. Extractive Fermentation – Integrated reaction and product recovery. Biotechnol Lett 9:425-430

Delgenes JP, Moletta R, Navarro JM. 1996. Effects of lignocellulose degradation products on ethanol fermtentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Phichia stipitis and Candida shehatae. Enzyme Microb Technol 19:220-225

Ezeji T, Qureshi N, Blaschek HP. 2007. Butanol production from agricultural residues: impact of degradation products on Clostridium beijerinckii growth and butanol fermentation. Biotechnol Bioeng 97:1460-1469

Grobben N.G., Eggink G., Cuperus F.P., Huizing H.J. 1993. Production of acetone, butanol and ethanol (ABE) from potato wastes: fermentation with integrated membrane extraction. Appl Microbiol Biotechnol 39:494-498

Hasmann FA, Santos VC, Gurpilhares, DB, Pessoa-Junior A, Roberto IC. 2008. Aqueous two-phase extraction using thermoseparating copolymer: a new system for phenolic compounds removal from hemicelullosic hydrolysate. J Chem Technol Biotechnol 83:167-173

Horváth IS, Taherzadeh MJ, Niklasson C, Lidén G. 2001. Effects of furfural on anaerobic continuous cultivation of Saccharomyces cerevisiae. Biotechnol Bioeng 75:540-549

Junqueira TL, Dias MOS, Maciel MRW, Maciel-Filho R, Rossell CEV, Atala DIP. 2009. Simulation and optimization of the continuous vacuum extractive fermentation for biotethanol production and evaluation of the influence on distillation process. Comput –Aided Chem Eng 26:827-832

Kapucu H, Mehmetoglu U. 1999. The effects of bioprocess parameters on the yield in extractive ethanol fermentation. Rev Chem Eng 15:307-318

Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant N-O. 1999. The generation of fermentation inhibitors during dilute acid hydrolyis of softwoo. Enzyme Microb Technol 24:151-159

Liu Z.L. 2011. Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Appl Microbiol Biotechnol 90: 809-825

Malinowski JJ, Daugulis AJ. 1993. Liquid-liquid and vapour-liquid behavior of oleyl alcohol applied to extractive fermentation processing. Can J Chem Eng 71:431-436

Martin C, Marcet M, Almazán O, Jönssen LJ. 2007. Adaptation of a recombinant xylose-utilizing Saccharomyces cerevisiae strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour Technol 98:1767-1773

Minier M., Goma G. 1982. Ethanol production by extractive fermentation. Biotechnology and Bioengineering. 24:1565-1579

Modig T, Lidén G, Taherzadeh MJ. 2002. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochem J 363:769-776

Oliva JM, Sáez F, Ballesteros I, González A, Negro MJ, Manzanares P, Ballesteros, M. 2003. Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast Kluyveromyces marxianus. Appl Biochem Biotechnol 105:141-154

Oliva, JM, Negro MJ, Sáez F, Ballesteros I, Manzanares P, González A, Ballesteros M. 2006. Effects of acetic acid, furfural and catechol combinations on ethanol fermentation of Kluyveromyces marxianus. Process Biochem 41:1223-1228

Pamlqvist E, Grage H, Meinander NQ, Hahn-Hägerdal B. 1999. Main and interaction effects of acetic acid, furfural, and p-Hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol Bioeng 63:46-55

Palmqvist E, Almeida J, Hahn-Hägerdal B. 1999. Influence of furfural on anaerobic glycolytic kinetics of Saccharomyces cerevisiae in batch culture. Biotechnol Bioeng 62:447-454

Palmqvist E, Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour Technol 74:17-24

Palmqvist E, Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol 74:25-33

Pampulha ME, Loureiro-Dias MC. 2000. Energetics of the effect of acetic acid on growth of Saccharomyces cerevisiae. FEMS Microbiol Lett 184:69-72

Rivera EC, Costa AC, Atala DIP, Maugeri-Filho F, Wolf-Maciel MR, Maciel-Filho R. 2006. Evaluation of optimization techniques for parameter estimation: Application to ethanol fermentation considering the effect of temperature. Process Biochem 41:1682-1687

Rossel CEV, Lahr Filho D, Hilst AGP, Leal MRLV. 2005. Saccharification of sugarcane bagasse for ethanol production using the Organosolv process. Int Sugar J 107:192-195

Taherzadeh MJ. 1999. Ethanol from lignocellulose: Physiological effects of inhibitors and fermentation strategies. Doctor Thesis, Chalmers University of Technology, Sweden ISBN 91-7197-780-5

Taherzadeh M, Niklasson C, Lidén G. 1997. Acetic acid- friend or foe in anaerobic conversion of glucose to ethanol. Chem Eng Sci 52:2653-2659

Vaisman B, Shikanov A, Domb AJ. 2008. The isolation of ricinoleic acid from castor oil by salt-solubility-based Fractionation for the biopharmaceutical applications. J Am Oil Chem Soc 85:169-184

Weilnhammer C, Blass E. 1994. Continuous fermentation with product recovery by in-situ extraction. Chem Eng Technol 17:365-373

Zautsen RRM, Maugeri-Filho F, Vaz-Rossell CE, Straathof AJJ, Wielen LAM, Bont JAM. 2008. Liquid-liquid extraction of fermentation inhibiting compounds in lignocelluloses hydrolysate. Biotechnol Bioeng 102: 1354-1360

Capítulo 5

Kinetics of ethanol fermentation and inhibition by hydrolyzed lignocellulosic biomass

5.1 Abstract

A kinetic model is proposed for the fermentation kinetics and interactions between temperature and key inhibiting substances in hydrolytic liquor from hydrolyzed biomass: furans and phenolic compounds in the presence of acetic acid. A series of experiments were carried out according to a Plackett-Burman experimental design, which identified temperature and the concentration of these compounds as most important factors influencing the severity of inhibition of ethanol fermentation. The impact of furfural on the reduction of furfural and vanillin was greater than the impact of vanillin and the impact of acetic acid was especially observed on the vanillin reduction rate. Further experiments were done according to a two-level complete center composite experimental design, and, based upon the observed variations in the fermentative behavior, a kinetic model was developed that includes the microbial conversion rates of the inhibitive compounds into their less toxic derivatives. The model accurately describes the reduction of furfural prior to the reduction of vanillin, under different conditions of temperature and initial inhibitor concentrations. Also the lag phase, during which yeast growth is surpressed because of the presence of the inhibitors, could be precisely predicted to up to 24 hours.

5.2 Introduction

5.2.a Context

Continuing interest in ethanol as biofuel and key component of polyethylene based bioplastics is leading to expanding production facilities and increasing demand for land used to grow crops like sugarcane or corn as raw material (Martin, 2010). For 'second generation biofuels', cellulosic and lignocellulosic material is converted into sugars as xylose and glucose and fermented (Sun *et al.*, 2002). Since lignocelluloses, which have a fortifying function for the physical structure of the used crop, often represent around 50% of total convertible dry mass (Zautsen, 2011), technologies that enable utilization of this raw material allow for greater efficiency of use of land and irrigation resources besides decreasing transportation costs of raw material and stillage per volume of end product. In the case of sugarcane, it can be estimated that by converting bagasse to ethanol in addition to sugarcane juice at the current state of art in terms of process efficiency, for one square hectometer of land yielding 80 wet-tons of sugarcane, potentially 9.9m^3 of ethanol can be produced rather than 7.3m^3 with only sugarcane juice. This corresponds to an increase in overall ethanol yield of 36% (based on data provided by Seabra *et al.*, 2010). With improved value for bagasse fiber as raw material, the total cane yield per land area can be further increased by shifting the current physical-economical optimum ratio of a minimum of strength providing fiber and maximal juice content towards relatively more fiber allowing for taller plants which can contain both more juice and more fiber in absolute terms. In the past, cultivars of such `energy canes` with yields of 265 and 307 (wet) tons per hectare per year have been reported (Giamalva *et al.*, 1984, Legendre *et al.*, 1995), more than four times the current yield averages albeit with inferior juice quality and sugar percentages. In an industrial context, the quality of the raw material and fluctuations thereof can lead to considerable changes in general kinetic behavior, impacting yield and productivity, as well as variations of dominant yeasts in the process and fluctuations in temperature that can dislocate the fermentation from optimal conditions (Rivera *et al.*, 2006). It is therefore important to gain more detailed insight in the kinetics of the reduction of furfural and other inhibitors, in addition to the kinetics of the conventional 'first generation' ethanol fermentation to be able to optimize the process conditions. For

example, Palmqvist *et al.* (1999) proposed a stoichiometry-based model including furfural reduction, NADH and ATP balance and Oliveira *et al.* (2006) proposed a polynomial model describing the effects of acetic acid, furfural and catechol on growth rate and ethanol and biomass yield.

5.2.b Fermentation inhibitors

To yield fermentable sugars from bagasse, pretreatment and hydrolysis steps are required. For example, using diluted sulfuric acid under high pressure and temperature in the presence of ethanol is an effective and fast organosolv hydrolysis process (Rossell *et al.*, 2005). Milder processes are also being developed that serve as mere pretreatment allowing further saccharification by enzymes (Lee and Jeffries, 2011). During such processes however, substances are generated and released that inhibit a subsequent ethanol fermentation process. These compounds comprise furans (furfural and hydroxyl-methyl-furfural), organic acids (e.g. acetic, formic and levulinic acid) and phenolic compounds (e.g. vanillin, 4-hydroxybenzaldehyde, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde) (Larsson *et al.*, 1999, Klinke *et al.*, 2004). As inhibitor, furfural decreases the growth rate (Oliva *et al.*, 2006), decreases the cell mass yield and ethanol productivity (Palmqvist *et al.*, 2000) and causes a lag phase in cell growth at concentrations typical for lignocellulosic hydrolysates (Almeida *et al.*, 2009, Oliva *et al.*, 2006, Palmqvist *et al.*, 1999). Acetic acid (pKa 4,75) is liposoluble in un-dissociated form and can thus diffuse across the plasma membrane at lower pH, dissociate inside the cell and decrease the growth rate and biomass yield due to toxicity of accumulating of its anion and ATP usage for proton excretion (Palmqvist *et al.*, 2000, Pampulah *et al.*, 2000, Almeida *et al.*, 2007). Phenolic compounds have a negative effect on ethanol yield and growth rate (Palmqvist *et al.*, 2000) by disturbing the structure membranes of the cell and its organelles.

5.2.c Biochemical and modeling complexity

These inhibiting effects are exhibited in a synergistic manner, such that their joint toxicity cannot be evaluated as the sum of the toxicity of each compound and its concentration (Palmqvist *et al.*, 1999, Oliva *et al.*, 2006). A review by Liu (2011) details

various mechanisms that contribute to a certain tolerance to the inhibitors, as increased defense against inhibitor influx to cytoplasm and organelles, increased employment of membrane repair mechanisms, shifts in the glycolytic pathway to tactically and strategically manage energy balances and promote NAD(P)H production, decreased toxicity of aggregation of damaged or misfolded proteins by higher chaperone production for enzyme refolding and ubiquitin production for enzyme degradation. Moreover, the toxicity of most inhibitors can be neutralized by many yeast strains providing sufficient cellular mass, adequate pH and sometimes light aeration, through mechanisms including aldehyde reduction by alcohol- and aldehyde dehydrogenases (Liu and Moon, 2009), double bond oxidation and polymerization triggered by laccase (Larsson *et al.*, 2000) and conversion of carboxilic acids to vinyl derivatives by phenylacrylic acid decaboxylase (Larsson *et al.*, 2001). As such, common ethanol fermentation yeast as *Saccharomyces cerevisiae* reduces furfural to furfuryl alcohol (Palmqvist *et al.*, 1999). Equally, vanillin is reduced to vanillyl alcohol by *S. cerevisiae* (Liu, 2011, Oliva *et al.*, 2003). The resulting derivatives of the compounds are less toxic, which is favorable for the overall performance of the fermentation, but attributes to the complexity of biochemical detoxification and fermentation kinetics. Since furfural is reduced by enzymes and the growth inhibition by furfural can be considered at the enzymatic level (Modig *et al.*, 2002, Liu 2011), the temperature may also be of importance to the furfural reduction rates. The same can be the case for vanillin reduction. Studies without inhibitors have shown that temperature does have an effect on growth rate, biomass yield and ethanol yield, as well as on maximal biomass and maximal ethanol concentration (Atala *et al.*, 2001, Rivera *et al.*, 2006).

5.2.d Scope of this work

Inhibitors as furfural, vanillin and acetic acid, other conditions as pH, initial biomass concentrations and maintenance level oxygen supply are all factors to take into account when predicting or optimizing the progress of fermentation of hydrolyzed lignocelluloses. For example, the presence of higher furfural concentrations induces a higher initial furfural reduction rate, but if the biomass concentration is too low and in the presence of acetic acid and at low pH, the health of the yeast becomes too compromised and the overall effect can

be cell death instead, unless cell maintenance is facilitated by small amounts of oxygen. In this context, only the relative influence of acetic acid, furfural, vanillin, temperature and pH to growth rate and aldehyde conversion rates were first studied according to a Plakett-Burman experimental design. After identifying the three factors that most influence the fermentation kinetics, a Central Composite Rotational Design was applied using only these factors to elaborate a kinetic model. Based on the results, a kinetic model was constructed for furfural reduction, vanillin reduction, growth and ethanol production and inhibition. Parameters were estimated by iterative parallel computations.

5.3 Materials and Methods

5.3.a Used materials

The following chemicals were used: peptone, bacto (Oxoid Ltd, Basingstoke Hampshire, England), yeast extract (Oxoid Ltd, Basingstoke Hampshire, England), agar (Merck kGaA, Darmstadt, Germany, extra pure), glucose (Labsynth, Diadema, SP Brazil, 98%), sulfuric acid (Labsynth, Diadema, SP Brazil, 95-98%), K_2HPO_4 (Ecibra, São Paulo, SP Brazil, 98%), furfural (J.T.Baker, Phillipsburg, NJ USA, 98.8%), furfuryl alcohol (Acros Organics, Geel, Belgium, 99%), vanillin (J.T. Baker, Phillipsburg, NJ USA), vanillyl alcohol (4-hydroxy-3-methoxy benzalyl alcohol, Acros Organics, Geel Belgium, 99%), acetic acid (Dinâmica, São Paulo, SP Brazil, 99.7%), glycerol (Aldrich, Milwaukee WI USA, 99.5%), ethanol (Ecibra, São Paulo, SP Brazil, 99.5%), Acetonitrile (J.T. Baker, Phillipsburg, NJ USA, 99.9%), mineral water (Bonafonte, Danone Ltda., Jacutinga MG Brasil).

5.3.b Experimental design

The experimental design consisted of two consecutive plans, where the first aimed to identify the most important factors between concentration of furfural, vanillin, acetic acid, pH and temperature for their influence on specific furfural and vanillin reduction and growth rate. This first design was according a screening design as originally proposed by

Plackett and Burman (1946) with 12 experiments and 2 central points as shown in Table 1, with concentrations of furfural, vanillin and acetic acid as they can be found in hydrolytic liquor with variations depending on the raw material and the applied hydrolysis process (Martinez *et al.*, 2001, Martin *et al.*, 2007, Silva *et al.*, 2005).

Table 1: Factor level values, real values (in brackets) and results of the Plackett-Burman screening design

test	Temp	Furfural	Vanillin	Acetic acid	pH	μ_{max}	$q_{F\,max}$	$q_{V\,max}$	$q_{FOH\,max}$	$q_{VOH\,max}$
#	°C	$g \cdot l^{-1}$	$g \cdot l^{-1}$	$g \cdot l^{-1}$	(-)	$mg \cdot g^{-1} \cdot h^{-1}$	$mg \cdot g^{-1} \cdot h^{-1}$	$mg \cdot g^{-1} \cdot h^{-1}$	$mg \cdot g^{-1} \cdot h^{-1}$	$mg \cdot g^{-1} \cdot h^{-1}$
1	1 (37)	-1 (0.2)	1 (3)	-1 (0.4)	-1 (4)	24	3	17	5	13
2	1 (37)	1 (3)	-1 (0.2)	1 (6)	-1 (4)	8	39	1	26	0
3	-1 (28)	1 (3)	1 (3)	-1 (0.4)	1 (5.2)	3	8	6	9	1
4	1 (37)	-1 (0.2)	1 (3)	1 (6)	-1 (4)	8	4	9	2	3
5	1 (37)	1 (3)	-1 (0.2)	1 (6)	1 (5.2)	4	19	2	15	1
6	1 (37)	1 (3)	1 (3)	-1 (0.4)	1 (5.2)	7	41	6	40	2
7	-1 (28)	1 (3)	1 (3)	1 (6)	-1 (4)	4	3	3	4	0
8	-1 (28)	-1 (0.2)	1 (3)	1 (6)	1 (5.2)	4	0	0	1	0
9	-1 (28)	-1 (0.2)	-1 (0.2)	1 (6)	1 (5.2)	93	2	3	2	0
10	1 (37)	-1 (0.2)	-1 (0.2)	-1 (0.4)	1 (5.2)	210	3	12	20	25
11	-1 (28)	1 (3)	-1 (0.2)	-1 (0.4)	-1 (4)	4	12	2	12	0
12	-1 (28)	-1 (0.2)	-1 (0.2)	-1 (0.4)	-1 (4)	220	4	7	4	0
13	0 (32.5)	0 (1.6)	0 (1.6)	0 (3.2)	0 (4.6)	160	29	26	23	28
14	0 (32.5)	0 (1.6)	0 (1.6)	0 (3.2)	0 (4.6)	220	31	31	39	33

The chosen temperature range, 28˚C to 37˚C, is a range frequently occurring in the ethanol fermentation industry and which has shown a linear relation with specific growth rate in absence of inhibitors (Rivera *et al.*, 2006). The pH was varied between 4.0 and 5.2, which was expected to be of great influence on the fermentation process due to the dissociation constant of acetic acid (pKa is 4.75) and the fact that acetic acid penetrates the cell membrane in undissociated form, especially in the presence of ethanol (Taherzadeh 1997, Casal *et al.*, 1997, Casey *et al.*, 2010, Oliva *et al.*, 2006). Preliminary experiments (data not shown) had confirmed that viability and overall aldehyde reduction rates are strongly related to the cell mass concentration in the medium. Too few yeast cells can

suffer irreversible decrease of metabolic activity and cell damage from the same concentration of inhibitors in the medium. Based on this experience, the initial biomass concentration was chosen as 1 $g \cdot l^{-1}$. The second design, based on the results of the screening design, was a Central Composite Rotational Design (CCRD) of 2^3, with six axial points and four central points, as shown in Table 2. Temperature, furfural and vanillin ranges were adjusted to the findings of the screening design experiments, whereas the acetic acid concentration, pH and initial biomass were fixed. Both designs were executed in two consecutive runs of 60 hours each at a minimal interval to assure resemblance of the prepared and frozen stock inoculum.

Table 2: Factor level values, real values (in brackets) and results of the Plackett-Burman screening design

test #	Temperature °C	Furfural $g \cdot l^{-1}$	Vanillin $g \cdot l^{-1}$
1	-1 (30)	-1 (0.4)	-1 (0.4)
2	1 (35)	-1 (0.4)	-1 (0.4)
3	-1 (30)	1 (1,5)	-1 (0.4)
4	1 (35)	1 (1,5)	-1 (0.4)
5	-1 (30)	-1 (0.4)	1 (1.5)
6	1 (35)	-1 (0.4)	1 (1.5)
7	-1 (30)	1 (1.5)	1 (1.5)
8	1 (35)	1 (1.5)	1 (1.5)
9	-1.68 (28.3)	0 (0.95)	0 (0.95)
10	1.68 (36.7)	0 (0.95)	0 (0.95)
11	0 (32.5)	-1.68 (0.03)	0 (0.95)
12	0 (32.5)	1.68 (1.87)	0 (0.95)
13	0 (32.5)	0 (0.95)	-1.68 (0.03)
14	0 (32.5)	0 (0.95)	1.68 (1.87)
15	0 (32.5)	0 (0.95)	0 (0.95)
16	0 (32.5)	0 (0.95)	0 (0.95)
17	0 (32.5)	0 (0.95)	0 (0.95)
18	0 (32.5)	0 (0.95)	0 (0.95)

5.3.c Inoculum preparation

Standard YEPD medium was composed of 2.5 g l^{-1} K_2HPO_4, 10 g l^{-1} peptone and 10 g l^{-1} yeast extract. Preliminary experiments had shown adaptation of yeast to increased levels of inhibitors throughout a limited number of generations, which was undone within 2 days without the presence of the inhibitors. Such apparent temporal inheritable adaptation of yeast to furfural, acetic acid and potentially vanillin in the first generations of the inoculum that might also have been noted by Palmqvist *et al.*, (1999) and Pampulha *et al.* (2000), was taken into account as follows. The yeast, an industrial strain of *Saccharomyces cerevisiae*, (*Santa Adélia Sugar Mill* strain), was first grown for at least two days on agar slants with standard YEPD medium and 20 g l^{-1} glucose at 34°C and transferred to a fresh slant every day. The yeast was then carried-over daily to fresh slants with gradually increasing inhibitors concentration to allow for adaptation of the yeast to the inhibitive environment until only minimal growth was observed. Furfural, acetic acid and vanillin were present in a ratio of 1:2.2:0.6, which was found representative for hydrolytic liquor hydrolyzed during weak acids treatment as deduced from Martin *et al.* (2007). Next, inoculum was grown in shaked flasks in two stages of 48 hours each. During the first stage, the biomass was increased by growth without inhibitors whereas in the second stage inhibitors were added and the yeast was further forced to adapt to the inhibitors and grown to a desired biomass concentration of 4.5 g l^{-1}. To finally assure a standard state of the initial inoculum, the thus prepared yeast was frozen in equal volumes and 15% glycerol at -18°C for several days before the execution of all experiments. Both incubation stages took place at 34°C in an incubator (Tecnallab TE420) at 150rpm. Incubation media had all been sterilized in an autoclave at 121°C for 15 min, while maintaining glucose as a separate solution to prevent browning. Also furfural and acetic acid had been sterilized in separate recipients to prevent partial evaporation or hydrolysis and added afterwards to the final medium in a laminar flux chamber. Optical density at 600nm and pH were monitored twice per day to verify and assure consistency (Beckman Coulter DU640 and Mettler Toledo AG CH8603).

5.3.d Shaked flasks fermentations

Both the shaked flask fermentations in the Plackett-Burman screening experimental design and the Central Composite Rotational Design contained the same YEPD medium as the inoculum with 2.5 g l^{-1} K_2HPO_4, 10 g l^{-1} peptone and 10 g l^{-1} yeast extract and had an initial glucose concentration of 65 g l^{-1}. The glucose concentration was chosen low enough to prevent any significant inhibition by substrate at the beginning or product or biomass towards the end of the fermentation (Rivera *et al.*, 2006). The pH was adjusted for each flask prior to autoclaving with either a 4M sulfuric acid solution or a 3M sodium hydroxide solution, such that the pH after autoclaving was according to the experimental design. Use of buffer solutions for each pH was avoided because of the practical difficulty to acquire a buffer for each desired pH of the experimental plan and to prevent unequal interference of the different buffer compounds with the fermentation results. Because glucose was autoclaved separately to prevent browning, the exact amount of acid or base per flask added to a concentrated YEPD solution was previously determined by establishing titration diagrams using complete media at the final concentrations, including acetic acid. Autoclaving and finalizing of the media was done according to the procedure of the inoculum preparation, using the inhibitor concentrations according to the experimental plans. After preparation of the media, the optical density of the inoculum was read at 600nm (Beckman Coulter DU640) to calculate the inoculum volume needed to obtain 1 g l^{-1} of yeast in the final experiment medium. This volume, approximately 40mL, was then centrifuged for each experiment at 4000rpm for 10 min at 20°C (Sorvall RC26 Plus with SLA1500 rotor). The supernatant was discarded and the pellet was resuspended in 150mL of the prepared media in 250mL Erlenmeyer flaks.

The flasks were then incubated in shakers (Innova 4430, Tecnallab TE420) at 150rpm and temperatures according to the experimental plans. Every three hours during 60 hours, samples of twice 2mL were taken and centrifuged in Eppendorf tubes at 2000rpm for 5 minutes. For each set of samples, 1 mL supernatant was diluted with 1mL Milli-Q water and filtered for HPLC analysis with 5.5cm^2·0.22µm filters with low death volume

(Microgon DynaGard®). The pellets of both Eppendorf tubes were washed twice with distilled water and centrifuged. The final pellets were dried at 50°C for at least 36 hours prior to measuring the dry weight on an analytical balance (Bel Engineering M214Ai). HPLC measurements for furfural, furfuryl alcohol, vanillin and vanillyl alcohol were done in 7 minute runs using a C-18 column (Merck Chromolith C18) with a precolumn (Bischoff Prontosil 120-5-C18 5µm) and quantified with UV detection (Varian 9095) at 229 nm. The mobile phase consisted of a 10% acetonitrile solution (A) and 100% Milli-Q H_20 (B), of which A was run at a proportion of 10% (1%ACN) for 1 min, then ramped up to 100% at 1.5 min until 3.5 min when it was ramped down again to 10% at 4 min until the end of the run at 7 min. Glucose and ethanol were retained using a HyperRez XP organic acids column (Thermo Scientific, 8µm 7,7·100mm), at 25˚C and 2 mM H_2SO_4 as a mobile phase at 1 ml min^{-1} and a runtime of 15 minutes; detection was done by a refractive index detection (ChromTech RI2000 RI) at 35˚C for glucose and ethanol and by UV detection (Varian 9095) at 215 nm. Due to high death volumes and limited allowed pH range of the said system, glycerol and acetic acid showed too much overlap for adequate quantification. All eluents were prepared with Milli-Q water, filtered with 0.22 µm filter (Millipore).

5.3.e Modeling

Construction of the model

Modeling of the fermentations was based on Jarzębski *et al.* (1989), previous work performed on the same yeast strain by Atala *et al.* (2001), Rivera *et al.* (2006), visualization of interaction patterns generated from the fermentation experiments, biological insights as summarized in the introduction and iterative acquisition of understandings while optimizing the model parameters. Kinetics concerning cell death were neglected because the experiments were carried out for limited time in a batch setup with the fraction of non-viable cells too low to determine correctly. Atala et al (2001) presented empirical estimates for temperature dependencies of, among others, μ_{max}, X_{max}, Y_{xs} and Y_{px}, using polynomial functions or a sum of two exponential functions, requiring 4 parameters for each formula, as did Rivera *et al.* (2006) based on Atala´s experimental data. However, using the values found by Rivera *et al.* (2006) within the temperature range of 28 and 37°C, as relevant to

this current work, these temperature dependencies could easily be linearized or described by simpler expressions without loss of regression, reducing the total number of parameters. Equation 1 represents the calculation of the specific growth rate μ as a product of the conventional Monod equation (with S as substrate concentration) and various inhibiting factors I_S, I_X, I_C, I_T: substrate, biomass, chemical and temperature inhibition, each limited between 0 and 1.

$$\mu = \mu_{max} \cdot \frac{S}{S + K_S} \cdot I_S \cdot I_X \cdot I_C \cdot I_T \qquad \text{h}^{-1} \qquad [1]$$

I_S, inhibition by substrate, is modeled as an inverse exponential function with constant K_i equal to 0.002 (Rivera *et al.*, 2006) as shown in equation 2.

$$I_S = e^{(-K_i \cdot S)} \qquad \textit{substrate inhibition} \qquad - \qquad [2]$$

I_X, inhibition by biomass, is modeled using a temperature-dependent maximal biomass concentration as in equation 3.

$$I_X = 1 - \left(\frac{X}{X_{max}(T)}\right)^m \qquad \textit{biomass inhibition} \qquad - \qquad [3]$$

Here, X stands for biomass in g·l^{-1}, T for temperature in °C, X_{max} for the biomass concentration in g·l^{-1} at which no further growth takes place. The exponent m, equal to 1.1, elevates the biomass ratio according to the work of Jarzębski *et al.* (1987). X_{max} as a function of temperature can be expressed as equation 4 by fitting an exponential to data published by Rivera *et al.* (2006) for temperatures between 28 and 37°C.

$$X_{max}(T) = 10587 \cdot e^{-0.145 \cdot T} \qquad \text{g·L}^{-1} \qquad [4]$$

I_C, chemical inhibition, is here proposed as a new factor (equation 5), replacing the often used independent product inhibition factor of Levenspiel (1980), i.e. a simple multiplication factor analogue to I_X, defined by the product concentration P and a P_{max} at

which growth stops. Instead, the product (ethanol) is regarded as one of the inhibitors present in the medium besides furfural, vanillin and acetic acid and can therefore be considered as part of an overall chemical inhibiting factor that models the toxicity caused by all these inhibitors in a synergistic manner. The biological explanation of this approach can be found in the argument that ethanol causes fluidity of the membrane (Heipieper and de Bont, 1994), resulting in activation and intensification of cell membrane repair and protecting mechanisms which is also the case for a fraction of furfural and vanillin, even while these disturb the cell membrane in other ways and also inhibit internal enzyme activities (Liu 2011). Including acetic acid, it can be readily expected that all these inhibitors eventually act synergistically in respect to increased requirements of ATP for repair, protection and excretion mechanisms which slows down or prohibits cellular growth (Almeida *et al.*, 2007, Liu 2011). Moreover, the total inhibition could intuitively be more severe at higher temperatures as it was shown for ethanol alone by Rivera *et al.* (2006), who noticed decreased tolerance for ethanol at higher temperatures. All these combined effects are expressed in equation 5 and adjusting of the parameters showed whether or not the assumptions were justified.

$$I_C = e^{\left[\frac{-(a\cdot F+b\cdot V)^c}{d^{(e\cdot q_F+f\cdot q_V)}}-(g\cdot P^h+i\cdot A)\cdot(T-j)^k\right]} \quad - \quad [5]$$

This chemical inhibition factor presented in this equation corresponds to the inverse of the exponent of a sum, so that this sum as a power weighs more than the sum of the elevation of individual factors to a power for each component. In other words, 2^{1+2} is more than $2^1 + 2^2$, which should cover the essence of the synergism of the effects. Being inversed, higher concentrations lead to lower I_C, with I_C approaching zero representing full inhibition. The components of the sum are functions of concentrations of furfural (F), vanillin (V), product (P), acetic acid (A) and temperature (T). The contribution of acetic acid (A) is linear with a factor i, and was not elevated to a power itself because its concentration was the same and constant for all experiments (2 $g \cdot l^{-1}$). Elevation to a power, as for non-constant state variables P by k, or the sum of $a \cdot F$ and $b \cdot V$ by c, with c limited between zero and 500, was done to allow for non-linear increase or reduction of the relative

weight of the inhibitor in the equation. The complexity of the function of *F* and *V* is a result of the iterative process of parameter estimation and will be explained in the results and discussion paragraph.

By virtue of consistency, rather than expressing the maximal specific growth rate μ_{max} as a function of temperature, the temperature dependency of the growth rate is included as a separate factor, I_T and to this end, again values found by Rivera *et al.* (2006) are interpreted as a linear function of the temperature between 28 and 37°C according to equation 6 with $a_{\mu T} = 0.53$ and $Topt_{\mu} = 37$°C.

$$I_T = 1 - a_{\mu T} \cdot \left(Topt_{\mu} - T\right) \qquad \textit{temperature inhibition} \qquad - \qquad [6]$$

Equations 7 and 8 are the state differential equations of biomass (*X*) and substrate (*S*).

$$\frac{dX}{dt} = \mu \cdot X \qquad g{\cdot}L^{-1}{\cdot}h^{-1} \qquad [7]$$

$$\frac{dS}{dt} = -\left(\frac{\mu}{Y_{XS} \cdot I_{TYxs}} + m_X\right) \cdot X \qquad g{\cdot}L^{-1}{\cdot}h^{-1} \qquad [8]$$

Here, *dS/dt* is programmatically limited to zero when *S* reached zero. If cell death kinetics would be considered, it would however make sense to include the lack of sugar needed for maintenance (m_x) in the calculation of this cell death. Because the biomass yield, *Yxs*, shows an approximately linear temperature dependency according to Rivera *et al.*, (2006), it is multiplied by I_{TYxs}, a linear function of the temperature described in equation Erro! Fonte de referência não encontrada..

$$I_{TYxs} = 1 - a \cdot (T - Topt_{Yxs}) \qquad - \qquad [9]$$

The maintenance coefficient (m_x) is chosen as a function of the sum of all inhibitors as in equation 10.

$$m_X = m_{X0} + m_{Xa} \cdot (F + V + P + A) \qquad - \qquad [10]$$

State differential equations for furfural and vanillin were initially based on visualizations of dynamic patterns that were generated from all fermentation results (data not shown). The iterative parameter estimation process finally led to equations 11 and 12.

$$\frac{dF}{dt} = -q_F \cdot I_{TqF} \cdot X \qquad g \cdot L^{-1} \cdot h^{-1} \qquad [11]$$

$$\frac{dV}{dt} = -q_V \cdot I_{TqV} \cdot X \qquad g \cdot L^{-1} \cdot h^{-1} \qquad [12]$$

With specific reduction rates q_F and q_V as functions of F and V:

$$q_F = \frac{a_{qF} \cdot F}{10^{-10} + \left(b_{qF} \cdot F + c_{qF} \cdot V\right)^{d_{qF}}} \qquad h^{-1} \qquad [13]$$

$$q_V = q_{Vmax} \cdot \frac{V}{K_V + V} \cdot {a_{qV}}^{-b_{qV} \cdot F} \qquad h^{-1} \qquad [14]$$

Inhibition by temperature of the specific furfural reduction rate is given by equation 15, where the optimum temperature $Topt_{qF}$ is always higher than T for the current experiments.

$$I_{TqF} = 1 - a_{qFT} \cdot \left(Topt_{qF} - T\right) \qquad \textit{with } T < Topt_{qF} \qquad - \qquad [15]$$

Inhibition by temperature of the specific vanillin reduction rate on the other hand has to be given by two equations, depending on the value of T relative to $Topt_{qV}$. Inhibiting factors I_{TqV} and I_{TqF} are always kept greater or equal than zero.

$$I_{TqV} = 1 - a_{qVT} \cdot \left(Topt_{qV} - T\right) \qquad for\ T <= Topt_{qV} \qquad - \qquad [16]$$

$$I_{TqV} = 1 - b_{qVT} \cdot \left(T - Topt_{qV}\right) \qquad for\ T > Topt_{qV} \qquad - \qquad [17]$$

As noted by Bai (2007) for high gravity fermentations, inhibition of growth (μ) and specific ethanol production (q_P) by ethanol concentration should be regarded as two distinct kinetic mechanisms. For the current experiments it was deliberately chosen to maintain a low enough substrate concentration to prevent both inhibition of μ and q_P by ethanol and biomass towards the end of the fermentation, so that q_P could be directly calculated from the growth rate and product yield. Note that in the executed batch fermentations, furfural and vanillin are converted by the yeast before growth and ethanol production even start, thus preventing these substances from contributing to any synergic effects with ethanol. Also acetic acid, of which the concentration is virtually constant throughout the fermentation, did not have to be considered as a factor that would increase sensitivity to ethanol because of the relatively low final concentration of the ethanol. It can be argued that in this light, a valid future model for continuous fermentation should however provide for synergic effects of inhibitors towards ethanol tolerance. For the current batch experiments, the model suffices with equations 18 and 19 for the state differential of ethanol and Y_{px} as a function of temperature.

$$\frac{dP}{dt} = (\mu \cdot Y_{PX} - m_P) \cdot X \qquad g \cdot L^{-1} \cdot h^{-1} \qquad [18]$$

$$Y_{PX} = a_{Ypx} - b_{Ypx} \cdot \left(Topt_{Ypx} - T\right) \qquad - \qquad [19]$$

Parameter estimation

Although the total number of unknown parameters could be greatly reduced by simplification of initial equations to above presented formulas, parameter estimation proved complex due to the presence of many local optima, partly caused by the standard deviation of the experimental data and despite the use of fitted curves and attempts to use sophisticated algorithms as Annealing, and Particle Swarm Optimization. A solution was found by breaking the problem down in stages by concentrating on furfural reduction first and subsequently on vanillin reduction, growth inhibition, substrate consumption and finally product yield. During each stage, a defined set of state variables was kept equal to fitted experimental data during the simulation, while only the remaining state parameters were allowed to follow the model dynamics. For example, during the first stage where furfural reduction related parameters were estimated, all other state variables (*V*, *X*, *S* and *P*) were constantly updated during the simulations according to the values of the fitted experimental data, except for the furfural concentration, which was calculated each step in time according to its corresponding differential equation and the values of the other state parameters at that moment. In the next stage, both furfural and vanillin were allowed to follow model kinetics and only *X*, *S* and *P* were updated according to fitted experimental data, and so on. To avoid most local optima, large sets of all possible parameter value combinations within a discrete domain were simultaneously evaluated, and the domain shifted each subsequent run around the combination that resulted in highest regression. Programming was done in Python. To increase computational efficiency, the dataset was first interpolated to represent samples at equal points in time for all experiments. For this purpose, the experimental data was fitted to sigmoid curves, according to equation 20. Here, *t* is the time in hours and p_1 to p_6 adjusted for fitting.

$$f(t) = p_1 + \frac{p_2 - p_1}{\left[1 + p_3 \cdot e^{-(p_4 \cdot (t - p_5))}\right]^{1/p_6}} \qquad \textit{general sigmoidal fit} \qquad \text{g·L}^{-1} \qquad [20]$$

The total number of sample points was then minimized by using the fitted curves to calculate a fixed series of time steps, with a maximum interval of 30 minutes, that accurately describes all state variables of all experiments with straight intersections between each time step. Finally, the actual kinetic model was fitted, per state variable, by simulating all combinations of a discrete range of the relevant parameters and choosing parameter values that resulted in the highest regression.

5.4 Results and discussion

5.4.a Experimental Design

The results of the Plackett-Burman screening experimental design are shown in Table 1 in terms of maximum growth rate (μ_{max}), maximum specific reduction of furfural (q_{Fmax}) and vanillin (q_{Vmax}), maximum specific production of furfuryl alcohol (q_{FOHmax}) and vanillyl alcohol (q_{VOHmax}). Table 3 shows the standardized effects of each of the factors acetic acid, furfural, vanillin, pH and temperature on the results and corresponding p-values.

Table 3: Standardized effects (t-values at 8 degrees of liberty) and p values. Significant effects are marked bold (p<0.2).

	μ_{max}		q_{Fmax}		q_{Vmax}		q_{FOHmax}		q_{VOHmax}	
	t	p	t	p	t	p	t	p	t	p
temperature	-0,34	0,7399	**2,08**	0,0713	**3,11**	0,0145	**2,78**	0,0240	**1,88**	0,0970
furfural	**-2,69**	0,0276	**2,72**	0,0262	**-3,04**	0,0161	**2,54**	0,0349	**-1,68**	0,1314
vanillin	**-2,49**	0,0376	-0,53	0,6078	**1,56**	0,1578	-0,65	0,5344	-0,36	0,7297
acetic acid	**-1,77**	0,1149	-0,12	0,9038	**-3,48**	0,0083	**-1,45**	0,1860	**-1,62**	0,1430
pH	0,27	0,7922	0,22	0,8296	-1,12	0,2967	1,20	0,2663	0,55	0,5993

Within the chosen ranges, furfural concentration and temperature showed to be the most important factors according to practically all indicators, whereas pH appeared of least importance. The initial furfural concentration had more influence on both furfural and vanillin reduction than vanillin, which was only slightly important for its own reduction rate with a p-value of 0.1578. The influence of vanillin was most pronounced for the

maximal specific growth rate achieved during the fermentations, with furfural being of approximately equal importance. The influence of acetic acid on the fermentation process was also confirmed to be of considerable importance, especially for the vanillin reduction rate. To limit complexity and prevent experimental and analytical complications, it was decided to concentrate only on three interaction effects in the second experimental design. It could be argued that within the chosen range, the acetic acid concentration is overall more significant than the vanillin concentration. However, because acetic acid does not show an explicit dynamic behavior in contrast to vanillin, the latter was preferred to study over acetic acid in order to model its conversion kinetics for vanillin concentrations that change over time. Thus, temperature, furfural and vanillin were chosen as independent factors for the second experimental design (CCRD), as shown in Table 2. Acetic acid concentrations and initial pH were fixed at 2 $g \cdot L^{-1}$ and 5 respectively. As results from experiments according to this design, which covers all possible high-low combinations and two extremes of each factor, the concentration profiles of the state variables biomass, glucose, ethanol, furfural and vanillin were used for estimation of model parameters are published separately (Zautsen *et al*., 2011) and listed in Appendix 1.

5.4.b Modeling and parameter estimations

Fitting of the concentration profiles of the state variables for all 18 experiments to sigmoid curves resulted in an average regression of fit of 0.971. For several experiments, experimental data is shown in Figure 1 and Figure 2 along with final model predictions of each state variable.

Furfural conversion rate

In Figure 1 and Figure 2 it can be seen that furfural is generally reduced at higher rates before the conversion rate of vanillin becomes substantial. The reason behind this is not clear, but it is plausible that furfural has competitive binding advantages over vanillin to

the involved alcohol and aldehyde reductases. The specific furfural reduction rate was modeled with equation 13. Here, the number 10^{-10} in the denominator prevents division by zero and replaces an originally allocated Monod-like constant K that consistently converged to zero. Parameter c_{qF} is 38% smaller than b_{qF}, reflecting the fact that the concentration of furfural is of more importance to the furfural reduction rate than vanillin, as noted before from the results of the Plackett-Burman experimental design for q_{Fmax}. The power d_{qF}, 1.12, is close to 1, meaning that the relation of q_F is almost, but not completely, linear to the inhibitor concentration.

Inhibition of q_F by temperature was found linear with an optimum temperature of 39.2 °C. Because this is higher than any temperature used in the experiments, the preciseness of this value for $Topt_{qF}$ as an optimum for the enzyme complex involved in the conversion of furfural can be disputed, but for the proposed model it served well to obtain desired regressions for experiments over the studied temperature range.

Vanillin conversion rate

Specific reduction rate of vanillin, q_V, was best approached by a Monod-like dependency of vanillin concentration multiplied by an inverse exponential of furfural concentration alone, rather than a sum of inhibition by furfural and vanillin or even acetic acid. This consideration resulted in equation 14 with four parameters q_{Vmax}, K_V, a_{qV} and b_{qV}. Inhibition by temperature was slightly more complex than for furfural because it required an optimum temperature of 32.5 °C, in between the minimum and maximum temperatures studied. Parameter a_{qVT} was almost three times higher than b_{qVT}, meaning that temperature inhibition was more severe below than above the optimum temperature. The fact that for q_V a different optimum temperature was found than for q_F, suggests the involvement of at least some different enzymes in the conversion of furfural and vanillin.

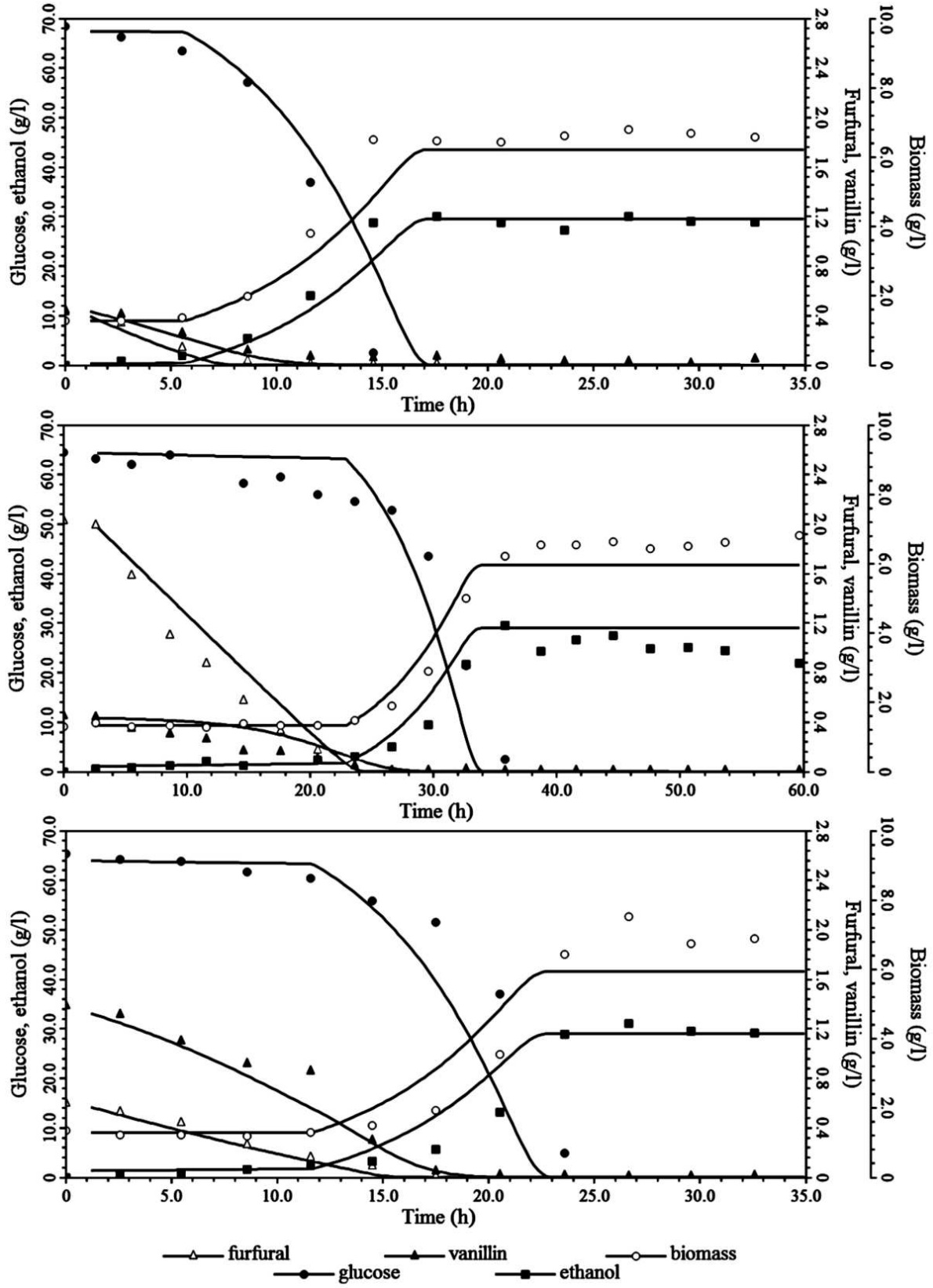


Figure 1: Profiles of state variables for different initial concentrations of furfural and vanillin at 35 °C by experimental data (symbols) and model (solid lines)

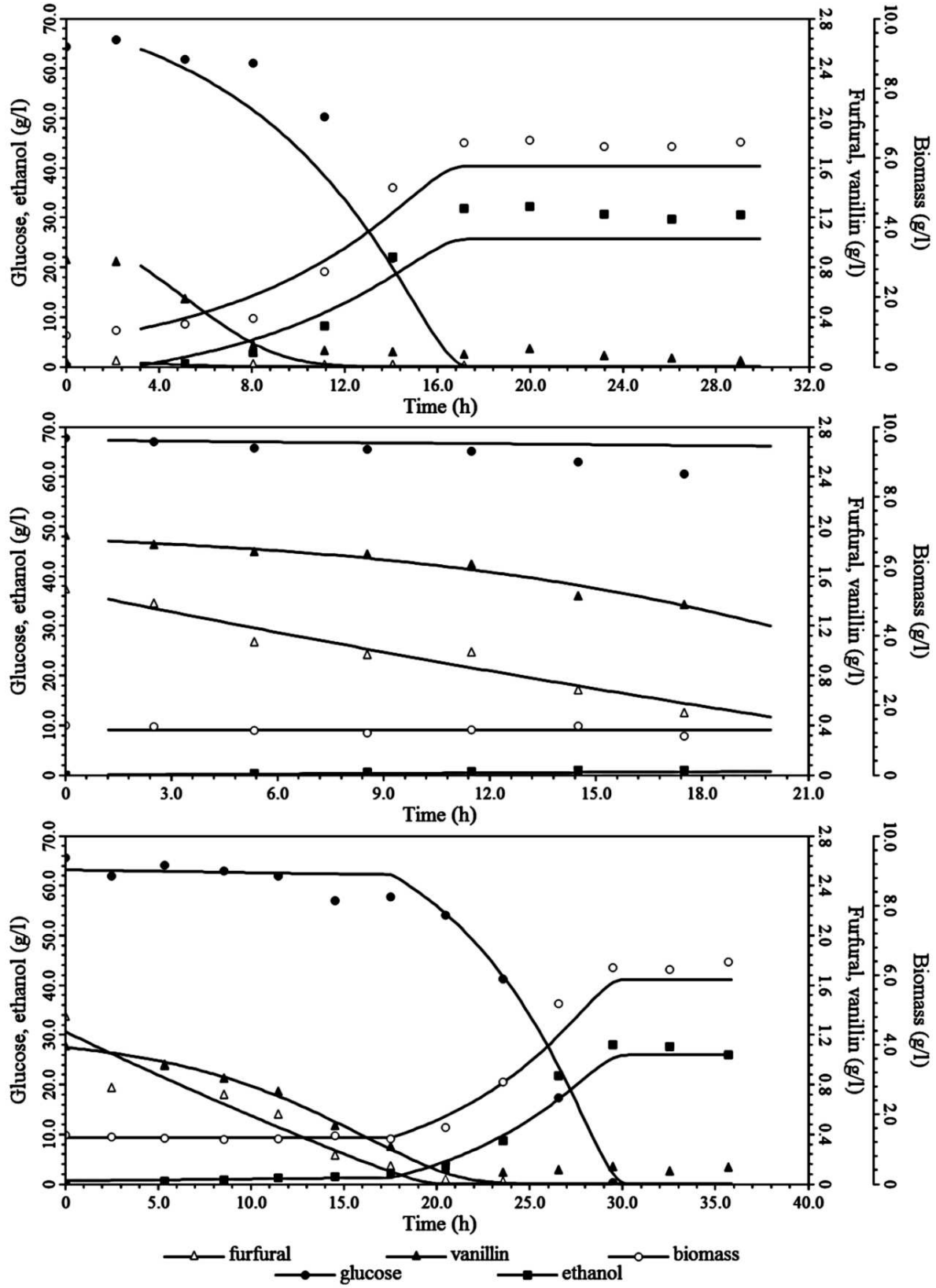


Figure 2: Profiles of state variables for different initial concentrations of furfural and vanillin at 32.5 °C by experimental data (symbols) and model (solid lines)

Growth rate

Yeast did not grow until inhibitor concentration was greatly reduced. This delayed growth can have been provoked by increased production and use of NAD(P)H for aldehyde reduction and stress protecting enzymes (Almeida *et al.*, 2009), use of ATP for maintenance and aldehyde excretion (Liu 2011) rather than biomass production, competitive inhibition of aldehyde- and alcohol dehydrogenases, non-competitive inhibition of pyruvate dehydrogenase (Modig *et al.*, 2002) or acetaldehyde accumulation (Palmqvist *et al.*, 1999). For the model, these effects were included in the proposed chemical inhibition factor I_C by means of an exponential function of the sum of furfural and vanillin. Convergence to a high number for the power c in equation 5 (180) resulted in a sharp tipping point for growth initiation.

However, it was also found that growth inhibition by furfural and vanillin was not only related to this elevated sum of F and V, but that I_C had to include a ratio with F and V in the numerator and specific reduction rates of the same (q_F and q_V) in the denominator. An explanation for this could be that when furfural and vanillin concentrations are low at the beginning of the fermentation, specific reduction is slow and growth is only allowed when the specific reduction rates are high. This is only the case close to the point of completion of the conversion, while when furfural and vanillin concentrations are high at the beginning of the fermentation, the specific reduction rates for these compounds are high by the time their concentrations reach the same low levels, allowing growth to start. In other words, two conditions have to be met for growth to start: the concentrations of both furfural and vanillin have to be low, and reduction of these compounds has to be active or completed. Adjustment regression of the model per state value and per experiment is shown in Table 4. Judging by the achieved regression for furfural and vanillin as state variables, the model succeeds in describing these conditions by equation 5.

Table 4: Regression of model per state variable and per experiment.

test #	Furfural %	Vanillin %	Biomass %	Glucose %	Ethanol %
1	99,2	89,3	62,4	83,5	66,1
2	92,9	88,6	92,2	82,3	95,7
3	87,2	69,7	69,4	68,9	68,4
4	93,9	85,0	95,8	90,4	93,9
5	73,6	62,5	51,1	60,5	55,7
6	96,8	95,8	86,0	88,1	92,1
7	58,8	11,2	4,0	34,7	50,0
8	75,0	38,6	3,1	40,5	57,1
9	90,5	86,0	76,5	74,7	80,4
10	94,9	90,2	94,9	94,1	90,1
11	56,6	84,2	89,2	81,9	82,5
12	57,8	43,9	2,2	14,0	51,5
13	95,7	68,0	80,1	51,1	74,9
14	90,7	93,8	4,4	37,9	53,8
15	92,1	83,8	84,0	72,6	85,8
16	92,8	96,0	86,4	93,8	94,8
17	93,3	97,1	96,8	97,7	97,3
18	95,1	98,4	89,3	91,0	92,0
total:	**95,7**	**94,8**	**81,5**	**74,4**	**85,4**

On the other hand, parameter $I_C g$ in equation 5 converged to zero, meaning that ethanol was not involved in growth inhibition which was expected because acetic acid and the final ethanol concentration were too low to inhibit the process substantially on their own. However, it would be interesting to see if a good regression can be found in continuous systems with $I_C g$ higher than zero and if growth inhibition by ethanol is indeed synergistically enforced by small amounts of furfural, vanillin and acetic acid.

Paramter $I_C k$ also converged to zero, meaning that the temperature has no influence on the severity of growth inhibition by acetic acid, which was not expected. Would the final ethanol inhibition have been higher, a temperature effect on inhibition by ethanol can still be expected since yeast metabolism becomes more sensible to ethanol at higher temperatures (Atala *et al.*, 2001, Rivera *et al.*, 2006).

Growth rate after conversion of furfural and vanillin resumed at higher rates than seen for fermentations with only acetic acid at the same concentration, a fact that can be atributed to the excess of ATP producing activity that was no longer needed at such extent for maintenance related mechanisms to counteract negative inhibitor effects, but could now be employed for biomass production. As can be understood from Palmqvist *et al.* (1999) and Almeida *et al.* (2009), due to competitive use of both aldehyde dehydrogenases (ALDH) and alcohol dehydrogenases (ADH) for furfural and vanillin conversion, even while over-expressed, acetaldehyde was now also excessively available for conversion to ethanol and acetate and the introduction of the latter into the TCA cycle and subsequent biomass production. In the model, this was expressed with a value of 0.50 h^{-1} for μ_{max} which is higher than commonly found for *Saccharomyces cerevisiae*, e.g. 0.43 h^{-1} (Rivera *et al.*, 2006). Note that for continuous fermentations, competitive inhibition of both ADH and ALDH enzymes remains, and maximal growth rate will thus be lower.

Final biomass concentrations in the experiments were purposely well beyond levels where biomass becomes an inhibitive factor. Therefore the biomass inhibition factor I_X can be considered unimportant and is merely included in virtue of completeness and can serve for construction of models for very high gravity (VHG) fermentation. In that case, it would however be advisable to re-estimate parameters used for the temperature dependence of X_{max}, because the above used parameters are derived from data published by Rivera *et al.* (2006) and are based on another model of biomass inhibition than used by Jarzębski *et al.* (1989). Also cell-death should be considered in this case and its rate might well depend on the temperature and severity of all inhibitors, including the product ethanol.

Substrate consumption rate

Substrate consumption rate depends on growth rate, biomass yield and maintenance. Previous studies have shown increase of overall biomass yield in the absence of acetic acid by up to certain amounts of furfural, but a decrease of the yield when both furfural and acetic acid are present (Palmqvist *et al.*, 1999). However, in this kinetic model of batch fermentation, where no growth takes place until both furfural and vanillin are depleted,

only a dynamic approach temporarily retaining information about previous inhibitor concentrations by means of integration would be able to describe such effect. Unfortunately the regression of the biomass was too poor to allow for this solution to be meaningful. This topic would in fact require a different experimental approach to be handled correctly and therefore a simpler assumption was made here that showed adequate for obtaining reasonable regressions for substrate for most experiments. In this assumption, yield was only considered depending on the temperature where Y_{xs} would be higher for lower temperatures as clearly seen by Atala *et al.* (2001) and Rivera *et al.* (2006). However, this effect, nor the contrary, could be concluded from this current work since factor I_{TYxs} consistently converged to 1, leaving Y_{xs} temperature independent. The dependence of the maintenance coefficient to the sum of all inhibitors as presented by equation 10 was revealed by parameter fitting and empirical optimization of the model, although Boyer *et al.* (1992) had not found an effect of only furfural concentrations on the maintenance coefficient.

Ethanol production rate

Like growth, ethanol production also only takes place after almost complete conversion of furfural and vanillin. This is a consequence of the competition for both NADH by alcohol dehydrogenase (ADH) and ADH itself by the aldehydes furfural, vanillin and intracellular acetaldehyde. Acetaldehyde is apparently the weakest competitor despite being accumulated and is even reported to be excreted from the cell rather than to be converted into ethanol (Palmqvist *et al.*, 1999). Calculation of product yield dependency on inhibitor concentration as done by Palmqvist *et al.* (1999) for furfural concentrations that do not inhibit growth completely, would require equal complexity for the current kinetic model as discussed previously for biomass yield and was therefore not considered. The resulting lack of fit might however be explained as a result of this decision. In contrast to Y_{xs}, temperature dependency of Y_{px} as noted by Atala *et al.* (2001) and Rivera *et al.* (2006) was confirmed by the found value of 0.2 for parameter b_{Ypx} resulting in higher product yields at higher temperatures.

Concluding

Furfural and vanillin kinetics could be described accurately. An overall satisfying regression for biomass could not be found, especially in respect to estimation of final biomass concentration. For cases where furfural or vanillin regression was not optimal, the model fails to correctly estimate the point at which growth starts due to the accumulative nature of the errors. For all other cases however, this point is correctly estimated. Too high furfural and vanillin concentrations can prohibit growth throughout at least 60 hours. During such time span cell death can be an important factor for sustaining absence of growth, but is not implemented in the model. As a consequence, predictions at large time spans and inhibitor concentrations using this model should be done with caution. Considering the complexity of the kinetics and the overall regression, the model suits well for describing the fermentation kinetics under different conditions of temperature and inhibitor concentrations. The regressions obtained for each state variable as listed in Table 4, were calculated according to equation 21. Note that for the calculation of these regressions, glucose, furfural and vanillin were only considered until their depletion and the total regressions were calculated over all data points for each state variable.

$$R^2 = 100\% \cdot \frac{R_{reg}}{R_{reg} + R_{res}} = 100\% \cdot \frac{\sum_i \left(\hat{y}_i - \bar{y}\right)^2}{\sum_i \left(\hat{y}_i - \bar{y}\right)^2 + \sum_i \left(y_i - \hat{y}_i\right)^2} \quad [21]$$

Optimal parameter values are listed in Table 5 along other values of constants used in the model. Conclusions drawn from the Plackett-Burman experiment were reflected by the relative values of these model parameters. For example, the vanillin concentration was indeed of minor importance to furfural reduction than the furfural concentration itself, as c_{qF} is 38% smaller than b_{qF}.

Table 5: Model parameter values per state differential. Estimated values are marked as bold.

dX/dt			dS/dt	dF/dt	dV/dt	dP/dt
μ_{max} **0,50**	I_{Cd} **860**	I_{Ck} **0,0**	Y_{xs} **0,08**	a_{qF} **5,9**	q_{vmax} **0,25**	a_{Ypx} **6,1**
K_S 4.1[a]	I_{Ce} **650**	$a_{\mu T}$ 0.053[b]	m_{x0} **0,01**	b_{qF} **40**	K_V **0,8**	b_{Ypx} **0,2**
K_i 0.002[b]	I_{Cf} **0,0**	$Topt_{\mu}$ 37[b]	m_{xa} **0,007**	c_{qF} **25**	a_{qV} **19,8**	$Topt_{Ypx}$ 37[b]
m 1.1[c]	I_{Cg} [d]			d_{qF} **1,12**	b_{qV} **0,7**	m_p **0,025**
I_{Ca} **4,0**	I_{Ch} [d]			a_{qFT} **0,007**	a_{qVT} **0,175**	
I_{Cb} **0,0**	I_{Ci} **0,44**			$Topt_{qF}$ **39,2**	b_{qVT} **0,063**	
I_{Cc} **180**	I_{Cj} **0,0**				$Topt_{qV}$ **32,5**	

a) Atala et al. (2001)
b) Rivera et al. (2006) or interpreted from data of the same
c) Jarzębski et al. (1989)
d) zero or unrelevant for low values of P

5.5 Conclusion

By means of a Plackett-Burman experimental design, the influence of several factors on growth rate and detoxification rates were studied for ethanol fermentation in a synthetic medium, which resembled liquor produced from hydrolyzed lignocelulose biomass. Within the studied ranges, between the factors pH, temperature and concentrations of the fermentation inhibitors furfural, vanillin and acetic acid, the pH was identified as the factor that least influences growth rate and aldehyde conversion. All other factors were significant for either the maximal growth rate, inhibitor conversion rate or both. Based on these results, a second experimental design was executed according to a 2^3 Central Composite Rotational Design, varying temperature and initial concentrations of furfural and vanillin. The concentration of acetic acid and the pH were kept constant to limit the complexity of the current work. From this latter set of experiments, concentrations of biomass, substrate and inhibitors were used to construct a kinetic model and describe the microbial detoxification and fermentation performance for all possible combinations of different temperatures and initial inhibitor concentrations within a plausible range. The resulting model gives an interesting general insight in the kinetics of fermentation in the presence of inhibitors in hydrolyzed lignocelluloses and the findings are discussed in detail for the conversion rates of furfural and vanillin, the growth rate, substrate consumption rate and ethanol production rate. Moreover, regression analyses showed that the obtained model accurately describes the conversion kinetics of furfural and vanillin and the suppressed growth during this

conversion. Therefore, the model can be used for the prediction of fermentation behavior in fed-batch fermentations and, for example, to optimize the substrate composition of a hydrolyzate with varying amounts of inhibiting components.

5.6 Nomenclature

α	(unit distance to) axial point	-
μ	specific growth rate	h^{-1}
μ_{max}	maximal specific growth rate	h^{-1}
A	acetic acid concentration	$g \cdot l^{-1}$
F	furfural concentration	$g \cdot l^{-1}$
I_C	chemical growth rate inhibition	-
I_C a…k	constants for calculation of I_C	-
I_S	growth rate inhibition by substrate	-
I_T	growth rate inhibition by temperature	-
I_{TqF}	inhibition of q_F by temperature	-
I_{TqV}	inhibition of q_V by temperature	-
I_{TYxs}	reduction factor of biomass yield	-
I_X	growth rate inhibition by biomass	-
K_s	modod constant	$g \cdot l^{-1}$
K_i	substrate inhibition power	-
K_V	half-value for vanillin reduction rate	$g \cdot l^{-1}$
P	product (ethanol) concentration	$g \cdot l^{-1}$
S	substrate concentration	$g \cdot l^{-1}$
T	temperature	°C
$Topt_{\mu}$	optimal temperature for growth	°C
$Topt_{Yxs}$	optimal temperature for biomass yield	°C
$Topt_{Ypx}$	optimal temperature for product yield	°C
$Topt_{qF}$	optimal temperature for biomass yield	°C
V	vanillin concentration	$g \cdot l^{-1}$
X	biomass	$g \cdot l^{-1}$
X_{max}	maximal biomass concentration	$g \cdot l^{-1}$
Y_{px}	product yield over biomass	$g \cdot g^{-1}$

Y_{xs}	biomass yield over substrate	$g \cdot g^{-1}$
$a_{\mu T}$	factor for growth rate inhibition by T	-
a_{qF}, $b_{qF,}$ c_{qF}, $d_{qF,}$	constants for calculation of q_F	-
a_{qFT}	constant for calculation of I_{TqF}	-
a_{qV}, b_{qV}	constants for calculation of q_V	-
a_{qVT}, b_{qVT}	constants for calculation of I_{TqV}	-
aY_{px}, bY_{px}	constants for calculation of Y_{px}	-
m	biomass inhibition power	-
m_p	constant specific ethanol production	-
m_x	maintenance coefficient for growth	h^{-1}
m_{x0}	constant maintenance coefficient	h^{-1}
m_{xa}	relative maintenance coefficient	h^{-1}
q_F	specific furfural reduction rate	h^{-1}
q_{FOH}	specific furfuryl alcohol formation rate	h^{-1}
q_V	specific vanillin reduction rate	h^{-1}
q_{VOH}	specific vanillyl-alcohol formation rate	h^{-1}
q_{Vmax}	maximal specific vanillin reduction rate	h^{-1}
$\hat{y}_i$	model estimate of state variable	$g \cdot g^{-1}$
y_i	experimental value	$g \cdot g^{-1}$
$\bar{y}$	mean of experimental values	$g \cdot g^{-1}$

5.7 References

Almeida J.R.M., Modig T., Petersson A., Hahn-Hägerdal B., Lidén G., Gorwa-Grauslund M.F. 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol. 82:340-349

Almeida JRM, Bertilsson M, Gorwa-Grauslund MF, Gorsich S, Lidén G. 2009. Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl Microbial Biotechnol 82:625-638

Atala D.I.P., Costa A.C., Maciel R., Maugeri-Filho F. 2001. Kinetics of ethanol fermentation with high biomass concentration considering the effect of temperature. Applied Biochemistry and Biotechnology 91-93:353-365

Bai F. 2007. Process oscillations in continuous ethanol fermentation with *Saccharomyces cerevisiae*. Doctoral thesis, Waterloo Ontario Canada.

Boyer L.J., Vega J.L., Klasson K.T., Clausen E.C., Gaddy J.L. 1992. The effects of furfural on ethanol production by *Saccharomyces cerevisiae* in batch culture. Biomass and Bioenergy 3:41-48

Casal M, Cardoso H, Leão C. 1997. Effects of ethanol and other alkanols on transport of acetic acid in *Sacharomyces cerevisiae*. Appl Environ Microbiol 64:665-668

Casey E, Sedlak M, Ho NWY, Mosier NS. 2010. Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of *Saccharomyces cerevisiae*. FEMS Yeast Res 10:385-393

Giamalva M.J., Clarke S.J., Stein J.M. 1984. Sugarcane hybrids of biomass. Biomass 6:61-68

Heipieper H.J., de Bont J.A.M. 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. Applied and Environmental Microbiology 60:4440-4444

Jarzębski A.B., Malinowski J.J., Goma G. 1989. Modeling of ethanol fermentation at high yeast concentrations. Biotechnol Bioeng 34:1225-1230

Klinke HB, Thomsen AB, Ahring BK. 2004. Inhibition of ethanol producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26.

Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant, N-O. 1999. The generation of fermentation inhibitors during dilute acid hydrolyis of softwood. Enzyme Microb. Technol 24:151-159.

Larsson S., Quintana-Sáinz A., Reimann A., Nilvebrant N.O., Jönsson L.J. 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 84-86:617-632

Larsson S., Nilvebrantt N.O., Jönsson L.J. 2001. Effect of overexpression of Saccharomyces cerevisiae Pad1p on the resistance of phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. Appl Microbiol Biotechnol 57:167-174

Lee J.W., Jeffries T.W. 2011. Efficiencies of acid catalysts in the hydrolysis of lignocellulosic biomass over a range of combined severity factors. Bioresour Technol 102:5884-5890

Legendre B.L., Burner D.M. 1995. Biomass production of sugarcane cultivars and early-generation hybrids. Biomass and Bioenergy 8:55-61

Levenspiel O. 1980. The monod equation: a revisit and a generalization to product inhibition situations. Biotechnol Bioeng 22:1671-1687

Liu Z.L., Moon J. 2009. A novel NADPH-dependent aldehyde reductase gene from *Saccharomyces cerevisiae* NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Gene 448:1-10

Liu Z.L. 2011. Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Applied Microbial Biotechnology 90: 809-825

Martin C, Marcet M, Almazán O, Jönssen LJ. 2007. Adaptation of a recombinant xylose-utilizing Saccharomyces cerevisiae strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour Technol 98:1767-1773

Martin M.A. 2010. First generation biofuels compete. New Biotechnology 27:597-607

Martinez A., Rodriguez M.E., Wells M.L., York S.W., Preston J.F., Ingram L.O. 2001. Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnol Prog 17:287-293

Modig T., Lidén G., Taherzadeh M.J. 2002. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochem J. 363:769-776

Oliva J.M. Sáez F., Ballesteros I., González A., Negro M.J., Manzanares P., Ballesteros M. 2003. Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. Appl Biochem Biotechnol 105-108:141-153

Oliva JM, Negro MJ, Sáez F, Ballesteros I, Manzanares P, González A, Ballesteros M. 2006. Effects of acetic acid, furfural and catechol combinations on ethanol fermentation of *Kluyveromyces marxianus*. Process Biochem 41:1223-1228.

Palmqvist E, Grage H, Meinander NQ, Hahn-Hägerdal B. 1999. Main and interaction effects of acetic acid, furfural, and p-Hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol Bioeng 63:46-55

Palmqvist E, Almeida J, Hahn-Hägerdal B. 1999. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnol Bioeng 62:447-454.

Palmqvist E, Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol 74:25-33.

Pampulha, ME, Loureiro-Dias MC. 2000. Energetics of the effect of acetic acid on growth of *Saccharomyces cerevisiae*. FEMS Microbiol Lett 18:69-72.

Plackett RL, Burmann JP. 1946. The design of optimum multifactorial experiments. Biometrika 33:305-325

Rivera EC, Costa AC, Atala DIP, Maugeri-Filho F, Wolf Maciel MR, Maciel-Filho R. 2006. Evaluation of optimization techniques for parameter estimation:Application to ethanol fermentation considering the effect of temperature. Process Biochem 41:1682-1687

Seabra J.E.A., Tao L., Chum H.L., Macedo I.C. 2010. A techno-economic evaluation of the effects of centralized cellulosic ethanol and co-products refinery options with sugarcane mill clustering. Biomass and Bioenergy 34:1065-1078

Silva S.S., Matos Z.R., Carvalho W. 2005. Effects of sulfuric acid loading and residence time on the composition of sugarcane bagasse hydrolysate and its use as a source of xylose for xylitol bioproduction. Biotechnol Prog 21:1449-1452

Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1-11

Taherzadeh M, Niklasson C, Lidén G. 1997. Acetic acid- friend or foe in anaerobic conversion of glucose to ethanol. Chem Eng Sci 52:2653-2659

Rossel CE, Lahr Filho D, Hilst AGP, Leal, MRLV. 2005. Saccharification of sugarcane bagasse for ethanol production using the Organosolv process. Int Sugar J 107:192-195.

Zautsen, R.R.M., 2011. Ethanol fermentation and simultaneous liquid-liquid extraction of ethanol and inhibitors derived from liquor of hydrolyzed lignocellulosic biomass. This PhD thesis, State University of Campinas, Campinas, Brazil.

Zautsen, R.R.M., Moço Gaudiosi, R., Maugeri Filho, F., 2011. Concentration profiles in batch fermentation of hydrolyzed lignocellulose. Dataset#771167 (DOI: http://doi.pangaea.de/10.1594/PANGAEA.771167)

5.8 Appendix 1

(Published as citable data table http://doi.pangaea.de/10.1594/PANGAEA.771167) Experimental data; time in hours, furfural, vanillin, biomass, glucose and ethanol concentrations in g L^{-1} per point in time (columns) and per experiment (set of six rows)

		Experimental data (g l^{-1})																			
exp#		**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1	*time*	*0*	*2,33*	*5,33*	*8,28*	*11,3*	*14,32*	*17,37*	*20,25*	*23,33*	*26,33*	*29,3*	*32,3*	*35,3*	*38,32*	*41,35*	*44,25*	*47,3*	*50,27*	*53,35*	*56,42*
	furfural	0,31	0,28	0,12	0,04	0,01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	vanillin	0,17	0,37	0,28	0,18	0,11	0,09	0,07	0,08	0,05	0,08	0,04	0,01	0,03	0	0,01	0,01	0,01	0,01	0,01	0,01
	biomass	0,93	1,11	1,09	1,31	2,28	4,41	6,35	6,5	6,25	6,47	6,44	6,62	6,78	7,21	7,18	7,59	7,76	8,21	8,5	9,18
	glucose	64,32	63,37	60,87	57,76	55,69	34,84	5,93	0,95	0	0	0	0	0	0	0	0	0	0	0	0
	ethanol	0	0,56	1,79	5,96	16,1	29,23	32,18	26,73	29,57	29,99	30,64	30,63	28,27	28,73	27,95	26,06	26,06	25,53	22,55	
2	*time*	*0*	*2,65*	*5,55*	*8,65*	*11,62*	*14,58*	*17,58*	*20,62*	*23,62*	*26,65*	*29,62*	*32,65*	*35,8*	*38,7*	*41,58*	*44,57*	*47,58*	*50,65*	*53,63*	*59,65*
	furfural	0,43	0,34	0,14	0,03	0,01	0,01	0,01	0,01	0,01	0	0	0	0	0	0	0	0	0	0	0
	vanillin	0,44	0,41	0,26	0,12	0,07	0,06	0,07	0,04	0,03	0,03	0,02	0,05	0,01	0,01	0,02	0,02	0,01	0,02	0,01	0,01
	biomass	1,27	1,28	1,36	1,98	3,8	6,5	6,48	6,44	6,61	6,79	6,69	6,58	6,56	6,98	7,24	7,47	7,7	8,16	8,75	9,85
	glucose	68,4	66,3	63,5	57,06	36,92	2,43	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	ethanol	0	0,81	1,89	5,39	14,06	28,78	30	28,82	27,2	30,11	29,03	28,85	28,76	23,92	28,01	24,94	25,02	21,76	25,68	21,45
3	*time*	*0*	*2,33*	*5,33*	*8,28*	*11,3*	*14,32*	*17,37*	*20,25*	*23,33*	*26,33*	*29,3*	*32,3*	*35,3*	*38,32*	*41,35*	*44,25*	*47,3*	*50,27*	*53,35*	*56,42*
	furfural	1,56	1,37	1,25	1,3	1,48	1,44	1,12	0,97	0,5	0,42	0,28	0,16	0,04	0	0	0	0	0	0	0
	vanillin	0,31	0,38	0,31	0,29	0,29	0,28	0,25	0,26	0,03	0,2	0,19	0,16	0,12	0,03	0,01	0	0,01	0,01	0	0,01
	biomass	1,38	1,1	1,08	1,24	1,09	0,97	1,2	1,17	1,7	1,14	1,18	1,17	1,24	1,38	1,75	2,65	4,89	6,83	7,31	7,5
	glucose	66,72	64,2	65,29	64,96	65,33	66,35	66,64	66,41	54,34	63,11	65,67	64,64	63,88	61,61	58,56	51,16	30,46	8,62	1,94	0
	ethanol	0	0,5	0,55	4,29	0,7	0,8	0,83	0,98	1,13	2,76	6,34	14,9	26,06	29,94	26,64					
4	*time*	*0*	*2,65*	*5,55*	*8,65*	*11,62*	*14,6*	*17,62*	*20,62*	*23,65*	*26,65*	*29,62*	*32,65*	*35,8*	*38,73*	*41,58*	*44,58*	*47,6*	*50,65*	*53,63*	*59,65*
	furfural	2,02	1,99	1,58	1,1	0,87	0,57	0,33	0,17	0,06	0,01	0	0	0	0	0	0	0	0	0	0
	vanillin	0,45	0,44	0,35	0,3	0,26	0,16	0,16	0,1	0,04	0,01	0,01	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01
	biomass	1,3	1,4	1,31	1,33	1,28	1,38	1,34	1,34	1,47	1,89	2,9	4,99	6,22	6,54	6,54	6,64	6,43	6,51	6,62	6,82
	glucose	64,42	63,23	62,03	63,98	76,92	58,24	59,56	56	54,58	52,85	43,45	21,37	2,43	0	0	0	0	0	0	0
	ethanol	0	0,56	0,83	1,15	2,13	1,25	2,37	3,05	4,96	9,47	21,68	29,5	24,34	26,55	27,47	24,83	25,15	24,46	21,9	
5	*time*	*0*	*2,2*	*5,22*	*8,15*	*11,22*	*14,2*	*17,23*	*20,07*	*23,25*	*26,2*	*29,17*	*32,17*	*35,17*	*38,18*	*41,22*	*44,08*	*47,17*	*50,13*	*53,23*	*56,28*
	furfural	0,42	0,27	0,23	0,22	0,19	0,1	0,05	0,01	0,01	0	0	0	0	0	0	0	0	0	0	0
	vanillin	0,94	0,8	0,84	0,86	0,88	0,77	0,52	0,24	0,01	0,03	0,03	0,02	0,02	0,01	0,01	0	0	0,01	0	0
	biomass	0,77	0,96	0,97	1,06	0,98	1,1	1,04	1,28	1,91	3,82	5,91	6,88	6,78	7,04	7	7,26	7,73	8	8,63	9,32
	glucose	65,61	61,82	61,96	63,06	64,53	67,02	63,61	62,74	55,24	40,55	16,26	0	0	0	0	0	0	0	0	0
	ethanol	0	0,52	0,85	1,02	1,38	1,94	4,04	10,34	23,25	30,29	29,63	28,74	28,28	28,8	25,75	27,06	23,85	22,61		
6	*time*	*0*	*2,57*	*5,47*	*8,58*	*11,58*	*14,5*	*17,5*	*20,53*	*23,58*	*26,62*	*29,58*	*32,58*	*35,73*	*38,67*	*41,48*	*44,48*	*47,48*	*50,58*	*53,58*	*59,58*
	furfural	0,6	0,53	0,44	0,26	0,16	0,09	0,01	0,01	0,01	0	0	0	0	0	0	0	0	0	0	0
	vanillin	1,39	1,31	1,1	0,92	0,86	0,3	0,05	0,02	0,01	0,01	0,01	0,01	0,01	0	0,01	0	0	0	0	0
	biomass	1,35	1,23	1,23	1,2	1,3	1,5	1,93	3,56	6,43	7,53	6,75	6,89	6,62	7,07	7,02	7,39	7,69	8,09	8,79	10,72
	glucose	65,36	64,2	63,81	61,73	60,41	55,86	51,51	37,01	4,87	0	0	0	0	0	0	0	0	0	0	0
	ethanol	0	0,61	1,01	1,58	2,31	3,23	5,7	13,2	28,87	31	29,54	29,15	27,71	18,28	7,15	18,54	25,1	25,01	23,18	18,15
7	*time*	*0*	*2,2*	*5,22*	*8,15*	*11,22*	*14,2*	*17,23*	*20,07*	*23,25*	*26,2*	*29,17*	*32,17*	*35,17*	*38,18*	*41,22*	*44,08*	*47,17*	*50,13*	*53,23*	*56,28*
	furfural	1,71	1,92	1,5	1,35	1,9	2,23	1,7	1,59	1,23	1,28	1,16	1,46	1,27	1,01	1,19	1,29	0,86	1,19	0,81	0,93
	vanillin	1,23	1,45	1,46	1,18	1,44	1,44	1,45	1,48	1,1	1,19	1,16	1,43	1,24	0,92	1,1	1,32	1,02	1,29	0,96	1,32
	biomass	0,84	1,02	1,16	0,95	1,01	1,03	0,93	0,94	1,06	1,03	0,96	1,11	1,08	1,07	1,11	1,09	1,09	1,17	1,05	1,16
	glucose	63,37	67,08	65,08	60,11	67,16	67,12	66,95	67,41	64,14	65,73	66,32	66,63	64,02	64,09	64,81	67,1	64,85	65,98	63,88	66,87
	ethanol	0	0,36	0,37	0,36	0,36	0,36	0,42													
8	*time*	*0*	*2,57*	*5,47*	*8,58*	*11,58*	*14,52*	*17,52*	*20,53*	*23,58*	*26,62*	*29,58*	*32,58*	*35,73*	*38,67*	*41,5*	*44,48*	*47,5*	*50,58*	*53,58*	*59,58*
	furfural	2,53	1,9	1,95	1,46	1,48	1,16	1,25	0,97	0,99	1,06	1,03	0,9	0,85	0,44	0,76	0,64	0,66	0,81	0,68	0,72
	vanillin	1,75	1,63	1,53	1,52	1,57	1,25	1,2	1,13	1,19	1,37	1,41	1,34	1,31	0,68	1,06	0,99	1,07	1,17	1,23	1,21
	biomass	1,4	1,45	1,35	1,28	1,27	1,31	1,37	1,32	1,41	1,38	1,43	1,38	1,21	1,32	1,34	1,44	1,28	1,27	1,32	1,26
	glucose	65,75	65,23	64,11	63,13	63,1	61,09	60,85	58,84	60,43	62,13	62,23	61,63	63,91	49,26	59,51	58,85	58,64	61,87	61,6	62,62
	ethanol	0,45	0,64	0,81	1,01	1,17	1,29	1,37	1,48	1,55	1,62	1,27	1,63	1,25	1,64	1,46	1,45	1,53	1,56	1,42	
9	*time*	*0*	*2,2*	*5,22*	*8,15*	*11,22*	*14,2*	*17,23*	*20,07*	*23,25*	*26,2*	*29,17*	*32,17*	*35,17*	*38,18*	*41,22*	*44,08*	*47,17*	*50,13*	*53,23*	*56,28*
	furfural	1,12	1,29	0,73	0,71	0,85	0,71	0,51	0,33	0,11	0,05	0,01	0	0	0	0	0	0	0	0	0
	vanillin	0,7	0,85	0,76	0,84	0,86	0,81	0,74	0,62	0,36	0,23	0,09	0,1	0,09	0,14	0,11	0,07	0,15	0,08	0,08	0,08
	biomass	0,79	0,95	0,82	1,11	1,03	0,92	1,02	1,14	1,07	1,24	2,01	3,45	5,57	6,57	6,74	6,8	6,88	6,79	7,08	7,31
	glucose	62,91	66,15	60,32	67,01	67,43	66,55	66,52	66,27	62,05	60,04	56,7	45,24	22,26	4,7	0	0	0	0	0	0
	ethanol	0	0,38	0,53	0,56	0,59	0,98	1,7	3,81	9,09	18,87	27,76	26,44	28,86	27,8	27,12	24,37	24,76			

Experimental data (g l^{-1})

exp #		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
10	*time*	*0*	*2,5*	*5,47*	*8,55*	*11,53*	*14,52*	*17,52*	*20,48*	*23,55*	*26,58*	*29,52*	*32,58*	*35,73*	*38,62*	*41,48*	*44,45*	*47,47*	*50,58*	*53,58*	*59,52*
	furfural	1,59	0,93	1,05	0,76	0,58	0,33	0,16	0,06	0,01	0	0	0	0	0	0	0	0	0,01	0	0
	vanillin	1,08	0,9	0,99	0,89	0,78	0,56	0,35	0,16	0,08	0,1	0,09	0,09	0,11	0,12	0,11	0,11	0,08	0,06	0,09	0,11
	biomass	1,29	1,17	1,14	1,27	1,23	1,28	1,28	1,33	2,07	3,34	5,57	5,57	5,63	5,92	5,86	5,92	5,63	5,7	5,7	5,68
	glucose	62,5	51,77	59,83	59,16	61,03	55,91	51,94	51,39	45,66	32,16	4,56	0,15	0,14	2,3	0	0	0	0	0	0
	ethanol	0	0,57	0,91	1,24	1,62	1,97	3,03	5,72	12,34	26,5	27,43	26,5	19,63	25,29	22,8	24,17	22,9	21,89	19,62	
11	*time*	*0*	*2,17*	*5,13*	*8,07*	*11,13*	*14,07*	*17,13*	*19,97*	*23,17*	*26,08*	*29,05*	*32,08*	*35,02*	*38,05*	*41,12*	*44,03*	*47,05*	*50,02*	*53,1*	*56,2*
	furfural	0,03	0,04	0,01	0,01	0,01	0,01	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	vanillin	0,85	0,83	0,54	0,17	0,12	0,11	0,09	0,13	0,08	0,06	0,04	0,02	0,02	0,04	0,03	0,02	0,04	0,02	0,02	0,03
	biomass	0,9	1,05	1,23	1,39	2,73	5,14	6,44	6,51	6,32	6,33	6,46	6,73	7,1	7,34	7,68	8,06	8,05	8,23	8,29	8,54
	glucose	64,41	65,77	61,85	61,01	50,22	21,78	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	ethanol	0	0,74	2,83	8,26	21,99	31,77	32,2	30,66	29,73	30,51	30,49	29,14	25,96	26,85	25,93	23,94	23,48	16,67	16,98	
12	*time*	*0*	*2,05*	*5*	*8*	*11,05*	*14*	*17,07*	*19,9*	*23,17*	*26*	*28,97*	*31,98*	*34,97*	*38*	*41,07*	*43,95*	*46,97*	*49,92*	*53*	*56,13*
	furfural	2,12	1,64	1,86	2,28	2,11	1,88	1,53	1,81	1,48	1,53	1,58	1,52	1,33	1,39	1,03	1,18	1,09	1,12	1,16	1,08
	vanillin	0,79	0,68	0,79	0,81	0,93	0,91	0,78	0,87	0,79	0,69	0,77	0,76	0,78	0,62	0,46	0,74	0,53	0,74	0,58	0,79
	biomass	1,05	1,04	0,96	1,05	1,13	1,06	1,06	1,08	1,06	1,15	1,12	1,25	1,17	1,06	1,04	1,08	1,17	1,12	1,12	1,31
	glucose	65,34	59,81	64,08	67,78	67,81	67,51	7,02	68,36	68,08	64,83	68,31	68,46	68,44	65,9	65,46	69,88	68,81	68,57	67,61	70,59
	ethanol	0	0,39	0,45	0,44	0,47	0,49	0,46	0,46	0,44	0,45	0,42	0,26	0,37	0,34						
13	*time*	*0*	*2,5*	*5,47*	*8,55*	*11,53*	*14,52*	*17,53*	*20,5*	*23,55*	*26,58*	*29,52*	*32,58*	*35,73*	*38,65*	*41,48*	*44,47*	*47,48*	*50,58*	*53,58*	*59,52*
	furfural	1,21	1,1	0,61	0,24	0,02	0,02	0,02	0,02	0,01	0	0	0	0	0	0	0	0	0	0	0
	vanillin	0,19	0,13	0,12	0,09	0,08	0,09	0,07	0,04	0,03	0,03	0,03	0,03	0,02	0,03	0,03	0,04	0,03	0,03	0,03	0,03
	biomass	1,34	1,33	1,29	1,47	1,95	3,1	5,54	6,11	6,24	6,07	6,11	6,42	6,15	6,39	6,45	6,54	6,75	6,89	7,65	8,87
	glucose	67,62	67,27	64,42	62,84	57,26	42,36	10,9	0,38	0	0	0	0	0	0	0	0	0	0	0	0
	ethanol	0	1,02	2,21	4,97	12,02	21,62	29,85	28,01	28,66	28,43	27,61	23,55	20,02	23,68	23,47	23,8	22,69	20,55	17,21	
14	*time*	*0*	*2,5*	*5,35*	*8,53*	*11,48*	*14,5*	*17,5*	*20,47*	*23,58*	*26,55*	*29,48*	*32,55*	*35,72*	*38,67*	*41,47*	*44,43*	*47,43*	*50,55*	*53,52*	*59,5*
	furfural	1,49	1,37	1,06	0,96	0,98	0,67	0,49	0,45	0,56	0,46	0,41	0,4	0,34	0,16	0,24	0,22	0,27	0,31	0,29	0,24
	vanillin	1,92	1,84	1,79	1,76	1,69	1,43	1,36	1,31	1,44	1,51	1,53	1,53	1,48	0,85	1,19	1,11	1,28	1,5	1,41	1,37
	biomass	1,42	1,39	1,29	1,2	1,29	1,41	1,11	1,25	1,32	1,27	1,33	1,37	1,27	1,22	1,33	1,17	1,2	1,18	1,3	1,05
	glucose	67,74	66,96	65,72	65,52	65,17	62,9	60,58	59,78	62,39	64,18	64,43	64,51	64,76	51,38	60,91	59,54	62,45	65,52	65,77	66,66
	ethanol	0	0,37	0,57	0,73	0,89	0,9	0,99	1,14	1,02	1,07	1,08	0,91	0,82	0,9	0,91	1,09	0,99	0,94	0,79	
15	*time*	*0*	*2,05*	*5*	*8*	*11,05*	*14*	*17,07*	*19,9*	*23,17*	*26*	*28,97*	*31,98*	*34,97*	*38*	*41,07*	*44,25*	*46,97*	*49,92*	*53*	*56,15*
	furfural	0,87	0,69	0,87	0,82	0,65	0,47	0,35	0,22	0,15	0,04	0,02	0	0	0	0	0	0	0	0	0
	vanillin	0,74	0,65	0,75	0,81	0,63	0,54	0,52	0,42	0,29	0,15	0,07	0,04	0,07	0	0,11	0,05	0,1	0,07	0,09	0,06
	biomass	0,92	1,01	1,1	1,25	1,22	1,07	0,99	1,08	1,07	1,26	1,66	3	5,64	6,44	6,38	6,57	6,61	6,69	6,97	7,02
	glucose	65,62	61,94	65,49	64,73	65,16	64,58	68,17	63,2	63,45	60,54	59,62	46,01	18,43	0	0	0	0	0	0	0
	ethanol	0	0,47	0,66	0,68	0,93	0,95	1,15	1,63	3	8,93	23,71	29,83	30,3	29,4	28,08	26,24	25,34	24,72		
16	*time*	*0*	*2,08*	*5*	*7,83*	*10,88*	*13,83*	*16,9*	*19,73*	*23*	*25,97*	*28,92*	*31,93*	*34,88*	*37,83*	*40,92*	*44,08*	*47*	*49,85*	*52,97*	*56*
	furfural	0,76	0,85	0,83	0,89	0,65	0,42	0,27	0,19	0,07	0,01	0	0	0	0	0	0	0	0	0	0
	vanillin	0,64	0,71	0,75	0,73	0,69	0,6	0,53	0,42	0,23	0,1	0,02	0,02	0,03	0,03	0,04	0,02	0,04	0,02	0,03	0,02
	biomass	0,94	0,99	1,01	1,02	1,02	1,01	0,99	0,98	1,04	1,18	1,92	3,54	6,14	6,58	6,68	6,77	6,74	6,93	6,92	7,41
	glucose	60,68	64,33	64,51	67,02	66,43	65,81	66,13	64,76	64,07	60,56	58,21	40,96	10,94	0	0	0	0	0	0	0
	ethanol	0	0,44	0,54	0,63	0,54	0,67	0,92	1,53	3,59	11,7	25,72	28,63	30,27	28,58	27,87	27,17	22,19	21,15		
17	*time*	*0*	*2,5*	*5,35*	*8,53*	*11,48*	*14,52*	*17,52*	*20,48*	*23,58*	*26,55*	*29,48*	*32,55*	*35,72*	*38,67*	*41,48*	*44,45*	*47,45*	*50,55*	*53,52*	*59,5*
	furfural	1,34	0,77	0,95	0,71	0,55	0,22	0,13	0,03	0,01	0	0	0	0	0	0	0	0	0	0	0
	vanillin	1,1	0,94	0,84	0,73	0,46	0,29	0,12	0,09	0,1	0,13	0,1	0,12	0,12	0,09	0,15	0,08	0,09	0,11	0,13	0
	biomass	1,41	1,36	1,32	1,27	1,29	1,39	1,29	1,63	2,93	5,19	6,21	6,16	6,39	6,44	6,56	6,28	6,44	6,4	6,66	7,17
	glucose	65,58	61,92	64,11	62,98	61,87	56,97	57,8	54,04	41,25	17,38	0,36	0	0	0	0	0	0	0	0	0
	ethanol	0	0,52	0,84	1,2	1,45	2,2	3,63	8,69	21,79	28	27,69	25,93	22,26	26,47	25,27	23,34	24,56	23,88	16,28	
18	*time*	*0*	*2,53*	*5,42*	*8,57*	*11,55*	*14,45*	*17,45*	*20,42*	*23,53*	*26,65*	*29,53*	*32,6*	*35,75*	*38,58*	*41,4*	*44,37*	*47,38*	*50,6*	*53,6*	*59,6*
	furfural	1,26	1,34	0,91	0,81	0,54	0,25	0,14	0,02	0	0	0	0	0	0	0	0	0	0	0	0
	vanillin	1,1	0,83	0,9	0,81	0,68	0,43	0,26	0,06	0,04	0,04	0,04	0,03	0,02	0,04	0,04	0,04	0,03	0,03	0,03	0,03
	biomass	1,27	1,3	1,19	1,36	1,33	1,37	1,36	1,59	2,75	5,03	6,57	6,37	6,5	6,69	6,79	6,54	6,84	7,07	7,47	8,56
	glucose	66,68	67,12	65,14	64,49	64,18	59,45	59,24	52,43	46,22	21,76	0	0	0	0	0	0	0	0	0	0
	ethanol	0	0,6	0,96	1,33	1,67	2,38	3,49	9,26	21,28	29,78	29,38	29,5	28,31	26,54	26,06	25,38	18,56	24,63	17,09	

Capítulo 6

Modeling and simulation of extractive ethanol fermentation combining *in-situ* product recovery, medium detoxification and cooling

6.1 Abstract

Production of biofuels as ethanol utilizing cellulose and hemicellulose fractions of for example sugarcane, can decrease costs of raw material and increase overall production efficiency. Inhibiting components in fermentation medium based on lignoceullulose hydrolyzate must be minimized to improve the fermentation rate. The model presented in this work proposes to remove these components *in-situ*, utilizing a biocompatible solvent as a secondary, organic phase in the fermentation process. The inhibiting effect of the product, for example ethanol, can be prevented simultaneously. Moreover, tuning flow-rates and temperatures, the same solvent can provide effective cooling for the fermentation. This work proposes a continuous fermentation scheme by means of a model and optimization of several input parameters to demonstrate the capabilities and advantages of such a system. Considerable gains in fermentation rate, up to 25% in production per substrate cost and other results are shown for a range of combinations of raw material, must concentration and solvent feed rate for a realistically sized modern ethanol plant.

6.2 Introduction

Oil prices are foreseen to be predominantly determined by global demand (Özbek and Özlale, 2010) which is expected to rise especially due to growing economies of increasingly populous emerging countries, bringing along an expected global duplication of number of vehicles within twenty years (Balat and Balat, 2009). Environmental implications, energy security and the negative influence of high oil prices on economic growth are valid arguments for shifting towards a bio-based economy. Use of holocelulose fractions of crops, a combination of cellulose and hemicellulose, for biofuels production will alleviate impacts on food prices and expand the availability of agricultural lands for increased food production (Martin 2010). Sugarcane is highly competitive to corn (Méjean *et al.*, 2010). The plant´s lignin fraction, which represents up to 25% of dry bagasse (Seabra *et al.*, 2010) and most of the bagasse caloric value, can remain available for on-site vapor and electricity production. Increased value of holoceluloses as raw material relative to sucrose compared to current standards encourages production of energy-canes which can be larger due to the structural support offered by lignocelluloses. In the past, sustained fresh cane yields have been reported as high as 307 tons per hectare for such energy canes with average fractions of total bio-convertible matter, i.e. soluble sugars and holocelulose, by dry weight (112 tons/ha) at 79.7% (Legendre *et al.*, 1995). Based on these yields, the potential of energy cane for ethanol production is almost tenfold compared to use of only the sugar fraction of conventional cane, 37.5%, with current yields at 24 tons/ha of cane dry weight (based on data from Seabra *et al.*, 2010, Mantelatto, 2005).

Deterioration of cane quality (Rossell 2006) can be reduced with shorter average distances from the cane fields to a mill of equal capacity and sugar in wet bagasse, which would otherwise be lost if not recovered (Finguerut 2006), can be readily fermented. The extra choice of raw material for biofuels production also allows for a more flexible optimization of source of substrate used in the process since the relative economic values of all types of raw material used in the same plant (e.g. juice, syrup, molasses and bagasse) are susceptible to fluctuations. Further, use of lignocelluloses can lead to less carbon dioxide

emitted per unit of product throughout the whole production process, making biofuels an even more environmental friendly alternative to gasoline.

Prerequisite of bioconversion of holoceluloses into biofuels is the hydrolysis of this raw material into convertible sugars as glucose and xylose. Hydrolysis also leads to the formation of fermentation inhibiting compounds as furfural (formed from pentoses), hydroxymethylfurfural (HMF, formed from hexoses), acetate (liberated from hemicellulose), levulinic acid (formed from HMF), formic acid (formed from furfural and HMF) and phenolic compounds like vanillin, released from lignin. Despite advances in enzymatic conversion (Szijártó *et al.*, 2008), organosolv hydrolysis (Olivério and Hilst, 2005) or less severe hydrolysis using carboxylic acids (Lee and Jeffries, 2011), formation of these components is unavoidable, because of a trade-off between sugar monomers yield and inhibitor formation. Enzymatic hydrolysis is an expensive alternative to acid-catalised hydrolysis, with prices amounting to half of the costprice of the raw material (Gonzalez *et al.*, 2011) and requires pretreatment to de-crystallize the lignocellulose structures and facilitate exposure to enzymatic attack. During this pretreatment inhibitors are also produced.

The presence of these compounds in fermentation media leads to inhibition of growth and product formation during the fermentation process (Palmqvist *et al.*, 1999). Pretreatment methods to remove these inhibitors from the fermentation media prior to fermentation have long been sought by means of overliming, adsorbtion, evaporation, biological treatment and other methods. However, pretreatment requires an extra processing step and is generally considered to be too expensive for a viable production economy (Liu 2011). In this work extractive fermentation is proposed for *in-situ* removal of both inhibitors and the fermentation product while using the extractive solvent as cooling agent.

Above certain concentrations, as in the case of ethanol, also the fermentation product becomes an inhibiting factor. This toxic effect, be it caused by for example ethanol, butanol, acetone or acetic acid, quickly becomes the limiting factor to the final concentration of the product in the medium, independent of other bottlenecks in the

metabolic pathway (Sriyudthsak and Shiraishi, 2010). Thus, only removal of the product during fermentation can enable higher volumetric productivities and reduction of fermentation time, even for yeast of which the metabolic pathway has been modified with this objective. For removal of the product, an organic biocompatible solvent that has a high boiling point can be applied. Product recovery becomes less energy intensive because of the ease of removal and distilling of the ethanol rich fraction from the solvent. As such, extractive fermentation by use of biocompatible solvents for solely extraction of fermentation product has previously been studied (Szewczyk and Zautsen, 2003) and by other authors (e.g. Minier and Goma, 1982, Kollerup and Daugulis, 1986, Malinowski and Daugulis, 1993) who demonstrated improvements in fermentation rates and energetic and economic advantages by decreased product recovery costs and decreased usage of process water.

Extractive removal of only inhibitors from hydrolytic liquor has also been studied (Cruz *et al.*, 1999, Hasmann *et al.*, 2008) and resulted in decrease of lag times and improved overall fermentation performance. Theoretically, product yield could be increased compared to fermentation in inhibitor-free media because the solvent acts as a buffer for the inhibitors and for small concentrations of furfural it was observed that ethanol yield improved at the cost of glycerol (Palmqvist *et al.*, 1999). Also, partial removal allows for the acting of the inhibitors as natural antibiotics, since their toxic effects also hold for viral and bacterial micro-organisms (Hassman et al, 2008).

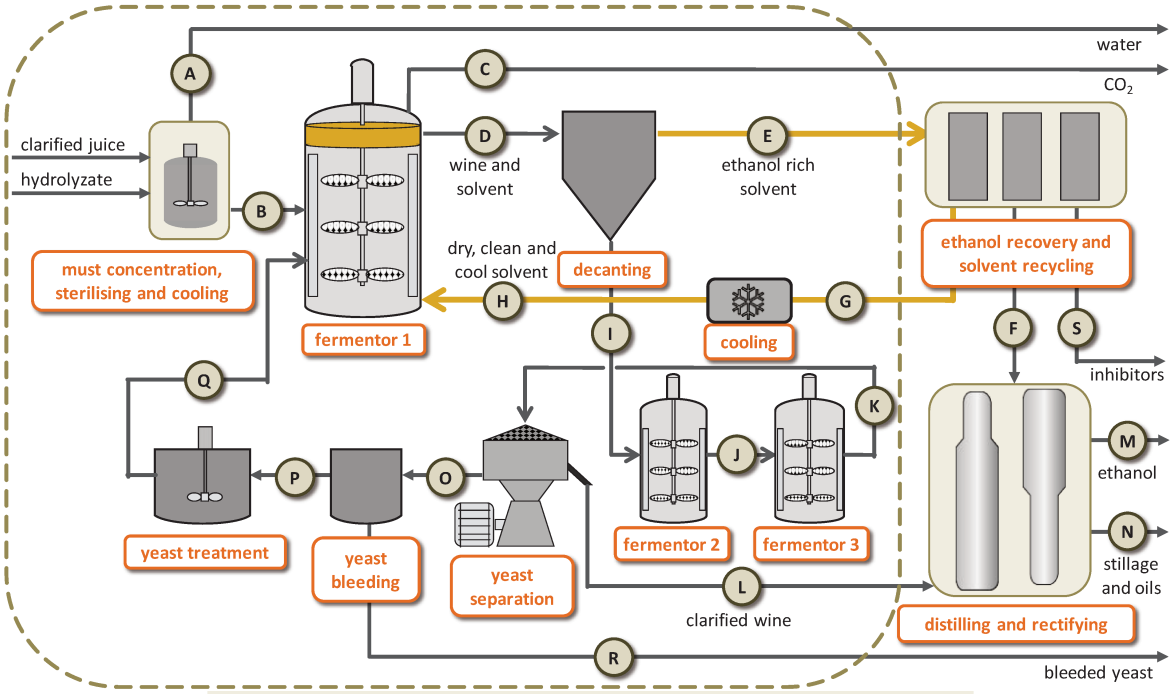
Simultaneous removal of both product and inhibitors as demonstrated in previous work (Zautsen *et al.,* 2011a) combines the positive aspects of both approaches to product inhibited fermentations of hydrolyzed liquor. Jassal *et al.* (1994) calculated for oleic acid as solvent, that the high selectivity for ethanol over water would lead to lower costs for distillation of the ethanol/water mixture retrieved from the solvent than conventional distillation of fermented wine. Extraction of water however, can lead to increased product yield due to resulting substrate concentration.

The solvent can be used as an effictive cooling agent for the fermentation. Dispersed in the broth, it can provide a direct, large contact surface for heat transfer between the fermentation broth and the cooling liquid, without the need to expose the broth to conventional heat exchangers. Currently, fermentation cooling is done by pumping a fraction of the fermentation broth through an external cooling device or inserting a cooling coil into the fermentation vessel. Direct cooling with the organic solvent, provided that this cooling is sufficient, eliminates the necessity of such devices, impacting investment and maintenance costs and decreasing risks of microbial contaminations as also suggested by Atala (2004) and Silva *et al.* (1999) for extractive fermentation by vacuum. Biodiesel has a low heat capacity (Conceição *et al.*, 2007) and its viscosity is higher than water especially at low temperatures (Zautsen *et al.*, 2011a), but the absence of undissolved solids is favorable compared to use of the fermentation broth itself as coolant because these tend to form crust layers, increase risc of contamination and maintenance costs of the recirculation system. Also mechanical stress to the yeast flowing at high rates through an external cooling apparatus can be avoided. The recycled solvent can be continuously retrieved from a process unit that uses heat for ethanol recovery, which further decreases the possibility of microbial infections as compared to conventional broth cooling.

Accumulation of inhibitors present in recycled stillage (Maiorella *et al.*, 1984) can partially be prevented by the solvent, thus making the practice of stillage recycling more viable and allowing increased overall water economy and avoiding the need for drying of the stillage. Biodiesel, as a model solvent selected for this process, additionally offers the possibility of using the solvent as a biofuel, for example for equipment involved in harvesting and transporting the raw material and produced stillage, which had also already been proposed by Grobben *et al.* (1993). Finally, oscillatory behaviour of continuous fermentations that can lead to unnecessary loss of substrate (Bai, 2007) could be counteracted by forward control of the organic face dilution rate.

6.3 Process Description

A continuous fermentation process is modeled with a series of three fermentors, representing a realistic industrial setup. Figure 1 shows a schematic overview of the process. In a first step, clarified juice and hydrolyzate are mixed, concentrated by evaporation, cooled and fed to the first fermentor as final must (B). The evaporated water (A) can be used for cleaning of the solvent after ethanol removal in the ethanol recovery unit, but this is not considered in the model. The first fermentor is the largest and is responsible for conversion of about 60% of the substrate, leaving the remaining 40% for the second and third fermenter. It holds an aqueous medium phase of 600m^3 and an organic phase of 20% of this volume.



A	excess water from must	K	wine fermentor 3
B	prepared must	L	clarified wine
C	carbon dioxide	M	ethanol
D	wine and solvent	N	stillage and oils
E	ethanol rich solvent	O	centrifuged yeast
F	ethanol and water	P	yeast to treatment
G	hot recycled solvent	Q	treated yeast
H	cool recycled solvent	R	bleeded yeast
I	decanted wine	S	inhibitors
J	wine fermentor 2		

Figure 1: Process schematics with streams and processing units of a continuous extractive fermentation system. Process steps within the dotted area are modeled

The aqueous phase contains all of the substrate and yeast. The organic phase is partly dispersed in the aqueous phase but tends to migrate to the top of the fermentor due to its lower density. A fraction of the produced ethanol, inhibitors and water from the aqueous phase is extracted to the organic phase, thus improving the fermentation rate and concentrating the substrate. The lower ethanol concentrations in the aqueous volume also allow for maximum partitioning of most of the hydrolyzate inhibitors to the organic phase (Zautsen *et al.*, 2011a). Other flows entering the fermentor are fresh yeast (Q) from the yeast treatment unit and dry, recycled solvent (H) that contains no inhibitors or ethanol. In practice, this stream could be injected at the bottom of the fermentor or through holes in the impellers and/or baffles, and it could contain entrapped sterile oxygen to aid microbial cell maintenance mechanisms. The temperature of the inflowing organic solvent is adjusted for optimal cooling of the fermentation medium while remaining three degrees above the solidification point of the solvent. In the model, a range of target fermentation temperatures is evaluated for its influence on fermentation performance. Pure carbon dioxide is assumed to be exiting the fermentor in stream (C).

A mixture of wine and solvent is romoved at the top of the fermentor where the solvent concentration is highest due to the density difference between solvent and the fermentation medium. This flow (D) is led to a decanting unit from which the solvent, rich in ethanol and water, is drawn from the top (E) while the decanted wine or aqueous phase (I) is recovered at the bottom of the decanter. With the solvent having a higher boiling point than both ethanol and water, the ethanol-rich solvent stream (E) is heated and ethanol and water are separated from the solvent by evaporation at low pressure, a step that is not included in the model. The ethanol and water fraction recovered from the solvent (F) is led directly to a rectifying unit in which the ethanol can be recovered at high purity (M). Decanted wine stream (I) is fed to a second and third fermentor, which are placed in series through flow (J). Both these fermentors contain only an aqueous phase of a third of the size of the aqueous phase of the first fermentor and serve for conversion of the remaining substrate (Eijsberg 2006). Because most of the ethanol produced in the first fermentor has been removed, inhibition by additional accumulation of the ethanol produced in these last two fermentors is limited. Further removal of hydrolyzate inhibitors is unnecessary because

the concentration of these inhibitors should already be at least low enough to enable fermentation in the first fermentor and low amounts of furfural have been shown to have a positive effect on the ethanol yield (Palmqvist *et al.*, 1999). Cooling of these second and third fermentors, in which the heat production is relatively low, is done conventionally, assuming fermentation temperatures equal to the target temperature of the first fermentor. From the exit stream of the third fermentor (K), yeast is separated by centrifugation and let to a yeast bleeding unit by flow (O), while the clarified wine (L) is pumped to distillation and rectifying units producing ethanol (M) and by-product streams consisting of stillage and oils (N), a process step that itself is not included in the model. A minor portion of the yeast is bleeded off (R) and the remaining fraction (P) is treated by aeration and dilute acid in a yeast treatment unit before re-entering the fermentor through flow (Q).

The choice of solvent, an ethyl-ester of ricinoleic acid or castor-oil based biodiesel, was elaborated in previous work (Zautsen *et al.*, 2011a) and based on the following desirable properties: favorable partition coefficients for product and inhibitors, high selectivity for these compounds compared to substrate, high biocompatibility, low solubility in the water phase, high water absorption capacity to concentrate the substrate in the fermentor, thermodynamic characteristics favorable for product removal from the solvent and regeneration of the solvent as high boiling point, no formation of stable emulsions, rapid separation from the aqueous phase, high chemical stability at high temperatures, low viscosity, low cost, possibly to be fabricated on-site and reusability for other purposes.

6.4 Model inputs and optimization objectives

The variable inputs to the system were chosen as the ratio of hydrolyzate to clarified juice, the sugar concentration of the must (B) by partial evaporation of its water fraction, rate of the solvent flow fed to the fermentor (H) and fermentation temperature. The fermentation temperature was controlled by the solvent flow entering the fermentor, of which the temperature was calculated to provide enough cooling to counteract heat

introduced by fermentation and stirring of the medium, limited to three degrees above its melting point. The ratio of juice to hydrolyzate was varied to optimize the composition of the must in terms of costs on one hand and production rate suffering from inhibitor concentrations on the other hand. As shown in Table 1, total sugar concentrations in cane juice are only slightly higher than in hydrolyzed bagasse.

Table 1: Composition of untreated cane juice and hydrolyzate in mass percentages.

	juice [a)]	hydrolyzate [b)]
sucrose	12.0	-
glucose	0.4	5.39
fructose	0.4	-
xylose	-	2.85
arabinose	-	0.77
total sugars	12.9	9.01
furans	-	0.12
phenols	-	0.27
organic acids	0.4	0.50

calculations based on data privided by:

a) Mantelatto 2005

b) Martinez et al. (2001), Aguilar et al. (2002)

The prices of these substrate sources however vary widely depending on fluctuations in market prices for sugar, cane variety, processing costs and need of the non-lignin portion of bagasse for *on-site* heat or electricity production. Therefore, relative costs were assumed for juice and hydrolyzate, allowing the calculation of substrate cost per volume of produced ethanol ($\kappa_{substrate}$) as an optimization objective by deviding the total substrate cost by the ethanol production rate as shown in equation 1. Here, r_{juice} and $r_{hydrolazate}$ are the mass rates of juice and hydrolyzate in the must, $cost_{juice}$ and $cost_{hydrolyzate}$ the cost of juice and hydrolyzate as relative, unit-less prices per kg, r_p is the ethanol production rate in kg·h^{-1} and ρ_P the density of ethanol in kg·m^{-3}.

$$\kappa_{substrate} = \frac{r_{juice} \cdot cost_{juice} + r_{hydrolyzate} \cdot cost_{hydrolyzate}}{r_P / \rho_P} \qquad \text{cost·m}^{-3} \qquad [1]$$

The assumption that hydrolyzate is the cheapest source of substrate would lead to the highest cost efficiency with a hydrolyzate fraction equal to 100% of the substrate stream. However, absence of essential nutrients in pure hydrolyzate and high concentrations of furans, phenols and acids in the hydrolyzate would impact the growth rate, yield and the final production rate due to the inhibitive effect of these compounds. High inhibitor concentrations would lead to incomplete fermentation, even in the third fermentor, decreasing final product yield Y_{ps} and consequently resulting in high values for $\kappa_{substrate}$. Optimal substrate composition is also not trivial because under certain conditions biomass yield can be increased at the expense of product yield over substrate, or because of insufficient cooling provided by the solvent at high metabolic activity due to the lower temperature limit of the solvent.

The volumetric ethanol production rate, r_P/V_{aq}, shown in equation 2, was chosen as a second optimization objective. As the total volume, only the aqueous volumes of the bioreactors where fermentation actually took place, were considered. Thus, for certain combinations of input variable values, where all substrate fermented in the first and second fermentor alone, the volume of the third fermentor could be disregarded, effectively elevating the volumetric production rate.

$$\frac{r_P}{V_{aq}} = \frac{r_p}{V_{aq_{fermentor1}}\left[+V_{aq_{fermentor2}}\right]\left[+V_{aq_{fermentor3}}\right]} \qquad \text{kg·m}^{-3}\text{·h}^{-1} \qquad [2]$$

6.5 Model development

The described fermentation process was modeled using kinetic functions of temperature and concentrations of substrate, ethanol, biomass and hydrolyzate inhibitors, which were elaborated in previous work (Zautsen *et al.*, 2011b). For most other process steps, only simplified mass balances were modeled. Ethanol recovery and solvent recycling, distilling and rectifying were not modeled. Energy introduced by stirring was only considered for the fermentors and heat introduced by exothermic dissolving of, for example

produced ethanol, was disregarded. Energy introduced by metabolic activity related to ethanol production was included in the model.

6.5.a Fermentation kinetic model

State differential equations of biomass (X), substrate (S) and product (P) are given by equations 3, 4 and 5. It must be noted that simulations were executed numerically such that variations in compositions and densities of the incoming and outflowing streams of each fermenter were taken into account, at each simulation step, prior to using listed step-differential equations.

$$\frac{dX}{dt} = \mu \cdot X \qquad \text{kg·m}^{-3}\text{·h}^{-1} \qquad [3]$$

$$\frac{dS}{dt} = -\left(\frac{\mu}{Y_{XS}} + m_x\right) \cdot X \qquad \text{kg·m}^{-3}\text{·h}^{-1} \qquad [4]$$

$$\frac{dP}{dt} = \left(\mu \cdot Y_{PS} + c_{qP}\right) \cdot X \qquad \text{kg·m}^{-3}\text{·h}^{-1} \qquad [5]$$

Here, the growth rate (μ) is given by equation 6 as a product of the Monod equation and inhibition by substrate (I_S), biomass (I_X), product (I_P), chemicals (I_C) and temperature (I_T). These inhibition factors are given by equations 7 to 12.

$$\mu = \mu_{max} \cdot \frac{S}{S + K_S} \cdot I_S \cdot I_X \cdot I_P \cdot I_C \cdot I_T \qquad \text{h}^{-1} \qquad [6]$$

$$I_S = e^{(-K_i \cdot S)} \qquad \textit{substrate inhibition} \qquad - \qquad [7]$$

$$I_X = 1 - \left(\frac{X}{X_{max}(T)}\right)^m \qquad \textit{biomass inhibition} \qquad - \qquad [8]$$

Here T stands for temperature in °C, X_{max} for the biomass concentration in kg·m^{-3} at which growth stops and exponent m, equal to 1.1, elevates the biomass ratio. X_{max} as a function of temperature is expressed by equation 9.

$$X_{max}(T) = 10587 \cdot \exp(-0.145 \cdot T) \quad \text{kg·m}^{-3} \quad [9]$$

$$I_P = 1 - \left(\frac{P}{P_{max}}\right)^n \quad \textit{product inhibition} \quad - \quad [10]$$

$$I_C = exp\left(\frac{-(a \cdot F)^b}{c^{(d \cdot q_F)}} - (e \cdot A)\right) \quad \textit{chemical inhibition} \quad - \quad [11]$$

$$I_T = 1 - a_{\mu T} \cdot \left(Topt_\mu - T\right) \quad \textit{temperature inhibition} \quad - \quad [12]$$

Equations 11 and 12 are discussed in more detail in previous work (Zautsen *et al.,* 2011b) and include the product concentration P, the maximal product concentration at which growth stops P_{max}, furfural concentration F, specific furfural reduction rate q_F, acetic acid concentration A, optimal and current temperatures $Topt_\mu$ and T.

For the substrate consumption rate differential, presented in equation 4, the maintenance factor m_x is a function of a standard maintenance coefficient, m_{x0} and a sum of all inhibiting compounds furfural F, vanillin V, ethanol P and organic acids A as given by equation 13.

$$m_x = m_{x0} + m_{xa} \cdot (F + V + P + A) \quad \text{h}^{-1} \quad [13]$$

The product yield Y_{px} is a function of the temperature according to equation 14.

$$Y_{PX} = a_{Ypx} - b_{Ypx} \cdot \left(Topt_{Ypx} - T\right) \quad - \quad [14]$$

State differential equations for furfural and vanillin are given by equations 15 and 16.

$$\frac{dF}{dt} = -q_F \cdot I_{TqF} \cdot X \qquad \text{kg·m}^{-3}\text{·h}^{-1} \qquad [15]$$

$$\frac{dV}{dt} = -q_V \cdot I_{TqV} \cdot X \qquad \text{kg·m}^{-3}\text{·h}^{-1} \qquad [16]$$

With specific reduction rates q_F and q_V as functions of F and V:

$$q_F = \frac{a_{qF} \cdot F}{10^{-10} + \left(b_{qF} \cdot F + c_{qF} \cdot V\right)^{d_{qF}}} \qquad \text{h}^{-1} \qquad [17]$$

$$q_V = q_{Vmax} \cdot \frac{V}{K_V + V} \cdot a_{qV}{}^{-b_{qV} \cdot F} \qquad \text{h}^{-1} \qquad [18]$$

Inibition factors of specific furfural and vanillin reduction rates by temperature are given by equations 19 to 21.

$$I_{TqF} = 1 - a_{qFT} \cdot \left(Topt_{qF} - T\right) \qquad \textit{with } T < Topt_{qF} \qquad - \qquad [19]$$

$$I_{TqV} = 1 - a_{qVT} \cdot \left(Topt_{qV} - T\right) \qquad \textit{for } T <= Topt_{qV} \qquad - \qquad [20]$$

$$I_{TqV} = 1 - b_{qVT} \cdot \left(T - Topt_{qV}\right) \qquad \textit{for } T > Topt_{qV} \qquad - \qquad [21]$$

The values of parameters used in the model are given in Table 2.

Table 2: Values of the fermentation kinetic model parameters per state variable differential

dX/dt		dS/dt	dF/dt	dV/dt	dP/dt
μ_{max} 0.5[a)]	I_{Cc} 860[a)]	Y_{xs} 0.08[a)]	a_{qF} 5.9[a)]	q_{vmax} 0.25[a)]	a_{Ypx} 6.1[a)]
K_S 4.1[b)]	I_{Cd} 650[a)]	m_{x0} 0.01[a)]	b_{qF} 40[a)]	K_V 0.8[a)]	b_{Ypx} 0.2[a)]
K_i 0.002[c)]	I_{Ce} 0.44[a)]	m_{xa} 0.007[a)]	c_{qF} 25[a)]	a_{qV} 19.8[a)]	$Topt_{Ypx}$ 37[c)]
m 1.1[d)]	$a_{\mu T}$ 0.053[c)]		d_{qF} 1.12[a)]	b_{qV} 0.7[a)]	c_{qP} 0.025[a)]
n 1.5[d)]	$Topt_{\mu}$ 37[c)]		a_{qFT} 0.007[a)]	a_{qVT} 0.175[a)]	
I_{Ca} 4[a)]			$Topt_{qF}$ 39.2[a)]	b_{qVT} 0.063[a)]	
I_{Cb} 180[a)]				$Topt_{qV}$ 32.5[a)]	

a) Zautsen et al. (2011b)
b) Atala et al. (2001)
c) Rivera et al. (2006) or interpreted from data of the same
d) Jarzębski et al. (1989)

6.5.b Partition coefficients

In previous work (Zautsen *et al.*, 2011a), partition coefficients for ethanol and various inhibitors were measured for different concentrations of ethanol in the aqueous phase. Equation 22 defines the partition coefficient (m_p) as the ratio of the mass fraction of a compound in the organic phase divided by the mass fraction of that compound in the aqueous phase, but also as a linear function of the ethanol concentration in the aqueous phase (w_{Paq}) with m_{pa} and m_{pb} as constants.

$$m_p = \frac{w_{org}}{w_{aq}} = m_{pa} \cdot w_{Paq} + m_{pb} \qquad \textit{partition coefficient} \qquad - \qquad [22]$$

It was found that for biodiesel, partition coefficients of phenolic compounds and aldehydes with low polarity, e.g. vanillin, tend to be smaller for higher ethanol concentrations in the aqueous phase while for other compounds with higher polarity as furfural, acetic acid and ethanol the contrary is true. Table 3 lists parameters m_{pa} and m_{pb} for furfural, vanillin, acetic acid and ethanol, based on the mentioned results of our previous work. Note that in practical applications, these relations should be re-evaluated for their dependence on temperature and actual composition of broth including salts.

Table 3: Linear constants used for the calculation of partition coefficients and regression to experimental data

	m_{pa}	m_{pb}	R^2
furfural	3.483	1.959	0.84
vanillin	-12.30	3.523	0.81
acetic acid	1.596	0.0939	0.91
ethanol	0.2831	0.1020	0.91
water	0.1804	0.00584	0.85

6.5.c Heat production

In the fermentor, heat is mainly introduced by two processes: the biologic activity of the yeast and the conversion of the kinetic energy of stirring into heat. Higher broth viscosities require an increased agitation speed to maintain certain turbulence, leading to more heat generated by stirring. Turbulence is measured by the Reynolds constant given by equation 23 (Riet and Tramper, 1991 p143). In this equation, N is the stirrer speed (s^{-1}), D the stirrer diameter (m), ρ_{broth} the broth density ($kg \cdot m^{-3}$) and η_{broth} the broth viscosity ($N \cdot s \cdot m^{-2}$).

$$Re = \frac{N \cdot D^2 \cdot \rho_{broth}}{\eta_{broth}} \qquad \textit{turbulance} \qquad - \qquad [23]$$

Heat introduced by stirring was calculated according to equation 24 (Riet and Tramper, 1991, p304), where N_p stands for the power number which depends on the Reynolds number Re and the type of stirrer.

$$Q_{stirring} = P_{stirring} = N_p \cdot \rho_{broth} \cdot N^3 \cdot D^5 \qquad \textit{heat or power} \qquad J \cdot s^{-1} \qquad [24]$$

The viscosity of fermentation broth is influenced by temperature, sugar, solids and biomass concentrations and the dispersed organic phase. Fermentation rate and the related biological heat production that elevates the temperature are also related to the sugar concentration. However, the fermentor is considered ideally mixed and the substrate concentration equal to the lower outflow concentration. Sugar and solids are not considered and viscosity of the fermentation broth is calculated as a weighted average of the viscosity of the aqueous phase and the solvent, where the viscosity of the aqueous phase is a function of temperature and biomass concentration X according to equations 25 and 26 (Malinowski *et al.*, 1987). Here, $\eta_w(T)$ is the viscosity of water depending on the temperature based on interpolated data from Korson *et al.* (1969).

$$\eta_{aq} = (\eta_w(T) + 0.04 \cdot X) \cdot 10^{-3} \qquad \textit{for } X <= 100\ g \cdot L^{-1} \qquad Pa \cdot s \qquad [25]$$

$$\eta_{aq} = (\eta_w(T) + 0.012 \cdot X)^2 \cdot 10^{-3} \qquad \textit{for } X > 100\ g \cdot L^{-1} \qquad \text{Pa·s} \qquad [26]$$

Viscosity of the solvent, biodiesel, was determined as in previous work (Zautsen *et al.*, 2011a) and given in equation 27, where η: viscosity (Pa·s), η_0: viscosity at infinite temperature, $3.126 \cdot 10^{-8}$ Pa·s, E_a: activation energy for viscous flow 33555.7 J·mol^{-1}, R: gas constant 8.314 J·mol^{-1}·K^{-1} and T: temperature (K).

$$\ln(\eta) = \ln(\eta_0) + \left(\frac{E_a}{R}\right)\frac{1}{T} \qquad \ln(\text{Pa·s}) \qquad [27]$$

Density and heat capacities of the aqueous and organic phases were calculated as relative sums of the densities and heat capacities of individual components. Heat capacity ($cp_{solvent}$) and density ($\rho_{solvent}$) of biodiesel were interpolated from data by Conceição *et al.* (2007) as in equations 28 and 29.

$$\mathrm{cp_{solvent}} = 0.005914 \cdot T - 0.0485 \qquad \textit{biodiesel heat capacity} \qquad \text{kJ·kg}^{-1}\text{·K}^{-1} \qquad [28]$$

$$\mathrm{\rho_{solvent}} = -0.68 \cdot T + 1124.84 \qquad \textit{biodiesel density} \qquad \text{kg·m}^{-3} \qquad [29]$$

Heat production by metabolic activity was estimated as a linear function of the glucose conversion rate (r_S) and a metabolic heat production coefficient (c_{Qmet}) as given in equation 30. Data on caloric measurements for anaerobic ethanol fermentations by Hoogerheide (1974) was used to calculate coefficient c_{Qmet}, estimating its value at $800 \cdot 10^3$ J/kg converted glucose.

$$\mathrm{Q_{met}} = -r_S \cdot c_{Qmet} \qquad \textit{metabolic heat production} \qquad \text{J·s}^{-1} \qquad [30]$$

6.5.d Dimensions and assumptions

The main fermentor had an aqueous phase volume of 600m^3 while the second and third fermentors both contained 200m^3 broth. The organic phase in the first fermentor had a volume of 20% of the aqueous phase. The dilution rate of the aqueous phase of the first fermentor was fixed to 1/8 h^{-1}, by adjusting the flowrates of the must feed ($kg \cdot h^{-1}$), acknowledging the different densities of all used substrate compositions and concentrations. The costs of the raw materials juice and hydrolyzate related as 3:1. All considered components in the model were substrate, biomass, ethanol, furfural, vanillin, acetic acid, water, biodiesel as solvent and carbon dioxide.

The model yeast strain was assumed to be able to convert both pentoses and hexoses without differentiation in conversion kinetics or metabolic heat generation. Sucrose, glucose, fructose and xylose were therefore considered as equal sugars of which the sum was denoted as substrate *S*. Juice and hydrolyzate composition and total sugar concentrations are listed in Table 1.

Inhibitors in the hydrolyzate were assumed not to evaporate during must preparation, although in a more realistic setup, the concentrations of some more volatile inhibitors as acetic acid and furfural may well be reduced in this step. Compounds present in molasses and syrup that inhibit fermentation such as sulfide, organic acids, high concentrations of salt, calcium and magnesium and insoluble solids (Rossell 2006), were disregarded.

Although biological conversion rates of inhibitors differ for each furanic or phenolic aldehyde, depending on distinctive functional groups (Larsson *et al.*, 2000), inhibition by furfural and HMF was considered accumulative and represented in the model by furfural as their sum, total furans, as was vanillin, a phenol aldehyde, chosen as representative for the sum of all phenols. Acetic acid represented all organic acids.

Densities of liquid volumes were assumed to be equal to the weighted average of the densities of all considered components and, where possible, with temperature dependence. Ethanol was recovered 100% from the exit solvent stream and the solvent stream was re-introduced dry into the fermentor and free of previously extracted inhibiting compounds. The lower temperature limit for solvent entering the fermentor was determined as three degrees above its melting point, which is approximately -5°C (Knothe 2008).

Transport kinetics of compounds from aqueous to organic phase was disregarded and mixing conditions in all involved equipment were considered ideal. Reynolds number *Re* was fixed to 10^6 as was the power number N_p to 5.5 for a turbine stirrer with baffles (Riet and Tramper, 1991 p305). For turbulence and power calculation, three stirrers were assumed per fermentor, each with a diameter equaling 30% of the vessel diameter, and the height to diameter ratio of the vessels was two for all fermentors. The resulting impeller tip speed was between 3.3 and 6.4 $m \cdot s^{-1}$, depending on the weighted average of the final viscosity and density of the broth and the organic phase.

The final distribution coefficients of the components over the organic and aqueous phase in the fermentor and decanter exit streams were equal to the partition coefficients as measured for 34°C at equilibrium for different ethanol concentrations in the aqueous phase (Zautsen *et al.*, 2011a). Cell viability was only dependent on the temperature, with a maximum temperature at 39°C (Atala *et al*., 2001) and a cell death rate relative to the positive deviation from this temperature. Only the viable fraction of the yeast contributed to cell growth and ethanol production, while the remaining fraction contributed to loss of product yield.

The carbon dioxide production rate was assumed equivalent to 95.7% of the ethanol production by weight and no ethanol was considered to be trapped in the CO_2 exhaust and neither water nor ethanol were assumed to evaporate from any of the fermentor vessels. Origination of water due to breakdown of substrate was not considered.

Yeast density was assumed 1200 $kg \cdot m^{-3}$ wet weight with a yeast cream volume of 7 $L \cdot kg^{-1}$ dry weight. From the yeast cream, 95% was treated and diverted back to the fermenter while the remaining part was discarded by the yeast bleeding unit. No heat was introduced by centrifuging or any other process step except fermentation. Heat transfer from the fermentation medium to the cooling solvent was assumed fast in all cases, such that the temperatures of the outflowing organic phase and the fermentation broth were equal.

6.5.e Calculation method

The fermentation process was modeled using Python as a computing language. Rather than solving differential equations for different input values, a numerical approach was chosen for which millions of input value combinations were evaluated in parallel using arrays of state variables, until these state variables were constant and equilibrium states for all value combinations were reached.

6.6 Results and discussion

Several surface response diagrams were constructed from the simulation data, showing the best possible fermentation performances obtained for different values of the independent parameters on the x and y axes, considering the best values of the remaining independent parameter(s) to obtain these results. Thus, except when specified differently, the remaining, unplotted parameter values were not constant, but their best values were chosen to construct each diagram in order to visualize optimized results for all values. State variables were periodically saved for various process streams to enable visualizing and evaluating evolution of states over time for individual parameter value combinations.

6.6.a Conventional fermentation

Fermentations without solvent were also simulated, using equal dimensions and assumptions for the purpose of comparison. Three input parameters were varied: the ratio of juice to hydrolyzate, the final concentration of substrate in the feeding must and the fermentation temperature. Figure 2A shows the dependence of the volumetric production rate and Figure 2B the inverse cost of substrate on the ratio of juice to hydrolyzate and the substrate concentration for a conventional fermentation without solvent.

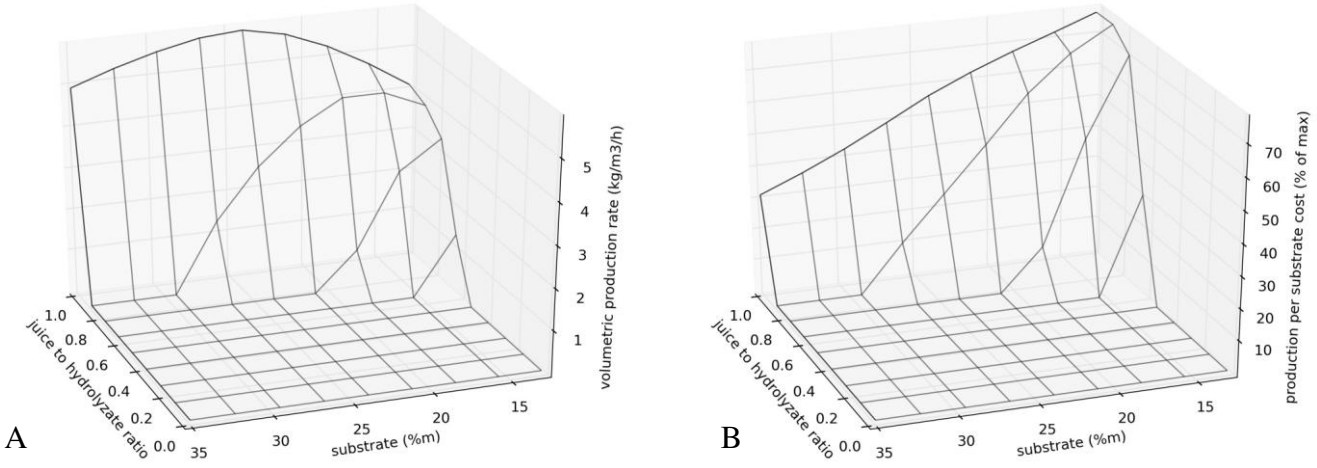


Figure 2: Volumetric production rate (A) inverse of substrate cost per volume produced ethanol (B) for fermentation without organic solvent

Because cooling was provided as conventionally for this fermentation, the amount of inhibitors in the final must was the only limiting factor for the feasibility of the fermentation. For low fractions of hydrolyzate, the growth rate was sufficiently high to prevent washout. For must containing only juice, the volumetric production rate could be maximized using a substrate concentration around 24%. Higher substrate concentrations reduced the growth rate and consequently the (volumetric) ethanol production rate. However at this optimum substrate concentration of 24%, the overall yield (data not shown) was merely 53% of the theoretical maximum due to a higher ratio of biomass to ethanol production rate. The production per substrate cost was consequently highest only for the lowest substrate concentrations used in the simulation but reached no more than 80% of the

highest value calculated for both conventional and extractive fermentation. Higher substrate concentrations caused incomplete sugar conversion and therefore decreased the overall yield, which is directly coupled to the cost of substrate per volume of produced ethanol.

Figure 3 shows a contour diagram for the optimal temperatures found, which were used to construct Figure 2A and 2B.

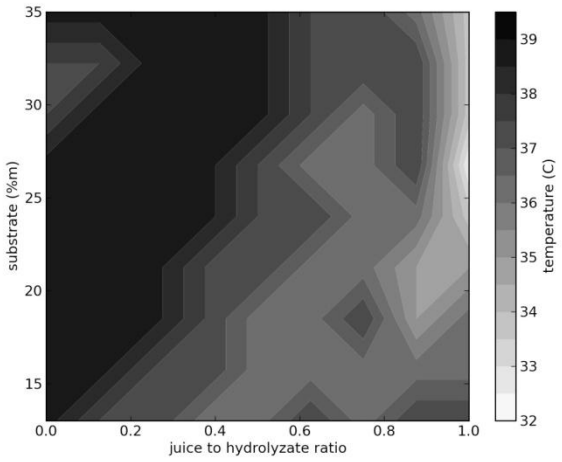


Figure 3: Fermentation without organic solvent; optimal fermentation temperatures.

With both r_p/V_{aq} and $\kappa_{substrate}$ optimal only for juice to hydrolyzate ratios close to one, optimal fermentation temperatures for maximal r_p/V_{aq}, found at substrate concentrations of 24%, were approximately 32°C. For optimal $\kappa_{substrate}$, found at lower substrate connectrations, the optimal temperatures were higher and close to 37°C.

6.6.b Extractive fermentation

For fermentations with solvent, the solvent flow rate was used as a fourth independent parameter and expressed as dilution rate of solvent over the aqueous volume. Figure 4A and 4B show the dependence of the volumetric production rate and the inverse substrate cost per volume produced ethanol on the ratio of juice to hydrolyzate and the dilution rate of the solvent. Final fermentation temperatures corresponding to these results are shown in Figure 5.

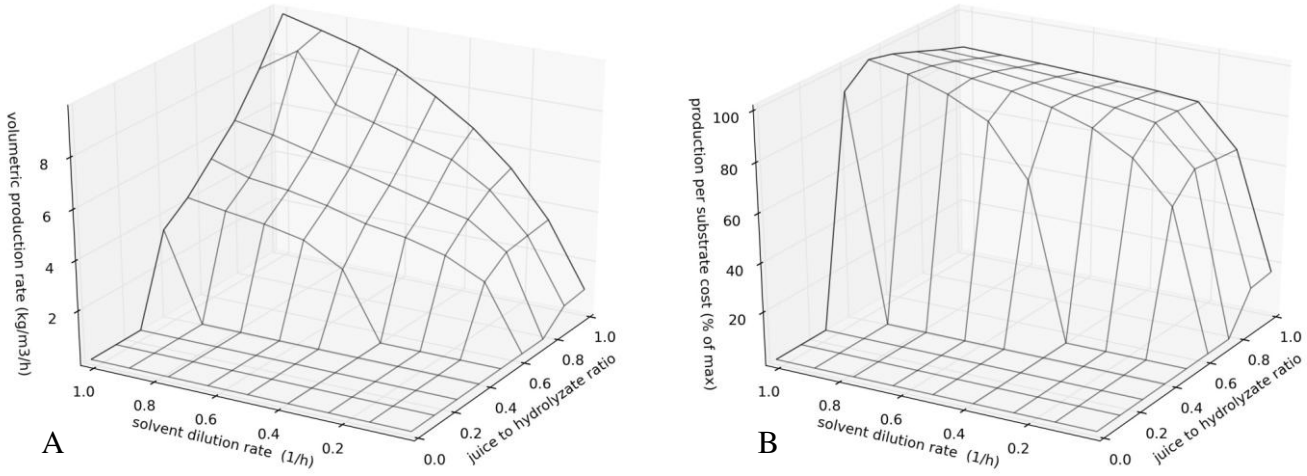


Figure 4: Volumetric production rate (A) inverse of substrate cost per volume produced ethanol (B) for fermentation with organic solvent

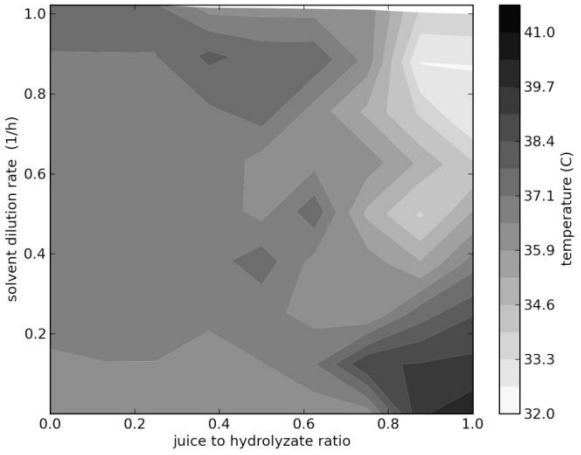


Figure 5: Fermentation with organic solvent; optimal fermentation temperatures.

The amount of inhibitors in the final must was a limiting factor for the feasibility of the fermentation for any given solvent dilution rate, causing washout at high concentrations as was the case for the conventional fermentation. Another limiting factor was cooling provided by the solvent. For low solvent dilution rates, both the volumetric production rate and the inverse substrate cost collapsed due to insufficient cooling. The final temperature

profile shows remnants of increased temperature values just after stabilization of the washed-out system below dilution rates of 0.25 h^{-1} and at hight juice-to-hydrolyzate ratios.

For higher solvent dilution rates however, the solvent provided sufficient cooling for all must compositions. Increased solvent rates increased the volumetric production rate due to the removal of ethanol and hydrolyzate inhibitors. Moreover, increased solvent rates allowed for higher fractions of hydrolyzate to be fermented and increased the maximal obtainable production per cost of substrate, although even a dilution rate of 1 h^{-1} was not enough to enable fermentation of pure hydrolyzate. However, the consequences of higher solvent dilution rates in terms of operating and energy costs were not calculated and are likely to increase the total production costs and limit feasibility of the use of very high solvent dilution rates.

For the substrate cost per volume of produced ethanol, with juice being three times as expensive as hydrolyzate, an optimum must composition could be found for each solvent flow dilution rate, with increasing hydrolyzate fractions for higher solvent rates. For example, as can be seen in Figure 4, for a dilution rate of 0.25 h^{-1}, the optimum ratio of juice to hydrolyzate is 0.75 and for a dilution rate of 1 h^{-1}, the optimum ratio is 0.5. It must be noted that, when the prices of the raw materials were assumed equal, the optimum was always one, because inhibition of growth and ethanol production by inhibitors in the hydrolyzate was not completely absent at any solvent dilution rate.

Figure 6 shows the same optimization objectives at a fixed ratio of juice to hydrolyzate of 0.7, for various substrate concentrations and optimal fermentation temperatures. It can be seen that for this composition of the must, substrate concentrations above 20% required considerable solvent dilution rates to obtain both attractive volumetric production rates and production rates per cost of substrate. For volumetric production rates, different optima of substrate concentrations were found for different solvent dilution rates. Optimal $\kappa_{substrate}$ on the other hand were only obtained for minimal substrate concentrations and increased slightly with increased solvent dilution rate beyond the tipping point of sufficient provision of cooling at around 0.25 h^{-1}. This tipping point was dependent on the

substrate concentration since the more concentrated the feed streams, the higher the fermentation rates and the higher the heat production by metabolic activity. Consequently, higher solvent dilution rates were necessary to provide sufficient cooling for higher substrate concentrations.

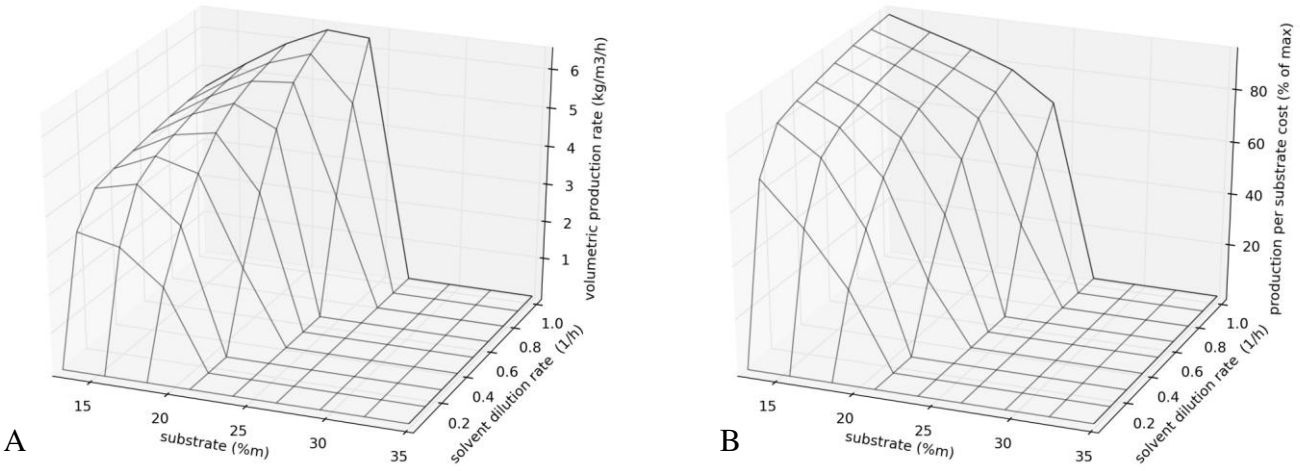


Figure 6: Volumetric production rate (A) and inverse of substrate cost per volume produced ethanol (B) for juice and hydrolyzate ratios fixed at 0.7

Figure 7 shows the volumetric production rate and the product yield for a solvent dilution rate fixed at 0.3. For the volumetric production rate, an optimal substrate concentration was found at 18%, which is lower than for the conventional fermentation and differs from an optimal substrate concentration for maximal yield or minimal substrate cost. Yield and inverse substrate cost were maximal at the lowest used substrate concentration of 15%. Higher fractions of hydrolyzate in the must did not influence the value of these optimal substrate concentrations but both r_p/V_{aq} and $\kappa_{substrate}$ were both optimal only for a juice to hydrolyzate ratio of one. Depending on the substrate concentration, which was directly coupled to the inhibitors concentration, a feasible hydrolyzate fraction was limited to a maximum around 30% for the fixed solvent dilution rate of 0.3. Beyond this fraction, the aqueous dilution rate exceeded the growth rate resulting in washout of the ferment. Decreasing the aqeous dilution rate by either decreasing substrate flowrate or increasing the fermentor volumes or number of fermentors would allow for higher hydrolyzate fractions

by increasing the overall residence time of the yeast. Volumetric production rate would however be decreased by such measure.

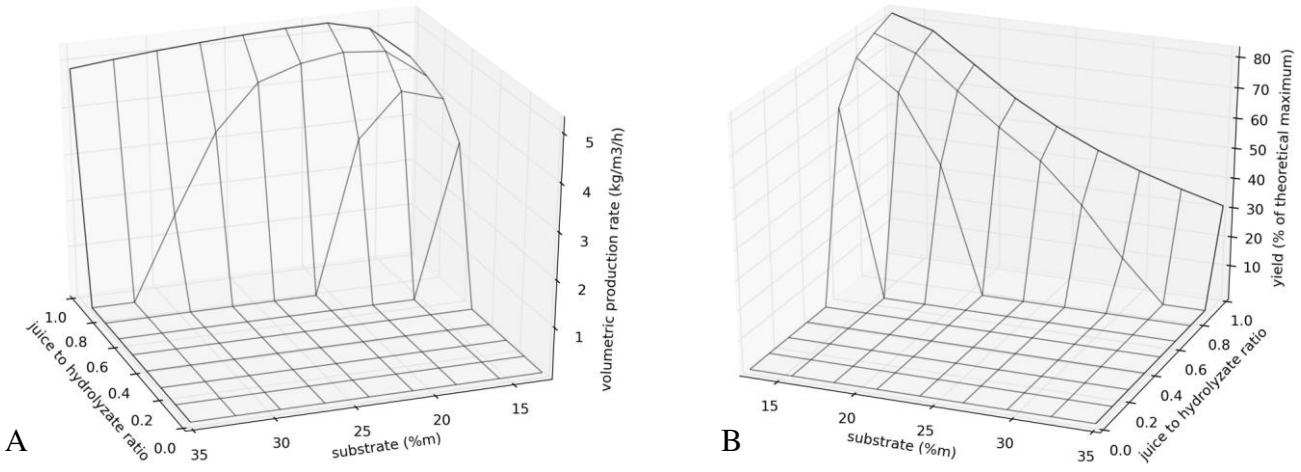


Figure 7: Volumetric production rate (A) and product yield (B) for a solvent dilution rate fixed at 0.3

6.7 Conclusion

An extensive mathematical model was constructed for a continuous two-phase fermentation that allowed for *in-situ* simultaneous extraction of inhibitors present in hydrolyzate and ethanol as the product of the fermentation, as well as effective cooling of the fermentation process by the same extractive agent. The model combined reduction of inhibitor concentrations by extraction and biological conversion affected by temperatures and composition of the fermentation broth and the extracting agent. Different values for the ratio of juice to hydrolyzate in the substrate must feed, substrate concentration, fermentation target temperatures and solvent dilution rates were evaluated, while including metabolic and mechanical heat generation, effects of ethanol concentration on partition coefficients of different compounds and effects of temperature on inhibitor conversion kinetics, fermentation rate and flow densities. Combined effects on several key fermentation performance indicators were revealed.

For the modeled fermentation, the solvent rate needed only to be high enough to assure yeast growth as fast as the aqueous phase dilution rate over all three fermentors, by preventing washout and providing sufficient cooling of the fermentation media. Before reaching washout conditions due to high inhibitor or substrate concentrations, low sugar conversion rates caused loss of substrate and low ethanol production rates. Higher solvent rates further reduced inhibition by both hydrolyzate inhibitors and ethanol, improving fermentation rates but also leading to lower concentrations of ethanol in the exit solvent flow. The latter is likely to increase operating and energy costs for ethanol and solvent recovery relative to the total amount of ethanol produced, although this has not been verified in the present work.

As also noted by Horvath *et al.*, 2001, washout occurred at relatively low inhibitor concentrations for continuous fermentations without removal of inhibitors. The synergistic toxic effect of vanillin and acetic acid in addition to furfural decreased the furfural concentration for which the furfural reduction rate was at maximum. Growth inhibition in turn was a limiting factor for the maximal specific inhibitor conversion rates by metabolic reduction. For the modelled continuous extractive fermentation however, furfural migrated to the organic phase as did vanillin and acetic acid, each with different partition coefficients depending on the actual ethanol concentrations, improving inhibitor conversion rates, growth rates and ethanol production rates, especially at increased solvent rates.

In the context of different fermentation regimes, the inhibitor concentrations can be kept low relative the biomass concentrations for fed-batch fermentations of hydrolyzed liquor by control of the feeding regime and for the presented liquid-liquid extractive fermentation by control of both the feeding regime and the dilution rate of the extracting agent. However, for batch fermentations or continuous fermentations without the application of an organic phase, this is not possible. Consequently, the conversion rates of furfural and vanillin were higher in the presented extractive fermentation than in conventional continuous fermentation, allowing for higher fractions of hydrolyzate to be fermented and generating higher volumetric production rates and lower substrate cost per

volme produced ethanol. These fermentation objectives were presented in detail by various surface response diagrams, revealing optimum solvent rates and substrate concentrations for assumed relative costs of juice and hydrolyzate.

It must be noted that the inhibitor concentrations in the original hydrolyzate were assumed not to decrease during must preparation, which represents a worse-case scenario considering the high volatility of especially acetic acid. Depending on the source of hydrolytic liquor and the method and severity of the hydrolysis of the raw material, it can be expected that final inhibitor concentrations in the must feed are lower than the assumed values in this work, leading to even more favourable results. In effect, this allows the complete omission of a detoxification step in the preparation of lignocelulosic biomass for fermentation. Overall, it can be concluded that for continuous fermentations, the proposed two-phase extractive liquid-liquid fermentation system is an efficient and promising integration of *in-situ* reduction of inhibitor concentration, product recovery and fermentation cooling.

6.8 Nomenclature

η_0	dynamic viscosity at infinite temperature	$N \cdot s \cdot m^{-2}$
η_{aq}	dynamic viscosity of the aqueous phase	$N \cdot s \cdot m^{-2}$
η_{broth}	dynamic viscosity of the broth	$N \cdot s \cdot m^{-2}$
η_w	dynamic viscosity of water	$N \cdot s \cdot m^{-2}$
$\kappa_{substrate}$	substrate cost per volume of product	$cost \cdot m^{-3}$
μ	specific growth rate	h^{-1}
μ_{max}	maximal specific growth rate	h^{-1}
ρ_{broth}	density of the broth	$kg \cdot m^{-3}$
$\rho_{solvent}$	density of the solvent	$kg \cdot m^{-3}$
A	acetic acid concentration	$kg \cdot m^{-3}$

D	stirrer diameter	m
E_a	activation energy	$J \cdot mol^{-1}$
F	furfural concentration	$kg \cdot m^{-3}$
F_{juice}	clarified juice flow	$kg \cdot h^{-1}$
$F_{hydrolyzate}$	hydrolyzate flow	$kg \cdot h^{-1}$
I_C	chemical growth rate inhibition	-
I_C $a...k$	constants for calculation of I_C	-
I_S	growth rate inhibition by substrate	-
I_T	growth rate inhibition by temperature	-
I_{TqF}	inhibition of q_F by temperature	-
I_{TqV}	inhibition of q_V by temperature	-
I_{TYxs}	reduction factor of biomass yield	-
I_X	growth rate inhibition by biomass	-
K_s	Modod constant	$kg \cdot m^{-3}$
K_i	substrate inhibition power	-
K_V	half-value for vanillin reduction rate	$kg \cdot m^{-3}$
N	stirrer speed	s^{-1}
N_p	power number	-
P	product (ethanol) concentration	$kg \cdot m^{-3}$
$P_{stirring}$	power used for stirring	$J \cdot s^{-1}$
Q_{met}	heat introduced by metabolic activity	$J \cdot s^{-1}$
$Q_{stirring}$	heat introduced by stirring	$J \cdot s^{-1}$
R	gas constant	$J \cdot mol^{-1} \cdot K^{-1}$
Re	Reynolds number	-
S	substrate concentration	$kg \cdot m^{-3}$
T	temperature	°C/K
$Topt_{\mu}$	optimal temperature for growth	°C
$Topt_{qF}$	optimal temperature for furfural conversion	°C
$Topt_{qV}$	optimal temperature for vanillin conversion	°C
$Topt_{Ypx}$	optimal temperature for product yield	°C
$Topt_{Yxs}$	optimal temperature for biomass yield	°C
V	vanillin concentration	$kg \cdot m^{-3}$
X	biomass	$kg \cdot m^{-3}$

X_{max}	maximal biomass concentration	$kg \cdot m^{-3}$
Y_{px}	product yield over biomass	$kg \cdot kg^{-1}$
Y_{px}	product yield over substrate	$kg \cdot kg^{-1}$
Y_{xs}	biomass yield over substrate	$kg \cdot kg^{-1}$
V_{aq}	aqueous phase volume	m^3
V_{org}	organic phase volume	m^3
$a_{\mu T}$	factor for growth rate inhibition by T	-
a_{qF}, $b_{qF,}$ c_{qF}, $d_{qF,}$	constants for calculation of q_F	-
a_{qFT}	constant for calculation of I_{TqF}	-
a_{qV}, b_{qV}	constants for calculation of q_V	-
a_{qVT}, b_{qVT}	constants for calculation of I_{TqV}	-
aY_{px}, bY_{px}	constants for calculation of Y_{px}	-
$cp_{solvent}$	heat capacity of pure solvent	$kJ^{-1} \cdot kg \cdot K^{-1}$
c_{Qmet}	metabolic heat production coefficient	$kJ \cdot kg^{-1}$
c_{qP}	constant specific ethanol production	h^{-1}
m	biomass inhibition power	-
m_p	partition coefficient	-
m_{pa}, m_{pb}	parameters for partition coefficient	-
m_x	maintenance coefficient for growth	h^{-1}
m_{x0}	constant maintenance coefficient	h^{-1}
m_{xa}	relative maintenance coefficient	h^{-1}
n	product inhibition power	-
q_F	specific furfural reduction rate	h^{-1}
q_{FOH}	specific furfuryl alcohol formation rate	h^{-1}
q_V	specific vanillin reduction rate	h^{-1}
q_{VOH}	specific vanillyl-alcohol formation rate	h^{-1}
q_{Vmax}	maximal specific vanillin reduction rate	h^{-1}
r_P	ethanol production rate	$kg \cdot h^{-1}$
$r_{hydrolyzate}$	total rate of hydrolyzate in unevaporated must	$kg \cdot h^{-1}$
r_{juice}	total rate of juice in unevaporated must	$kg \cdot h^{-1}$
r_S	substrate conversion rate	$kg \cdot h^{-1}$
w_{Paq}	mass fraction of ethanol in aqueous phase	$kg \cdot kg^{-1}$

6.9 References

Aguilar R., Ramírez J.A., Garotte G., Vázquez M. 2002. Kinetic study of the acid hydrolysis of sugar cane bagasse. Journal of Food Engineering 55:309-318

Atala D.I.P., Costa A.C., Maciel R., Maugeri-Filho F. 2001. Kinetics of ethanol fermentation with high biomass concentration considering the effect of temperature. Applied Biochemistry and Biotechnology. 91-93:353-365

Atala D.I.P. 2004. Montagem, instrumentação, controle e desenvolvimento experimental de um processo fermentativo extrativo de produção de etanol. Doctoral thesis. State University of Campinas UNICAMP-FEA/DEA

Balat M., Balat H. 2009. Recent trends in global production and utilization of bio-ethanol. Applied Energy 86:2273-2282

Bai F. 2007. Process oscillations in continuous ethanol fermentation. Doctoral thesis. University of Waterloo, Canada.

Conceição M.M., Candeia R.A., Silva F.C., Bezerra A.F., Fernandes V.J., Souza A.G. 2007. Thermoanalytical characterization of castor oil biodiesel. Renew Sust Energ Rev 11:964-975

Cruz J.M., Domínguez J.M., Domínguez H., Parajó J.C. 1999. Solvent extraction of hemicellulosic wood hydrolysates: a procedure useful for obtaining both detoxified fermentation media and polyphenols with antioxidant activity. Food Chemistry 67:147-153

Eijsberg, R. 2006. The design and economic analysis of a modern bio-ethanol factory located in Brazil. Master Thesis, Delft University of Technology, The Netherlands and State University of Campinas, Brazil.

Finguerut J. 2006. I Workshop of ethanol production technology. Lorena EEL/USP Brasil. Available via http://www.apta.sp.gov.br/cana.

Gonzalez R., Treasure T., Phillips R., Jameel H., Saloni D. 2011. Economics of cellulosic ethanol production: green liquor pretreatment for softwood and hardwood, greenfield and repurpose scenarios. Bioresources 6:2551-2567

Grobben N.G., Eggink G., Cuperus F.P., Huizing H.J. 1993. Production of acetone butanol and ethanol (ABE from potato wastes: fermentation with integrated membrane extraction. Applied Microbiology and Biotechnology 39:494-498

Hasmann F.A., Santos V.C., Gurpilhares D.B., Pessoa-Junior A., Roberto I.C. 2008. Aqeous two-phase extraction using thermoseparating copolymer: a new system for phenolic compounds removal from hemicelullosic hydrolyzate. Journal of Chemical Technology and Biotechnology 83:167-173

Horváth I.S., Taharzadeh M.J., Niklasson C., Lidén G. 2001. Effects of furfural on anaerobic continuous cultivation of Saccharomyces cerevisiae. Biotechnology and Bioengineering 75:540-549

Hoogerheide J.C. 1974. Studies on the energy metabolism during anaerobic fermentation of glucose by baker's yeast. Rad. And Environm. Biophys. 11:295-307

Jarzębski A.B., Malinowski J.J., Goma G. 1989. Modeling of ethanol fermentation at high yeast concentrations. Biotechnology and Bioengineering 34:1225-1230

Jassal D.S., Zhang Z., Hill G.A. 1994. In-situ extraction and purification of ethanol using commercial oleic acid. The Canadian Journal of Chemical Engineering 72:822-826

Kollerup F., Daugulis A.J. 1986. Ethanol production by extractive fermentation – solvent identification and prototype development. The Canadian Journal of Chemical Engineering 64:598-606

Korson L., Drost-Hansen W., Millero F.J. 1969. Viscosity of water at various temperatures. Journal of Physical Chemistry 73:34-39

Knothe G. 2008. Designer biodiesel: optimizing fatty ester composition to improve fuel properties. Energy and Fuels 22:1358-1364

Larsson S., Quintana-Sáinz A., Reimann A., Nilvebrant N-O., Jönsson L.J. 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae. Appl Biochem Biotechnol 84-86:617-632

Lee J-W., Jeffries T.W. 2011. Efficiencies of acid catalysis in the hydrolysis of lignocellulosic biomass over a range of combined severity factors. Bioresource Technology 102:5884-5890

Legendre B.L., Burner D.M. 1995. Biomass production of sugarcane cultivars and early-generation hybrids. Biomass and Bioenergy 8:55-61

Liu Z.L. 2011. Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Applied Microbial Biotechnology 90:809-825

Maiorella B.L., Blanch H.W., Wilke C.R. 1984. Feed component inhibition in ethanolic fermentation by Saccharomyces cerevisiae. Biotechnology and Bioengineering. 26:1155-1166

Malinowski J.J., Lafforgue C., Goma G. 1987. Rheological behavior of high density continuous cultures of *Saccharomyces cerevisiae*. Journal of Fermentation Technology 65: 319-323

Malinowski J.J., Daugulis A.J. 1993. Liquid-liquid and vapour-liquid behaviour of oleyl alcohol applied to extractive fermentation processing. The Canadian Journal of Chemical Engineering 71:431-436

Mantelatto P.E.. 2005. Estudo do processo de cristalização de soluções impuras de sacarose de cana-de-açúcar por resfriamento. Master thesis, Federal University of São Carlos.

Martin M.A. 2010. First generation biofuels compete. New Biotechnology 27:596-608

Martinez A., Rodriguez M.E., Wells M.L., York S.W., Preston J.F., Ingram L.O. 2001. Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnol Prog 17:287-293

Méjean A., Hope C. 2010. Modelling the costs of energy crops: a case study of US corn and Brazilian sugar cane. Energy Policy 38:547-561

Minier M., Goma G. 1982. Ethanol production by extractive fermentation. Biotechnology and Bioengineering 24:1565-1579

Olivério J.L., Hilst A.G.P. 2005. DHR-DEDINI Rapid Hydrolysis – Revolutionary process for producing alcohol from sugar cane bagasse. Guatemala XXV ISSCT Congress.

Özbek L., Özlale, Ü. 2010. Analysis of real oil prices via trend-cycle decomposition. Energy Policy 38:3676-3683

Palmqvist E., Almeida J.S., Hahn-Hägerdal B. 1999. Influence of furfural on anaerobic glycolytic kinetics of Saccharomyces cerevisiae in batch culture. Biotechnology and Bioengineering 62:447-454

Riet K., Tramper J. 1991. Basic bioreactor design. Marcel Dekker, New York. ISBN 0.8247.8446.4

Rivera E.C., Costa A.C., Atala D.I.P., Maugeri-Filho F., Wolf-Maciel M.R., Maciel-Filho R. 2006. Evaluation of optimization techniques for parameter estimation: application to ethanol fermentation considering the effect of temperature. Process Biochemistry 41:1682-1687

Rossell C.E.V. 2006. Quality of raw material. I Workshop of ethanol production technology. Lorena EEL/USP Brasil. Available via http://www.apta.sp.gov.br/cana.

Seabra J.E.A., Tao L., Chum H.L., Macedo I.C. 2010. A Techno-economic evaluation of the effects of centralized cellulosic ethanol and co-products refinery options with sugarcane mill clustering. Biomass and Bioenergy 34:1065-1078

Silva F.L.H., Rodrigues M.I., Maugeri-Filho F. 1999. Dynamic modelling, simulation and optimization of an extractive continuous alcoholic fermentation process. Journal of Chemical Technology and Biotechnology 74:176-182.

Sriyudthsak K., Shiraishi F. 2010. Investigation of the performance of fermentation process using a mathematical model including effects of metabolic bottleneck and toxic product on cells. Mathematical Biosciences 228:1-9

Szewczyk K.W., Zautsen R.R.M. 2003. The energetics and economics apects of extractive ethanol fermentation. Przemysł Chemiczny 82:2-4

Szijártó N., Siika-aho M., Tenkanen M., Alapuranen M., Vehmaanperä J., Réczey K., Viikari L. 2008. Hydrolysis of amorphous and crystalline cellulose by heterologously produced cellulases of Melanocarpus albomyces. Journal of Biotechnology 136:140-147

Zautsen R.R.M, Sato A.C.K., Perrechil F.A., Vaz-Rossell C.E., Straathof A.J.J., van der Wielen L.A.M., Maugeri-Filho F. 2011. Comparison of vegetable oil and vegetable oil based biodiesel as organic solvent for in-situ extraction of fermentation inhibitiors in hydrolysed bagasse.

Zautsen R.R.M., Moço Gaudiosi R., Maugeri-Filho F. 2011. Kinetics of ethanol fermentation and inhibition by hydrolyzed lignocellulosic biomass

Capítulo 7

Conclusões e sugestões

7.1 Conclusões principais

As principais conclusões obtidas através da pesquisa apresentada nos capítulos anteriores podem ser resumidas a seguir:

Entre alkanos e álcoois, um menor valor log P_{ow} do solvente orgânico resulta em maior coeficiente de partição para inibidores da fermentação provenientes do caldo hidrolítico. Portanto, melhores propriedades extrativas, principalmente para inibidores mais polares, podem ser encontrados para álcoois de cadeia curta. Porém, para estes álcoois o número mínimo de carbonos na cadeia é limitado pela biocompatibilidade, que diminui com menores valores de log P_{ow}. O estresse gerado por agitação prejudica a biocompatibilidade de alcoóis com cadeias de carbono iguais ou menores que 12.

Álcool oléico, um álcool com 18 carbonos, mostra uma biocompatibilidade de 100% e os coeficientes de partição dos componentes inibidores são bastante elevados para este solvente. Fermentações bateladas em frasco agitado na presença de furfural e álcool oléico mostraram elevada produção de etanol e maior biomassa final.

Comparando óleo de mamona e biodiesel deste óleo como solventes, mostra que ambos têm uma alta biocompatibilidade, capacidades semelhantes de absorção de etanol e seletividade baixa para substrato. Por outro lado, o biodiesel tem propriedades melhores em termos de absorção de água, viscosidade e coeficientes de partição de inibidores provenientes de caldo hidrolítico. Em sistema bifásico, valendo para ambos solventes, maiores concentrações de etanol na fase aquousa leva a coeficientes de partição mais elevados, assim como a razão entre absorção de etanol e água, especialmente para o biodiesel, que absorve relativamente mais água, resultando em maior capacidade de concentração de substrato no meio da fermentação.

Fermentações em fermentadores de bancada, em regime de batelada, mostraram o efeito positivo do uso de biodiesel como solvente na fermentação extrativa líquido-líquido, na presença dos inibidores furfural, baunilha e ácido acético no mosto. As taxas de redução

biológica de furfural e baunilha em produtos sem efeito inibidor foram mais elevadas na presença do solvente, diminuindo a fase lag e aumentando a taxa de crescimento e produtividade específica e volumétrica, enquanto que as mesmas concentrações iniciais destes inibidores, na ausência do solvente, frequentemente inibiram a fermentação, diminuindo a viabilididade e até levando a morte completa do inóculo antes do mesmo sair da fase lag.

Uma série de experimentos permitiu a construção de um modelo cinético da redução de furfural e baunilha, na presença de ácidio acético e para várias temperaturas e concentrações destes inibidores. O modelo descreve, entre outros, as taxas de conversão de furfural e baunilha, a taxa de crescimento e taxa de produção de etanol em função da temperatura, substrato e concentração de inibidores e produto, ajustado por estimativa de parâmetros e validado para várias possíveis combinações chaves das variáveis independentes envolvidas. O modelo pode ser usado principalmente para fermentações em regime batelada e batelada alimentada.

A modelagem e simulação de um sistema integrado de fermentação extrativa líquido-líquido com recuperação de solvente e resfriamento do meio de fermentação pelo próprio solvente, permitiu a otimização da taxa de diluição de solvente, a concentração de substrato, a temperatura e a fração de caldo hidrolítico. Utilizando um modelo de custos relativos das matérias primas, obteve-se uma relação importante entre os custos da mistura do mosto e a produtividade do processo, baseado no rendimento total do mesmo e admitindo o limite para a utilização de caldo hidrolítico. Sobretudo, mostrou-se a vantagem da utilização do biodiesel como agente extrativo de inibidores, provenientes do caldo hidrolisado, e do etanol, assim como agente resfrigerante para o processo fermentativo, permitindo a fermentação de maiores frações de caldo hidrolítico e elevando a produtividade e rendimento do processo integrado.

7.2 Sugestões para pesquisas posteriores

Baseando-se nesta tese, surgem as seguintes ideias e estudos desejáveis para avaliação mais detalhada e aperfeiçoamento da tecnologia de fermentação por extração líquido-líquido:

Um estudo econômico da viabilidade da tecnologia e outras tecnologias propostas neste item das sugestões.

Um estudo técnico e econômico do uso da tecnologia para a produção de outros produtos fermentativos, por exemplo, butanol, acetona, ácido láctico.

Melhores estimativas de concentrações de inibidores no caldo hidrolítico para processos modernos de hidrólise, a variação das concentrões destes inibidores em ambiente industrial, e o impacto relativo dos mesmos na cinética da levedura possibilitará uma avaliação mais exata e detalhada da tecnologia proposta.

Avaliação e ajuste do modelo cinético é ainda necessário para fermentação em regime contínuo e batelada alimentada, porque, após de um período de adaptação, a levedura é capaz de crescer e converter inibidores simultâneamente com taxas que podem ser diferentes das taxas descritas pelo modelo apresentado nesta tese, sendo que o modelo é principalmente adequado para fermentação em regime batelada.

No desenvolvimento do modelo cinético ajustado para a fermentação contínua, como sugerido no item acima, necessita um estudo para incluir os efeitos sinérgicos do etanol e dos demais inibidores presentes no meio, em diversas concentrações. A temperatura pode aumentar a gravidade da inibição total no crescimento, na produção e no rendimento.

Incorporação do fator pH no modelo cinético, que é de maior importânca, especificamente em relação à concentração de ácido acético, cujo efeito inibidor é mais severo em condições mais ácidas.

Um estudo da influência da temperatura e pH nos coeficientes de partição e fenômenos de transporte de etanol e inibidores, entre meio e solvente, para meios sintéticos e meios industriais.

Incorporação da taxa de produção de glicerol e dióxido de carbono no modelo cinético, para fechar o balanço de massa da produção e avaliar efeitos da presença de pequenas concentrações de inibidores, como furfural, no rendimento de produção.

A construção de um modelo dinâmico, considerando oscilações na taxa de conversão de substrato e inibidores, a partir de cinética com tempos de atraso, resultará em um modelo mais adequado e pode revelar mais vantagans da tecnologia em termos de controle de rendimento.

Heipieper e de Bont (1994) mostram que na defesa do micro-organismo contra o etanol, ácidos graxos em conformação *cis* são convertidos em conformação *trans*, provavelmente para diminuir a fluidez da membrana e anular o efeito contrário causado pelo etanol. Neste contexto, pode ser interessante estudar a utilização de uma versão *trans* de ácido ricinoleíco ou a conversão de biodiesel de ácido ricinoleico -*cis* para -*trans* pela levedura durante a fermentação, e se o próprio solvente pode aumentar a resistência da levedura contra etanol, fator muito importante para maximização da taxa de produção.

Um estudo da viabilidade econômica e técnica da tecnologia para regimes de fermentação batalada e batelada alimentada, sem ou com reciclo contínuo do solvente e levando em conta a produção de glicerol. Mesmo sem reciclo do solvente, as concentrações dos inibidores na fase aquosa são menores na presença da fase orgânica, o que acelera a conversão destes inibidores em componentes sem efeito inibidor. O rendimento de etanol pode ser mais elevado na presença moderada de aldeídos, como furfural, em detrimento da produção de glicerol.

Um estudo empregando um ambiente de micro-oxigenação na fermentação, para detalhar o conhecimento sobre a influênça de oxigênio na taxa de produção e na viabilidade

da levedura em função da temperatura, pH, concentrações de inibidores. Finalmente, a taxa de respiração pode ser otimizada para um melhor rendimento e/ou taxa de produção.

Um estudo para desenvolver tecnologia adequada para a recuperação do solvente e a recuperação dos produtos extraídos do mesmo.

Um estudo de resfriamento econômico do solvente, por exemplo, utilizando energia solar e o armazenamento do solvente à baixa temperatura.