Universidade de Aveiro Departamento de Química
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Cláudio José Remédios Frazão

Desafios na produção de etanol a partir de biomassa lenhocelulósica

Challenges of ethanol production from lignocellulosic biomass

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Challenges of ethanol production from lignocellulosic biomass

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia – Ramo Industrial e Ambiental, realizada sob a orientação científica da Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, Portugal, e da Doutora Marie Gorwa-Grauslund, Professora da Divisão de Microbiologia Aplicada da Universidade de Lund, Suécia.

Aos meus pais e avô

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Palavras-chave

Bioetanol, licor de cozimento ao sulfito ácido, *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, inibidores lenhocelulósicos, engenharia evolutiva, contaminação microbiana, bactérias ácido-lácticas, *Lactobacillus pentosus*.

Resumo

A presente dissertação tem como objetivo abordar dois dos maiores desafios na produção de bioetanol a partir de biomassa lenhocelulósica: (i) elevada tolerância de microrganismos a inibidores, e (ii) prevenção de contaminação microbiana.
Os inibidores lenhocelulósicos são uma fração relevante do licor de cozimento ao sulfito ácido (SSL), um subproduto das indústrias do papel e pastas. O SSL de folhosas (HSSL) é rico em pentoses, principalmente xilose, que podem ser fermentadas em etanol pela levedura *Scheffersomyces stipitis*. Neste estudo, utilizou-se uma população de *S. stipitis* previamente adaptada a 60 % (v/v) HSSL, e avaliou-se a sua estabilidade na ausência de inibidores durante dez transferências sequenciais. Comparando com a estirpe original, todos os clones isolados exibiram taxas de consumo de xilose e ácido acético superiores e produtividades em etanol inferiores. O clone que demonstrou a maior taxa de consumo de xilose (0,558 g L^{-1} h^{-1}) foi designado isolado C4, e o efeito de adaptação de curta duração no seu desempenho fermentativo foi investigado através do seu pré-cultivo na presença ou ausência de 60 % (v/v) HSSL. Nas duas condições, as taxas de consumo de glucose e xilose foram idênticas, contudo, atingiu-se maior taxa de consumo de ácido acético (0,101 g L^{-1} h^{-1}) e maior concentração máxima de etanol (4,51 g L^{-1}) foram atingidas na ausência do processo de adaptação de curta duração. Tais resultados demonstram a robustez do isolado C4.
A maioria dos processos de produção industrial de bioetanol é realizada na ausência de esterilidade, favorencendo a contaminação por microrganismos. Neste estudo, investigou-se o mecanismo responsável pela contaminação com *Lactobacillus pentosus* na indústria de SSL. Para tal, utilizou-se um hidrolisado sintético mimetizando a composição média de açúcares e inibidores de SSL de resinosas (SSSL) e averiguou-se o impacto de vários fatores na viabilidade de *L. pentosus* e *S. cerevisiae*. A presença de extrato de levedura foi responsável pelo aumento da produção de ácido lático (9 vezes) e da viabilidade bacteriana quando *L. pentosus* foi cultivado na ausência de levedura. Diferentes proporções de inóculo de levedura/bactéria não afetaram a produção de etanol após 48 h de fermentação, e *L. pentosus* foi incapaz de ser a estirpe dominante durante os ensaios de co-cultura. A presença de inibidores retardou o crescimento da levedura, mas a bactéria foi de novo incapaz de se a espécie dominante. Ajustando o valor de pH para o ótimo *de L. pentosus* nos ensaios de co-cultura, a viabilidade celular da bactéria diminuiu mais lentamente. Os resultados demonstram que *L. pentosus* não foi a espécie dominante nos ensaios de co-cultura. A presença de extrato de levedura e de valores de pH favoráveis a *L. pentosus* podem desempenhar um papel importante no mecanismo responsável pela contaminação bacteriana nas indústrias de produção de bioetanol.

Keywords

Bioethanol, spent sulphite liquor, *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, lignocellulosic inhibitors, evolutionary engineering, microbial contamination, lactic acid bacteria, *Lactobacillus pentosus*

Abstract

The present work aimed to tackle two of the major challenges in bioethanol production from lignocellulosic feedstocks: (i) high tolerance of microorganisms to lignocellulosic inhibitors, and (ii) microbial contamination avoidance.

Lignocellulosic inhibitors are an important fraction of spent sulphite liquor (SSL), a by-product of the pulp and paper industries. Hardwood SSL (HSSL) is rich in pentose sugars, mainly xylose, which can be converted to ethanol by the yeast *Scheffersomyces stipitis*. In this work, a population of *S. stipitis* previously adapted to 60 % (v/v) of HSSL was used, and its stability on the absence of inhibitors during ten sequential transfers was investigated at single-clone level. During the screening trials, all the isolated clones showed higher xylose and acetate uptake rates and lower ethanol productivities than the parental strain. The clone exhibiting higher xylose uptake rate (0.558 g L^{-1} h^{-1}) was named isolate C4. The effect of short-term adaptation on isolate C4 fermentation performance was evaluated by pre-cultivating the clone in the presence or absence of 60 % (v/v) of HSSL. The uptake rates of glucose and xylose were similar under both conditions, but a higher acetate consumption rate (0.101 g L^{-1} h^{-1}) and maximum ethanol concentration (4.51 g L^{-1}) were achieved without pre-adaptation step, suggesting the robustness of isolate C4.

The industrial bioethanol production is mostly carried out under non-sterile conditions, which favours microbial contamination. In this work, the mechanism that triggers *Lactobacillus pentosus* contamination in SSL plants was investigated. A simulated synthetic hydrolysate mimicking the average composition of sugars and inhibitors of softwood SSL (SSSL) was used and the impact of different factors in bacterial and *Saccharomyces cerevisiae* viability was analysed. The presence of yeast extract led to an increase in lactate production (9-fold higher) and *L. pentosus* viability when only bacteria was inoculated. Using different inoculation ratios of yeast/bacteria, the ethanol production rates were not affected after 48 h, and *L. pentosus* failed to overtake *S. cerevisiae*. The presence of inhibitors delayed yeast growth, but the bacteria did not outcompete *S. cerevisiae*. When the pH was optimal to *L. pentosus* in co-culture experiments, the bacterial cell viability decreased slower. The results indicate that *L. pentosus* was unable to overtake *S. cerevisiae*. The presence of yeast extract and favourable pH to bacteria are important factors that can play a role in the mechanism that triggers the bacterial contamination in ethanol plants.

Contents

Contents i
List of figures ... v
List of tables ... vii
Abbreviations ... ix

1 Introduction **1**
1.1 General context ... 1
1.2 Objectives ... 2

2 Background **3**
2.1 Lignocellulosic biomass ... 3
2.1.1 Wood ... 4
2.1.2 Pulp and paper industry ... 4
2.1.2.1 Acidic sulphite pulping ... 5
2.1.2.2 Spent sulphite liquor ... 5

2.2 Biofuels ... 6
2.2.1 Bioethanol ... 7
2.2.1.1 Microorganisms in bioethanol production ... 7
2.2.1.1.1 *Saccharomyces cerevisiae* ... 8
2.2.1.1.2 *Scheffersomyces stipitis* ... 8
2.2.2 Biorefinery concept ... 9
2.2.2.1 Energy-driven biorefinery ... 9
2.2.2.2 Product-driven biorefinery ... 10

2.3 Lignocellulosic inhibitors ... 11
2.3.1 Improving yeast tolerance ... 12
2.3.1.1 Detoxification of lignocellulosic hydrolysates ... 13
2.3.1.2 Development of inhibitor-tolerant organisms ... 14
2.3.1.2.1 Rational metabolic engineering ... 14
2.3.1.2.2 Evolutionary engineering ... 14

2.4 Microbial contamination during ethanol fermentation ... 16
2.4.1 Lactic acid bacteria ... 17
2.4.2 Avoiding microbial contamination ... 18

3 Materials and methods **19**
3.1 Evolutionary engineering study 19
3.1.1 Strains and maintenance 19
3.1.2 Cultivation media 19
3.1.2.1 Hardwood spent sulphite liquor 19
3.1.2.2 Chemically defined medium 20
3.1.2.3 Solid growth media 20
3.1.3 Selection of tolerant clones 21
3.1.4 Screening trials 21
3.1.4.1 Pre-culture 21
3.1.4.2 Intermediate step 21
3.1.4.3 Shake flask fermentation 21
3.1.5 Clone characterisation 22
3.1.5.1 Pre-culture 22
3.1.5.2 Intermediate step 22
3.1.5.3 Batch reactor fermentation 22
3.1.6 Analytical methods 22
3.1.6.1 Metabolites determination 22
3.1.6.2 Cell viability 23
3.1.6.3 Cell dry weight 23

3.2 Microbial contamination study 24
3.2.1 Strains and maintenance 24
3.2.2 Cultivation media 24
3.2.2.1 Solid and liquid media 24
3.2.2.2 Simulated synthetic hydrolysate 25
3.2.3 Cell cultivation 25
3.2.3.1 Pre-culture 25
3.2.3.2 Intermediate step 25
3.2.3.3 Anaerobic batch fermentation 26
3.2.4 Analytical methods 27
3.2.4.1 Metabolites determination 27
3.2.4.2 Cell viability 27

4 Results and discussion **29**
4.1 Evolutionary engineering study 29
4.1.1 Screening trials 29
4.1.2 Characterisation of isolate C4 31
4.1.2.1 Overall comparison 33

4.2 Microbial contamination study 35
4.2.1 Initial trials using SSSL 35
4.2.2 Viability of yeast and bacterial contaminant in SSH 35
4.2.3 Co-cultivation of yeast and bacterial contaminant 38
4.2.4 Overall comparison – understanding the tested effects 44

5 Conclusions 47

6 Future work 49

7 References 51

List of figures

Figure 1. Schematic representation of a group of lignocellulosic biomass (wood) and its composition. Adapted from [19] 3

Figure 2. Process diagram of acidic sulphite pulping with spent sulphite liquor release. Adapted from [2, 26] 5

Figure 3. Total biofuel and bioethanol production worldwide according to U.S. Energy Information and Administration (EIA) [42] 7

Figure 4. Schematic diagram for bioethanol production according to the energy-driven biorefinery approach. Adapted from [7] 10

Figure 5. Schematic diagram for bioethanol production according to the product-driven biorefinery approach. Adapted from [7] 10

Figure 6. Summary of the main antifungal inhibitors produced by lactic acid bacteria [88] 17

Figure 7. Fermentation performance of the parental strain *S. stipitis* Y-NRRL 7124 (O), adapted population (A) and isolated clones (1-10), named C1-10, cultivated in HSSL/CDM (60/40 % v/v) 30

Figure 8. Ethanol yields of the parental strain *S. stipitis* Y-NRRL 7124 (O), adapted population (A) and isolated clones (1-10), named C1-10, obtained after 41 h of culture in HSSL/CDM (60/40 % v/v) 31

Figure 9. Fermentation performance of the pre-adapted isolate C4 cultivated in HSSL/CDM (60/40 % v/v). The intermediate step was performed in the same conditions 32

Figure 10. Fermentation performance of the non-pre-adapted isolate C4 cultivated in HSSL/CDM (60/40 % v/v). The intermediate step was performed in 100 % (v/v) of CDM 33

Figure 11. Cell viability (upper panels), sugars consumption and product formation (lower panels) during cultivation of *S. cerevisiae* TMB3500 in SSH, pH 5.0 without (A) and with (B) yeast extract.

Values showed are a representative profile of each fermentation, and biological duplicates were performed 36

Figure 12. Cell viability (upper panels), sugars consumption and product formation (lower panels) during cultivation of *L. pentosus* in SSH, pH 5.0 without (A) and with (B) yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed 37

Figure 13. Cell viability (upper panels), sugars consumption and product formation (lower panels) during co-cultivation of *S. cerevisiae* TMB3500 and *L. pentosus* at a ratio of 1:1 in SSH, pH 5.0 without (A) and with (B) yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed 38

Figure 14. Cell viability (upper panels), sugars consumption and product formation (lower panels) during co-cultivation of *S. cerevisiae* TMB3500 and *L. pentosus* at a ratio of 1:100 in SSH, pH 5.0 without (A) and with (B) yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed 40

Figure 15. Cell viability (upper panels), sugars consumption and product formation (lower panels) during co-cultivation of *S. cerevisiae* TMB3500 and *L. pentosus* at a ratio of 1:1 in SSH without inhibitors, pH 5.0 in the absence (A) and presence (B) of yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed 42

Figure 16. Cell viability during co-cultivation of *S. cerevisiae* TMB3500 and *L. pentosus* at a ratio of 1:100 in SSH without inhibitors, pH 6.5 in the absence (A) and presence (B) of yeast extract. Values showed are a representative profile of each fermentation 43

List of tables

Table 1. General chemical composition of HSSL and SSSL. Adapted from [26] 6

Table 2. Summary of the main advantages and disadvantages of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* for ethanol fermentation. Adapted from [44] 9

Table 3. Summary of the most common lignocellulosic inhibitors [2] 11

Table 4. Fermentation performance of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in various lignocellulosic hydrolysates under different detoxification methodologies 14

Table 5. Adaptation effect on the fermentation performance of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in various lignocellulosic hydrolysates 16

Table 6. Microbial contaminants usually found in ethanol plants 17

Table 7. Composition of hardwood spent sulphite liquor provided by Caima-Indústria de Celulose S.A. (Constância, Portugal) [99] 20

Table 8. Composition of chemically defined medium (CDM) 20

Table 9. Composition of solid growth media [98] 21

Table 10. Composition of solid and liquid growth media [13, 102] 24

Table 11. Composition of simulated synthetic hydrolysate (SSH) medium 25

Table 12. General scheme and parameters of anaerobic batch fermentations performed 26

Table 13. Fermentation parameters of isolate C4 cultivation with and without a pre-adaptation step 34

Table 14. General scheme and parameters of anaerobic batch fermentations performed 45

Abbreviations

∆log (Bacteria)	–	Difference between the final and initial bacteria viability, in log units
∆log (Yeast)	–	Difference between the final and initial yeast viability, in log units
μ_{max}	–	Maximum growth rate, in h^{-1}
CDM	–	Chemically defined medium
CFU	–	Colony-forming units
$Ethanol_{max}$	–	Ethanol maximum concentration, in g L^{-1}
Furfural	–	2-furaldehyde
HMF	–	5-hydroxymethyl-2-furaldehyde
HPLC	–	High performance liquid chromatography
HSSL	–	Hardwood spent sulphite liquor
I	–	Inhibitors
LAB	–	Lactic acid bacteria
LCB	–	Lignocellulosic biomass
MRS	–	De Man, Rogosa and Sharpe
OD_{620}	–	Optical density at 620 nm
$P_{ethanol}$	–	Ethanol productivity, in g L^{-1} h^{-1}
$P_{lactate}$	–	Lactate productivity, in g L^{-1} h^{-1}
$r_{acetate}$	–	Acetate uptake rate, in g L^{-1} h^{-1}
$r_{glucose}$	–	Glucose uptake rate, in g L^{-1} h^{-1}
r_{xylose}	–	Xylose uptake rate, in g L^{-1} h^{-1}
SSH	–	Simulated synthetic hydrolysate
SSL	–	Spent sulphite liquor
SSSL	–	Softwood spent sulphite liquor
YE	–	Yeast extract
YMG	–	Yeast Mold supplemented with glucose
YMX	–	Yeast Mold supplemented with xylose
YMXH	–	Yeast Mold supplemented with xylose and 60 % (v/v) of HSSL
YNB	–	Yeast nitrogen base
$Y_{ethanol/S}$	–	Ethanol yield, in g ethanol g $substrate^{-1}$
$Y_{lactate/S}$	–	Ethanol yield, in g ethanol g $substrate^{-1}$
YPD	–	Yeast peptone dextrose

Chapter 1

Introduction

1.1 General context

Lignocellulosic biomass, that is the most abundant renewable resource on Earth, can be divided into several groups. Among them, by-products from pulp and paper industry, like spent sulphite liquor (SSL), are typically non-valued. SSL is the side product of acidic sulphite pulping process, and can be generated from hardwoods or softwoods (HSSL or SSSL, respectively). Both HSSL and SSSL are composed by high amounts of sugars (mainly glucose, mannose and xylose), and thus can be used for the production of biofuels [1].

Biofuels, which consist of fuels produced from biomass, are suitable alternatives to conventional and non-renewable fuels (e.g. gasoline, diesel), which pose serious adverse effects to the environment and society. Bioethanol and biodiesel are currently the most promising liquid biofuels, and can be produced from various raw materials, like lignocellulosic biomass and its derivatives [2]. The baker's yeast *Saccharomyces cerevisiae* is the preferred organism for industrial bioethanol production due to its robustness, high bioethanol productivity and tolerance towards high concentrations of sugar [3-5]. However, it lacks the ability to convert pentose sugars into ethanol. *Scheffersomyces stipitis* (formerly *Pichia stipitis*), a well-studied organism, can ferment a wide variety of sugars present in lignocellulose, including hexoses, pentoses and cellobiose [6].

Regarding the bioethanol production from lignocellulosic biomass, there are two important challenges: (i) high tolerance of microorganisms to fermentation inhibitors, and (ii) microbial contamination avoidance [7].

Microbial inhibitors (e.g. acetate, phenolics) can severely affect microbial growth, thereby limiting the economic feasibility of bioconversion processes from SSL [8]. The adaptation process is a possible strategy to enable significantly inhibitors-tolerance, and is based on the effect of selective pressure for a short- or long-term [7, 9]. As a result, an adapted population is obtained, and stability tests over time in the absence of selective pressure should be performed at single-clone level. This approach is of great importance and aims to verify if the tolerance towards inhibitors and better fermentation performance was definitely acquired or not.

At industrial scale, bioethanol production is carried out under non-sterile conditions, due to high sterilization costs. This favours the introduction of microbial contaminants, which can affect negatively the yeast performance and pose serious economic consequences. Lactic acid bacteria are one of the most frequent contaminants of ethanol plants [3, 10]. However, its effect on the viability of *S. cerevisiae* is not clear.

1.2 Objectives

The present work aimed to tackle two of the major challenges in bioethanol production from lignocellulosic feedstocks: (i) high tolerance of microorganisms to lignocellulose inhibitors, and (ii) microbial contamination avoidance.

The first project was included in an evolutionary engineering study, and the main goal was to investigate, at single clone level, the stability of an adapted population of *S. stipitis* to HSSL, and if improved tolerance to lignocellulosic inhibitors was acquired.

In another study, related with microbial contamination during ethanol fermentations, the main purpose was to understand the mechanism responsible by *Lactobacillus* sp. contamination on ethanol plants. In addition, the process conditions which can simultaneously optimise the fermentation of *S. cerevisiae* and suppress growth and product formation of *Lactobacillus* were investigated.

Chapter 2

Background

2.1 Lignocellulosic biomass

Lignocellulosic biomass (LCB), a typically non-edible plant material, has been recognized as the most abundant and cheapest sustainable carbon source throughout the world [11, 12]. In general, LCB can be classified into four different groups: (i) wood; (ii) non-food agricultural crops and residues; (iii) municipal solid wastes, and (iv) by-products and/or wastes from pulp and paper industries [13].

Cellulose, hemicelluloses and lignin are the three major components of LCB (Figure 1) [14]. Since cellulose and hemicellulose are polysaccharides, both can be hydrolysed to fermentable sugars that can be converted into bulk chemicals (e.g. ethanol, succinic acid) [15, 16]. Whilst cellulose is a linear polymer of D-glucose subunits, hemicellulose consists of a heterogeneous polymer composed by pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids. The composition of hemicelluloses varies widely across biomass sources [17]. Lignin is a large complex polymer consisting of phenylpropane units that are held together by different types of linkages. It is extremely resistant to degradation, and may be used for the production of bulk chemicals (e.g. phenols, vanillin) [15, 18].

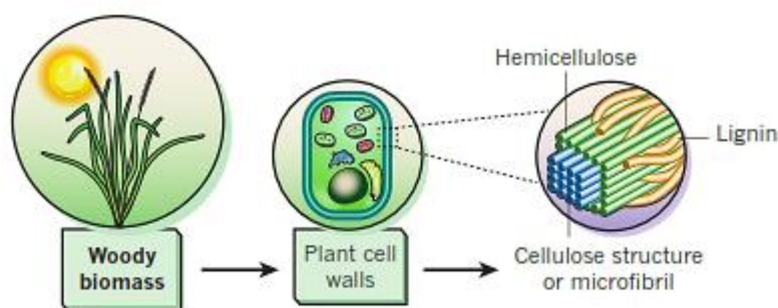

Figure 1. Schematic representation of a group of lignocellulosic biomass (wood) and its composition. Adapted from [19].

Typically, LCB is composed of 33-51 % cellulose, 19-34 % hemicellulose and 20-30 % lignin. However, the composition and content of each polymer can vary widely among plant species [7].

LCB is traditionally used as a source for pulp and paper production, fertilisers, biomass fuels, and other goods and services [20]. More recently, the interest in lignocellulose as a feedstock for the production of high-value compounds, like biofuels (e.g. bioethanol) and chemicals, has been growing [21, 22].

2.1.1 Wood

Wood is a complex biological structure, which comprises numerous cell types acting together to serve the needs of the plant [23]. It is mainly composed by cellulose, hemicelluloses and lignin. At a lesser extent, inorganic compounds (ash) and other low molecular-weight substances, mainly extractives (e.g. terpenes, fatty acids, phenols), can also be found in wood species [24, 25].

Taxonomically, woods can be divided in two general classes – softwoods and hardwoods. Whereas softwoods are those woods coming from gymnosperms (e.g. pine, spruce), hardwoods are woods that come from angiosperms (e.g. birch, eucalyptus). The chemical composition and abundance of celluloses are relatively uniform in softwoods and hardwoods, however the same does not occur with lignin and hemicelluloses. Hardwood hemicelluloses are richer in pentoses (xylose), while softwood hemicelluloses contain more hexoses (glucose, mannose) [23, 24, 26].

2.1.2 Pulp and paper industry

Currently, the global paper industry is, definitely, one of the world's largest industrial sectors [27]. According to *Lucintel*, it is expected to reach a total market value of about €190 billion in 2017 [28]. Worldwide, paper is mostly produced from cellulose fibers, of which less than two-thirds come from wood, one-third from recycled paper and approximately 5 % from non-wood sources (e.g. bagasse, cereal straw) [29].

The paper and paperboard manufacturing can be divided into four main phases: (1) raw material production, storage and preparation; (2) pulping; (3) bleaching, and (4) papermaking [30, 31]. One of the key steps in the paper production process is the pulping. During that stage, lignin is removed from the raw material and cellulose fibers (pulps) are released. Pulps produced are then further used for paper manufacture [26, 30]. Besides, they may be used to produce cellulose derivatives or chemical compounds [13]. The process of pulping can be accomplished mechanically, chemically or by a combination of both methods [24]. Chemical pulping (e.g. Kraft and acidic sulphite process) is the most common process, and is performed using aqueous chemical solutions at extreme pH with high temperature and pressure [31, 32].

2.1.2.1 Acidic sulphite pulping

The acidic sulphite pulping process is based on the use of a cooking liquor, which is mainly composed by sulphur dioxide and bisulphite, as well as a counter ion (calcium, magnesium, sodium or ammonium). This process is carried out in batch digesters under acidic conditions (pH 1-2) at high temperatures [2, 26, 33].

During the pulping stage, the main reaction occurring is the lignin sulphonation, leading to its hydrolysis [33]. As a result of wood cooking, cellulose fibers are held together, forming unbleached pulps, while most of the lignin and hemicelluloses are dissolved in spent sulphite liquor (SSL). Subsequently, unbleached pulp is submitted to bleaching stage for lignin residues removal. SSL, the side-product from the acidic sulphite pulping, is usually concentrated by evaporation and a thick liquor is generated. Then, the thick liquor is burned for chemical and energy recovery (Figure 2).

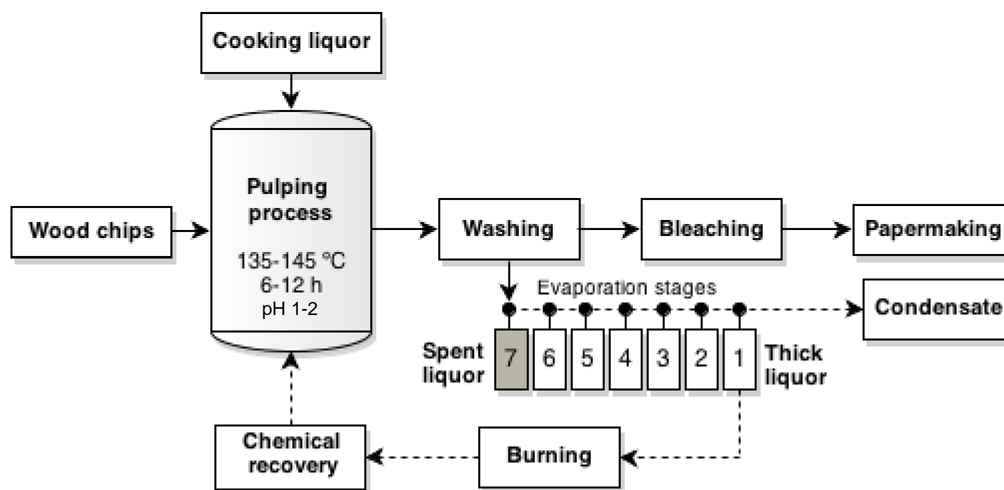

Figure 2. Process diagram of acidic sulphite pulping with spent sulphite liquor release. Adapted from [2, 26].

2.1.2.2 Spent sulphite liquor

Spent sulphite liquor (SSL) has high amount of sugars and is produced annually in large quantities, and thus may be instead used for bulk chemical production associated to the biorefinery concept (e.g. bioethanol) [2, 33]. Its chemical composition varies widely and depends on the wood species used as raw-materials, pulping reagent and pulping conditions [34]. According to the wood type used for pulping, SSL can be classified as hardwood or softwood sulphite spent liquor (HSSL and SSSL, respectively) [2]. The general composition of HSSL and SSSL is presented in Table 1.

Table 1. General chemical composition of HSSL and SSSL. Adapted from [26].

	Concentration (g L^{-1})	
Components	HSSL	SSSL
Hexose sugars		
Mannose	7.6–9.4	21.0–27.0
Glucose	2.2–2.4	7.0–9.7
Galactose	4.4–4.6	4.7–6.0
Pentose sugars		
Xylose	24.1–25.1	9.0–11.0
Arabinose	7.5–8.1	0.7–2.0
Lignosulphonates	77.6–78.8	110.0–120.0
Acetic acid	9.5–9.7	3.0
2-furaldehyde (furfural)	Traces	0.2

Due to the acidic sulphite pulping process, numerous compounds are generated and retained in the liquor. Most of SSL organic content arises from the lignin and hemicelluloses degradation. As a result, lignosulphonates and monosaccharides (e.g. xylose, glucose, arabinose, mannose) are formed [35]. The sugars composition of SSSL and HSSL is quite different. While SSSL contains a high proportion of hexoses (glucose, mannose and galactose), HSSL possesses a high amount of pentoses (mainly xylose). Since hexoses bioprocessing is less complex than that of pentoses, the development of strategies to ferment simultaneously and efficiently both sugar types is highly recommended. In addition to lignosulphonates, HSSL and SSSL contain other microbial inhibitory compounds, such as acetic acid, 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF) [36]. These features make SSL a complex media, which constitutes a challenge for a quick and efficient ethanol fermentation.

2.2 Biofuels

In 2008, fossil fuels (e.g. oil, natural gas) accounted for 88 % of the world primary energy consumption [37]. However, they are non-renewable resources and have several negative environmental and social impacts [38]. Therefore, research on more sustainable and renewable energy resources is highly required.

Biofuels, a form of renewable energy, are used to generate heat and/or power. They are expected to play a key role in the future, particularly in the transport sector [7, 39]. Biofuels may be derived from different biomass types, namely (i) forest products; (ii) agricultural products; (iii) fishery products, and (iv) by-products and wastes from forestry, pulp and paper, agriculture and food industries [21, 39]. Liquid biofuels include bioalcohols, like bioethanol and biodiesel. Biogas and biohydrogen are examples of gaseous biofuels [40].

2.2.1 Bioethanol

Bioethanol is referred as ethanol produced from renewable biomass [41]. From 2000 to 2011, the world bioethanol production increased around 400 % to 545 million barrel per year, representing 79 % of the total biofuel production by 2011 (Figure 3).

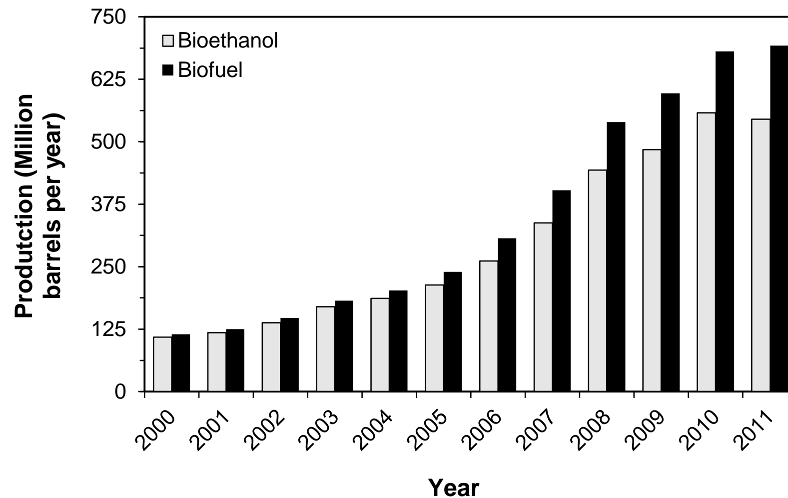

Figure 3. Total biofuel and bioethanol production worldwide according to U.S. Energy Information and Administration (EIA) [42].

United States of America and Brazil are the biggest bioethanol producers worldwide and most of it is obtained by using sugarcane or corn as feedstock – first generation bioethanol [7, 42]. This class of feedstocks is generally part of the typical human and animal food chains. Therefore, the usage of food-related feedstock can be problematic and raise some ethical, social and economic issues [41].

In order to avoid food competition, new feedstocks are being studied for bioethanol production. Second generation bioethanol is produced from agricultural lignocellulosic biomass and industrial wastes, and thus direct competition between fuel and food is absent. Depending on the biorefinery type, bioethanol can be generated as a main product or co-product from this raw material, what implies different configuration processes (Section 2.2.2) [7].

2.2.1.1 Microorganisms in bioethanol production

Bioethanol is mainly produced through fermentation of sugars, what implies the usage of microorganisms. To be considered for industrial scale bioprocesses, the microorganisms should meet some requirements: (i) high bioethanol yield, productivity and tolerance; (ii) fast growth in inexpensive media, and (iii) high-tolerance to microbial inhibitors. To prevent contamination, the microorganisms should also present growth ability under extreme conditions, such as acidic pH or high temperatures [43].

There is a wide variety of microorganisms able to produce bioethanol, like *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Scheffersomyces stipitis* and *Kluyveromyces marxianus*. However, most of them remain limited in term of sugars co-fermentation, ethanol yield and tolerance to fermentation inhibitors [44]. Therefore, the microorganism's choice for ethanol fermentation should be taken considering the origin and composition of raw material [13].

2.2.1.1.1 *Saccharomyces cerevisiae*

The baker's yeast *Saccharomyces cerevisiae* is one of the organisms of choice in industrial microbiology, and is widely studied for bioethanol production [45]. This microorganism produces ethanol at a high yield (higher than 0.45 g ethanol g sugars^{-1}) and specific rate (up to 1.3 g ethanol g cell mass^{-1} h^{-1}) under anaerobic or aerobic conditions [13, 46]. Besides, *S. cerevisiae* has proven to be tolerant towards inhibitors and high concentrations of ethanol (over 100 g ethanol L^{-1} in some strains) and sugars [7, 46].

Most of *S. cerevisiae* strains are able to metabolise the hexose sugars present in lignocellulose, but at different rates [7]. Glucose, the preferred hexose sugar, and mannose are efficiently fermented by *S. cerevisiae*. On the other hand, galactose fermentation is strain dependent, and the genes responsible for galactose utilisation are repressed by the presence of glucose, leading to a sequential metabolism of sugars [7, 46, 47]. In addition to hexoses, pentoses are also an important sugar fraction of lignocellulosic biomass and are the main source of fermentable sugars in HSSL [7, 26]. However, *S. cerevisiae* cannot naturally convert pentoses, like xylose and arabinose, into bioethanol. The simultaneous fermentation of hexoses and pentoses constitutes a challenge in ethanol fermentation. Several strategies have been applied to overcome this challenge, including rational metabolic engineering and use of microorganisms with the ability of consuming both sugar types [48].

2.2.1.1.2 *Scheffersomyces stipitis*

Naturally xylose-fermenting microorganisms can be advantageous to use when biomass contains high amounts of pentose sugars [46]. *Scheffersomyces stipitis* (formerly *Pichia stipitis*) is a haploid and homothallic yeast with the highest-native capacity for xylose fermentation of any known microorganism. Fed-batch cultures of *S. stipitis* produced ethanol with high yields (0.35-0.44 g ethanol g xylose^{-1}), but its fermentation rate is considerably lower than that of *S. cerevisiae* [49, 50]. A summary of the main advantages and disadvantages of *S. stipitis* and *S. cerevisiae* for ethanol fermentation is shown in Table 2.

Table 2. Summary of the main advantages and disadvantages of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* for ethanol fermentation. Adapted from [44].

Yeast species	Advantages	Disadvantages
Saccharomyces cerevisiae	• Naturally adapted to ethanol fermentation • High ethanol yield (90 %) • High tolerance to ethanol and chemical inhibitors • Amenability to genetic modifications	• Not able to ferment pentose sugars
Scheffersomyces stipitis	• Best performance for xylose fermentation • Ethanol yield (82 %) • Able to ferment most of lignocellulose sugars	• Not-tolerant to ethanol at high concentrations • Does not ferment xylose at low pH • Sensitive to chemical inhibitors • Requires microaerophilic conditions to reach best performance

Apart from xylose, this yeast has also the capacity to ferment other important sugars, like other pentoses (arabinose), hexoses (glucose, mannose, galactose, rhamnose) and oligomers (cellobiose, xylans, mannans) [50]. The consumption rate of pentose sugars by *S. stipitis* is also repressed by the presence of hexoses, similarly to *S. cerevisiae* [13]. Moreover, a very low and well-controlled supply of oxygen is required for an efficient fermentation, and its tolerance towards fermentation inhibitors and ethanol has proven to be low. In fact, yeast growth may be completely inhibited under acetate concentrations higher than 3.0 g L^{-1} and negatively affected by ethanol concentrations above 30 g L^{-1} [51]. Considering these drawbacks, evolutionary and rational metabolic engineering have been applied to improve *S. stipitis* performance and these methodologies are revised in Section 2.3.1.2.

2.2.2 Biorefinery concept

The term biorefining has been in focus in the past recent years, and is defined as the *"sustainable processing of biomass into a spectrum of marketable products and energy"*. Thus, in analogy to petroleum refinery, the biorefinery concept aims to separate the biomass resources (e.g. wood, grass) into building blocks (carbohydrates, proteins), which can be converted in high-value products (e.g. biofuels, chemicals) [52]. Two main types of biorefineries can be distinguished: energy-driven and product-driven.

2.2.2.1 Energy-driven biorefinery

In this approach, the biomass is primarily used for the production of energy carriers (biofuels, power and heat), and the process residues are sold or upgraded to added-value bio-based products [53]. Thus, bioethanol can be generated as a main-product in this case. The bioethanol

production process in such biorefinery type includes three main stages: (1) pretreatment, (2) hydrolysis and (3) fermentation (Figure 4).

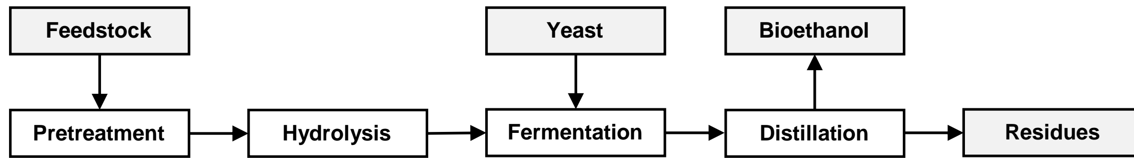

Figure 4. Schematic diagram for bioethanol production according to the energy-driven biorefinery approach. Adapted from [7].

Considering LCB as a feedstock, the pretreatment is designed to degrade the complex lignocellulose matrix, occurring the separation of its main components [41]. Besides, the pretreatment leads to total or partial hydrolysis of hemicelluloses, being monosaccharides (e.g. xylose, arabinose) and oligosaccharides released. Different strategies have been employed to pretreat LCB, including physical, chemical, physico-chemical and biological processes [2, 54]. The hydrolysis aims to degrade the cellulose released during the pretreatment stage. The cellulose can be degraded into fermentable sugars (i.e. glucose) by either acidic or enzymatic hydrolysis. As a result, a lignocellulosic hydrolysate is generated and it can be fermented to ethanol, which is usually recovered by distillation [55].

2.2.2.2 Product-driven biorefinery

The purpose of a product-driven biorefinery is the production of various added-value bio-based compounds from biomass. The process residues, like SSL, are then used for power and heat production [53]. In this approach, bioethanol can be produced as a co-product, thus resulting from the obtained residues (Figure 5). In these biorefineries, both the pretreatment and hydrolysis are optimised for the main product generation, and thus the fermentation media used for bioethanol production may contain undesired compounds.

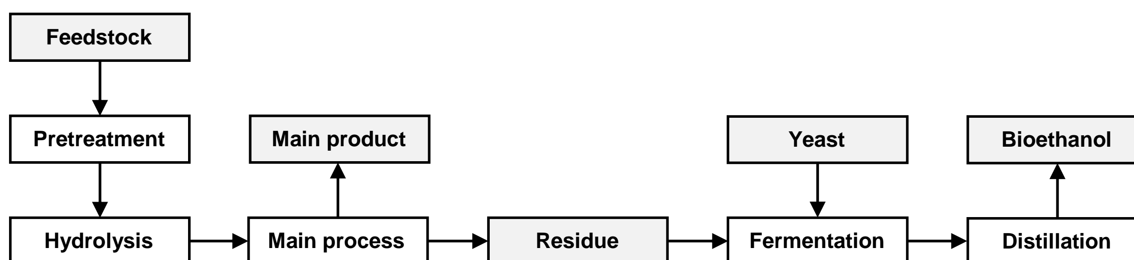

Figure 5. Schematic diagram for bioethanol production according to the product-driven biorefinery approach. Adapted from [7].

2.3 Lignocellulosic inhibitors

In the fermentation step, there are three main challenges regarding the bioethanol production from lignocellulose: (i) efficient simultaneous fermentation of hexoses and pentoses; (ii) high tolerance of microorganisms to toxic compounds, and (iii) microbial contamination avoidance [7].

Microbial inhibitors are particularly undesirable compounds for fermentation systems, thus limiting the economic feasibility of bioconversion processes [8]. Their toxicity depends on: (i) the concentration of inhibitors; (ii) type of fermentative organism; (iii) cultivation mode, and (iv) cultivation conditions (e.g. pH, temperature) [56]. Microbial inhibitors may drastically affect the viability, growth and fermentative ability of microorganisms [8].

Lignocellulosic hydrolysates in general and SSL in particular contain fermentation toxic compounds, which are called lignocellulosic inhibitors [2]. The amount and nature of the inhibitors depend primarily on the raw material and hydrolysis procedure [57]. According to their origin, lignocellulosic inhibitors can be divided into four groups: (i) sugar degradation products; (ii) weak acids; (iii) lignin degradation products; (iv) extractive-derived compounds, and (v) heavy metal ions (Table 3) [2, 56].

Table 3. Summary of the most common lignocellulosic inhibitors [2].

Inhibitor's group	Compound	Inhibitor's group	Compound
Sugar-derived	a) Furfural b) HMF	Extractives	a) Acidic resins b) Terpenic acids c) Tannin acids
Weak acids	a) Acetic acid b) Formic acid c) Levulinic acid	Heavy metal ions	a) Iron b) Chromium c) Nickel d) Copper
Lignin-derived	a) Aromatics b) Polyaromatics c) Phenolics d) Aldehydes e) Lignosulphonates		

During the hydrolysis of LCB, the furan aldehydes furfural and HMF are formed from dehydration of pentose and hexose sugars, respectively [8, 36]. These sugar-derived products have been found to negatively affect cell growth and ethanol productivity by interfering with glycolytic enzymes [8]. However, HMF is not so toxic to microorganisms as furfural [2]. Interestingly, Lohmeier-Vogel et al. [58] suggested the occurrence of a synergistic effect between furfural and HMF, even at low concentrations of each inhibitor (below 1.0 g L^{-1}). Considering ethanol-producing yeasts, Hanly et al. [59] reported that the furan aldehydes have stronger inhibitory effects on *S. stipitis* than on *S. cerevisiae*.

The most common weak acids found in lignocellulosic hydrolysates are acetic, formic and levulinic acid. Acetic acid is formed by hydrolysis of hemicelluloses, while formic and levulinic acids arise

from the degradation of HMF. Formic acid may also be generated from furfural degradation [60]. Weak acids inhibit cell growth. At low pH medium, weak acids are undissociated and can diffuse across the plasma membrane, thus decreasing the neutral intracellular pH and affecting negatively cell proliferation and viability. The concentration of undissociated acids in lignocellulosic hydrolysates is highly dependent on pH, and therefore this is an important variable during fermentation [36]. In general, a pH of at least 5.5 improves fermentation performance by reducing the toxicity of weak acids [8]. Despite their severe effects, a low concentration of weak acids have been shown to stimulate ethanol production by yeasts [36]. Among the referred weak acids, acetic acid is the most toxic to cell growth and no synergistic effects occur between them [13, 60].

Lignin degradation products are formed during the hydrolysis of lignocellulose, and include aromatic, polyaromatic, phenolic and aldehydic compounds. Phenolic compounds are the most toxic substances found in lignocellulosic hydrolysates for microorganisms and severely affect biological membranes, reducing cell growth and sugar uptake [2]. Some few examples of lignin-derived compounds are vanillin, syringaldehyde, 4-hydroxybenzoic acid and catechol. Vanillin was found to be the strongest inhibitor for both xylose- and hexose-fermenting yeasts [56]. In SSL, lignin-degradation substances are commonly found in the sulphonated form due to the cooking process, thus generating lignosulphonates [2]. Hernandez-Pérez et al. [61] suggested that also lignosulphonates have a partial microbial inhibiting effect, resulting in slower growth kinetics.

Another group of lignocellulosic inhibitors includes extractives (e.g. acidic resins, tanninic acid and terpenic acids), which are less toxic to cell growth than lignin-degradation products. Some hardwoods contain high amounts of hydrolysable tannins, leading to phenolics formation during the pulping process [26].

Heavy metal ions (e.g. iron, chromium, nickel, copper) are usually found in hydrolysates as a result of equipment corrosion during LCB hydrolysis. These compounds are commonly present in SSL, having an inhibitory effect on microbial metabolism [26].

According to Almeida et al. [62], an inhibitory synergistic effect occurs when furan derivatives, weak acids and phenolic compounds are present simultaneously [63]. Therefore, the development of strategies to counteract the negative effects of lignocellulosic inhibitors is highly required to improve the fermentation performance [26]. Those strategies are crucial to achieve an economically viable bioprocess of ethanol fermentation [13].

2.3.1 Improving yeast tolerance

According to the literature, two main alternative measures can be taken to enhance the efficiency of fermentation process and avoid the negative effects caused by microbial inhibitors: (i)

detoxification of the lignocellulosic hydrolysate before fermentation, and (ii) development of inhibitors-resistant microorganisms.

2.3.1.1 Detoxification of lignocellulosic hydrolysates

A wide variety of physical, chemical and biological methods can be used to detoxify lignocellulosic hydrolysates, and they are based on the removal or conversion of inhibitors, and should not degrade the sugars [8, 13]. A common objection against detoxification is based on the assumption that it would require a separate process step, increasing the cost of bioethanol production [60].

The physical methods are based on the adsorption process, thus promoting the removal of inhibitors from hydrolysates without changing their chemical structure. These methods include the use of: (i) activated charcoal; (ii) ion exchange resins, and (iii) phase transfer processes [64].

The main chemical detoxification treatments are based on the addition of reductive substances (e.g. dithionite treatment) and pH modification (e.g. alkali treatment). While reductive substances lead to the conversion of inhibitors into less toxic compounds, pH alteration to high values (pH 9-10) may promote the precipitation and/or decomposition of toxics [2, 64].

Biological detoxification methods are more feasible and environmentally friendlier than the physical and chemical treatments, and involve the use of enzymes and/or microorganisms, which act on inhibitors found in hydrolysates by changing their chemical structures [56, 64]. Lignolytic enzymes from the white-rot fungus *Trametes versicolor* (e.g. laccase, peroxidase) have the capability to oxidise weak acids and phenolic compounds, allowing improved fermentability [2]. There are numerous microorganisms with the inhibitor-removing ability from hydrolysates. Lopéz et al. [65] used the fungus *Coniochaeta ligniaria* (obtained from furfural contaminated soil) to detoxify a pretreated corn stover hydrolysate. As a result, 97 % of furfural and 78 % of HMF were converted into non-inhibitory substances. Besides, Pereira et al. [66] demonstrated that HSSL can be successfully biodetoxified using the fungus *Paecilomyces variotii*, allowing improved ethanol production. This methodology resulted in the decrease of acetic acid and phenolics content in HSSL. Table 4 summarizes some of the detoxification strategies adopted by research groups to improve ethanol fermentation.

Table 4. Fermentation performance of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in various lignocellulosic hydrolysates under different detoxification methodologies.

Organism	Feedstock	Detoxification	Process parameters		
			$Y_{ethanol/S}$ (g g^{-1})[a]	$P_{ethanol}$ (g L^{-1} h^{-1})[b]	Ref.
S. cerevisiae					
Baker's	Spruce hydrolysate	None	0.42	0.21	[67]
Baker's	Spruce hydrolysate	Alkali treatment	0.45	0.34	[67]
Baker's	Spruce hydrolysate	Anion-exchange resins	0.46	1.71	[67]
RLJY-019	HSSL	Overliming	0.47	0.45	[68]
TMB 3720	SSSL	None	0.37	–	[3]
S. stipitis					
DSM 3651	Sugarcane bagasse	None	0.20	0.04	[69]
DSM 3651	Sugarcane bagasse	Ion-exchange resins	0.30	0.16	[69]
Y-NRRL 7124	HSSL	None	0.16	0.01	[68]
Y-NRRL 7124	HSSL	Overliming	0.30	0.11	[68]
Y-NRRL 7124	HSSL	*Paecylomyces variotii*	0.24	0.09	[66]

[a] Ethanol yield (g ethanol g consumed sugars^{-1}).
[b] Ethanol productivity (g ethanol L^{-1} h^{-1}).

2.3.1.2 Development of inhibitor-tolerant organisms

Unlike hydrolysate detoxification, this approach has microorganism as the main target and is based on the selection of microbial species and strains tolerant to inhibitory compounds. According to this approach, inhibitor-resistant microorganisms may be obtained by two main strategies: (i) rational metabolic engineering, and (ii) evolutionary engineering [60, 70].

2.3.1.2.1 Rational metabolic engineering

Rational metabolic engineering refers to the engineering of metabolic pathways (namely enzymes, transporters, regulatory proteins) based on the available information. Based on this information, a methodology is typically designed to improve the metabolic flux and/or a phenotypic trait. This methodology is strongly based on systems biology and genetic engineering, and has been successfully used for strain improvement in a wide range of applications [71-73]. For instance, Petersson et al. [74] proved that *S. cerevisiae* strains overexpressing the ADH6 gene (encoding for alcohol dehydrogenase 6) showed increased HMF reduction capacity. Besides, Fujitomi and colleagues [75] showed that deleting the PHO13 gene (encoding for *p*-nitrophenylphosphatase), *S. cerevisiae* improved the ethanol fermentation in the presence of three important inhibitors – furfural, and acetic and formic acids.

2.3.1.2.2 Evolutionary engineering

By definition, the term evolutionary engineering includes all the methods for empirical strain improvement, such as: (i) adaptation; (ii) induced mutagenesis, and (iii) genome shuffling. This approach can be very effective, and is based on sequential and multiple cycles of random genetic

perturbation and selection, resulting in various genetic alterations. However, all the evolutionary engineering methods depend on suitable screening methods for the improved trait, what can constitute a major drawback. Therefore, the applications of this approach are restricted to few phenotypic improvements, like higher growth rate or substrate consumption [73].

Adaptation

The adaptation of fermenting microorganisms to lignocellulosic hydrolysates is a natural mutagenesis technique and has been appointed as an alternative to the detoxification strategy. It may reduce bulk chemical production cost, and avoid loss of fermentable sugars. Moreover, adaptation has been found to increase yeast tolerance to inhibitors, decrease fermentation time, and increase ethanol production [2, 8, 76]. This approach is based on the fact that microorganisms can be adapted by constant exposure to sublethal inhibitor concentrations in hydrolysates in fed-batch or continuous cultivation [76]. At these conditions, cells are under selective pressure, and the variants of cell population with selective advantage will take over the initially dominating cells [77].

The adaptation process can be divided in short- and long-term [76]. Short-term adaptation is performed immediately prior to fermentation by cell cultivation on the hydrolysate or on a defined media supplemented with synthetic inhibitors. Sànchez i Nogué et al. [78] showed that the short-term adaptation of an industrial strain of *S. cerevisiae* at pH 5.0 in the presence of acetic acid (at least 4 g L^{-1}) enhanced aerobic growth and fermentation performance under low pH (3.7) and inhibitory levels of acetic acid (6 g L^{-1}).

Long-term adaptation is usually realised by cell cultivation (i) in increasing concentrations or (ii) in a dilution of the lignocellulosic hydrolysate or inhibitors-containing defined media [8, 77]. After this process, an adapted population is obtained, and stability tests over time in the absence of selective pressure should be performed at single clone level. This approach is of great importance and aims to verify if the phenotype improvement was definitely acquired or not. For instance, Nigam [68] has found that long-term adaptation of *S. stipitis* on non-detoxified dilute acid hydrolysate of wheat straw resulted in an improved ethanol yield. On the other hand, Sànchez i Nogué [7] reported that after a long-term adaptation of *S. cerevisiae* in mineral medium containing inhibitors, a stable population was obtained, but the acquired tolerance to inhibitors was transient, since the population was not able to grow in their presence after being stored as glycerol stock. Table 5 summarizes some of the work done by research groups to improve the ethanol fermentation using the adaptation strategy.

Table 5. Adaptation effect on the fermentation performance of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in various lignocellulosic hydrolysates.

Organism	Feedstock	Strain improvement	Process parameters		
			$Y_{ethanol/S}$ ($g\ g^{-1}$)[a]	$P_{ethanol}$ ($g\ L^{-1}\ h^{-1}$)[b]	Ref.
S. cerevisiae					
Y-265	Synthetic medium	None	0.15	0.09	[79]
Y-265A-3	Synthetic medium	Adaptation	0.24	0.13	[79]
S. stipitis					
Y-NRRL 7124	Wheat straw	None	0.36	0.30	[80]
Y-NRRL 7124	Wheat straw	Adaptation	0.41	0.54	[80]
Y-NRRL 7124	HSSL	None	0.16	0.01	[68]
Y-NRRL 7124	HSSL	Adaptation	0.28	0.07	[68]

[a] Ethanol yield (g ethanol g consumed sugars^{-1}).
[b] Ethanol volumetric productivity (g ethanol $L^{-1}\ h^{-1}$).

Induced mutagenesis

Induced mutagenesis is another approach of evolutionary engineering, and can be divided in two steps. The first stage includes the treatment of microorganism with mutagens (e.g. N-nitroguanidine, UV-light), leading to the generation of random mutations. The second step is of enormous relevance, and comprises the screening/selection for the mutants with the desired characteristics. This is an efficient, quick and widely used method to obtain a desired microbial strain [8, 81]. Bajwa et al. [82] found that UV irradiation combined with a screening on HSSL was a good strategy to improve inhibitors-resistance and fermentation efficiency of *S. stipitis*.

Genome shuffling

This approach can be used to combine phenotypes conferring tolerance to various inhibitors, since it can accelerate directed evolution by facilitating recombination between members of a distinct population. The genome shuffling is a process that combines the advantage of multiparental crossing through DNA shuffling, along with the recombination of entire genomes [7, 83]. Bajwa et al. [84] used this method to obtain two different *S. stipitis* strains, GS301 and GS302, with improved tolerance to HSSL when compared to the parental strain. These strains were able to consume all the glucose and xylose and produce higher amounts of ethanol.

2.4 Microbial contamination during ethanol fermentation

The industrial ethanol fermentations of sugar-based feedstocks are not designed to run under sterile conditions, due to the high sterilization costs. This favours microbial contamination, which can occur in any stage of the process and pose serious economic consequences. A wide variety of yeast and bacteria have been isolated and identified from ethanol plants (Table 6) [3, 10, 40]. Microbial contaminants can compete for nutrients with the fermenting organism and also produce toxic compounds, thus affecting considerably the yeast fermentation performance [44].

Table 6. Microbial contaminants usually found in ethanol plants.

Microbial contaminant	Feedstock	Reference
Bacteria		
Lactobacillus buchneri	Corn	[85]
Lactobacillus plantarum	Spent sulphite liquor	[10]
Lactobacillus fermentum	Corn	[86]
Lactobacillus pentosus	Corn	[85]
Acetobacter tropicalis	Spent sulphite liquor	[10]
Acetobacter syzygii	Spent sulphite liquor	[10]
Yeast		
Candida sp.	Sugar cane	[87]
Dekkera bruxelensis	Sugar cane	[87]
Pichia sp.	Sugar cane	[87]

2.4.1 Lactic acid bacteria

Lactic acid bacteria (LAB), the most common bacterial contaminants found in ethanol facilities, are gram-positive, neutrophile and non-sporulating microaerophilic [88-90]. They can assimilate sugars by a homofermentative and/or heterofermentative pathway, and the resulting main fermentation end-product is lactate [89]. The most known inhibitory compounds formed by LAB are lactate and acetate, which lower the pH of the media, contributing to a non-optimal pH range for *S. cerevisiae* and other ethanol-producing microorganisms [40]. Moreover, at low pH, such acids are in undissociated form and can diffuse across the plasma membrane, decreasing the intracellular pH and the cell proliferation and viability [88]. Other compounds with antifungal activity, like reuterin and hydrogen peroxyde, are also formed by LAB (Figure 6).

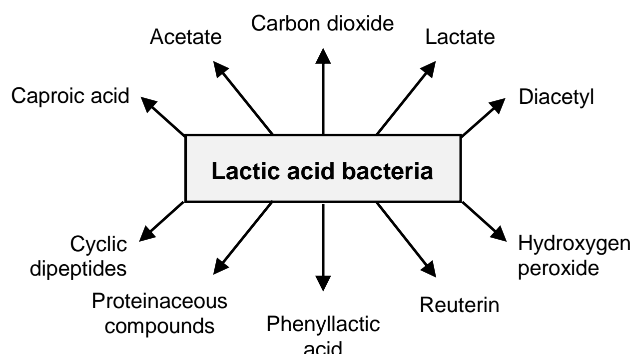

Figure 6. Summary of the main antifungal inhibitors produced by lactic acid bacteria [88].

Different studies have shown that pure cultures of *S. cerevisiae* are clearly affected by the addition of lactate and acetate to the fermentation media, resulting in decreased growth rates and ethanol productivities [91, 92]. The effects of contaminating organisms are less clear [85]. Thomas et al. [93] demonstrated that growth of *Lactobacillus fermentum* for 24 h in corn mash prior to inoculation of *S. cerevisiae* resulted in a 22 % loss of ethanol production. However, when the

yeast and bacteria were both inoculated at the same time, the ethanol production decreased only 3 % if compared to the control. Albers et al. [10] reported that the presence of *L. pentosus*, *L. plantarum* or *L. buchneri* had no negative effect in *S. cerevisiae* viability during ethanol fermentation of SSL. In other study, *L. paracasei* was introduced as a contaminant in a defined medium (with no pH control) at ratios of 1:100, 1:1 and 70:1 with *S. cerevisiae*, but failed to overtake the yeast. However, when medium pH was increased up to 6.0, *L. paracasei* was able to compete with *S. cerevisiae*, resulting in 44 % loss of ethanol production [94]. More research studies are needed to understand what triggers the contamination by *Lactobacillus* sp. in ethanol plants.

2.4.2 Avoiding bacterial contamination

Several strategies are being evaluated to reduce the levels of bacterial contamination in ethanol facilities. They can be controlled by the use of antibiotics, acid washing, among others [88]. The use of antibiotics (e.g. penicillin, virgiamycin) should be avoided, due to economic and environmental reasons. Additionally, they only reduce the contamination levels temporarily and not permanently [7]. The acid washing treatment can be a feasible alternative to the antibiotics' usage. In this process, cells are collected from the fermentation broth and sulphuric acid is used to adjust the pH. The acid treatment can be successfully used in batch processes, but not in continuous cultures with cell recirculation [95]. Other strategies, such as modification of the fermentation parameters can be further investigated to solve the bacterial contamination in ethanol plants. Changing process conditions like pH and temperature during ethanol fermentation process can avoid the growth of bacterial contaminants [10, 96]. This is a simple, economic and effective measure, which can contribute to the development of more economically viable ethanol plants. However, the effect on ethanol-producing strains also needs to be evaluated.

Chapter 3

Materials and methods

As previously outlined, this work is composed of two studies concerning different challenges of ethanol fermentation: (i) evolutionary engineering for yeast improvement, and (ii) microbial contamination. For a better understanding of each topic, the chapters 3 and 4 are thus divided in two sections.

3.1 Evolutionary engineering study

3.1.1 Strains and maintenance

Scheffersomyces stipitis NRRL Y-7124, denoted parental strain, was gently supplied by ARS Culture Collection (NCAUR, Peoria, IL, USA) [97]. The parental strain was cultivated in increasing concentrations of HSSL (0-60 % v/v) supplemented with CDM by operating a continuous stirred-tank reactor during 68 days with a working volume of 1 L. The cells collected from the final medium, designated as adapted population, were stored for further studies [98].

The parental strain and adapted population were grown at 28ºC and maintained in YMG Agar plates. The detailed composition of the mentioned media is shown below (Section 3.1.2).

3.1.2 Cultivation media

3.1.2.1 Hardwood spent sulphite liquor

HSSL from the magnesium-based acidic sulphite pulping of *Eucalyptus globulus* was kindly provided by Caima-Indústria de Celulose S.A. (Constância, Portugal). Pre-evaporated HSSL was collected, and its composition is shown in Table 7. The pretreatment of HSSL consisted in pH adjustment to 7.0 with KOH, followed by aeration with compressed air (2 h L^{-1}) and centrifugation at 5,000 rpm for 20 min, 4ºC. The precipitated colloids were filtered off using a 1.0 μm glass microfiber filter (Filtres Fiorini, France).

Table 7. Composition of hardwood spent sulphite liquor provided by Caima-Indústria de Celulose S.A. (Constância, Portugal) [99].

Components	Conc. (g L^{-1})	Components	Conc. (g L^{-1})
Hexose sugars		Lignosulphonates	78.2 ± 0.6
D-Mannose	8.5 ± 0.9	Acetic acid	8.2 ± 0.3
D-Glucose	2.3 ± 0.1	Furfural	< 0.1
D-Galactose	4.5 ± 0.1	Ash	Traces
Pentose sugars			
D-Xylose	24.6 ± 0.5		
L-Arabinose	7.8 ± 0.3		
L-Rhamnose	1.6 ± 0.3		
L-Fucose	0.4 ± 0.3		

3.1.2.2 Chemically defined medium

The composition of chemically defined medium (CDM) [100] is shown in Table 8. CDM was formulated in order to mimic the sugars composition (xylose and glucose) of HSSL, and supplemented with salts, vitamins and trace elements. A buffer solution at pH 5.5 was also part of CDM.

Table 8. Composition of chemically defined medium (CDM).

Components	Conc. (g L^{-1})	Components	Conc. (mg L^{-1})	Components	Conc. (mg L^{-1})
Sugars		Vitamins		Trace elements	
D-Glucose	2.3	Biotin	0.05	$ZnSO_4 \cdot 7H_2O$	4.50
D-Xylose	24.6	Panthothenic acid calcium salt	1.00	$MnCl_2 \cdot 4H_2O$	1.00
Salts				$CoCl_2 \cdot 6H_2O$	0.30
$(NH_4)_2SO_4$	5.0	Nycotin acid	1.00	$CuSO_4 \cdot 5H_2O$	0.30
$MgSO_4 \cdot 7H_2O$	0.5	Myo-inositol	25.0	$Na_2MoO \cdot 2H_2O$	0.40
KH_2PO_4	3.0	Thiamine·HCl	1.00	$CaCl_2 \cdot 2H_2O$	4.50
Buffer		Piridoxine·HCl	1.00	$FeSO_4 \cdot 7H_2O$	3.00
$KHC_8H_4O_4$	5.1	ρ-Aminobenzoic acid	0.20	H_3BO_3	1.00
KOH	1.1			KI	0.10

3.1.2.3 Solid growth media

The composition of solid media used (YMG Agar, YMX Agar, YMXH Agar) during the study of evolutionary engineering of *S. stipitis* is shown in Table 9.

Table 9. Composition of solid growth media. Abbreviations: YMG, Yeast Mold supplemented with glucose; YMX, Yeast Mold supplemented with xylose; YMXH, Yeast Mold supplemented with xylose and 60 % (v/v) of HSSL [98].

	Concentration (g L^{-1})		
Components	YMG Agar	YMX Agar	YMXH Agar[a]
Malt extract	3.00	3.00	1.20
Yeast extract	3.00	3.00	1.20
Peptone from casein	5.00	5.00	1.60
HSSL	–	–	60 % (v/v)[b]
D-Glucose	10.0	–	–
D-Xylose	–	10.0	4.00
Agar	20.0	20.0	20.0

[a] Solid medium composed of HSSL/YMX Agar (60/40 % v/v). Agar was added as 100 % (v/v).
[b] HSSL concentration expressed in volume percent (% v/v).

3.1.3 Selection of tolerant clones

The pre-culture of the adapted population of *S. stipitis* was performed by taking colonies from YMG Agar plates into 5 mL of CDM. Cells were grown in 50 mL conical tubes until late exponential phase at 28ºC, 180 rpm. The pre-grown cells were then streaked in YMXH Agar plates and incubated at 28ºC for 5 days. Ten of the originated colonies (i.e. clones) were randomly selected and their phenotypic stability was evaluated. For that, ten sequential transfers of each clone were performed under non-selective conditions in YMX Agar plates incubated at 28ºC for 48 h per transfer. In the final of the process, the clones-containing plates (of the 10th transfer) were stored at 4ºC for further studies.

3.1.4 Screening trials

3.1.4.1 Pre-culture

The yeast pre-cultures were performed by taking colonies of *S. stipitis* parental strain, adapted population or clones from the respective agar plates into 5 mL of CDM. Cells were grown in 50 mL conical tubes until late exponential phase at 28ºC, 180 rpm.

3.1.4.2 Intermediate step

The intermediate step was performed in 5 mL of HSSL/CDM (60/40 % v/v). The vitamins and trace elements solution of CDM were added as 100 % (v/v). The biomass needed to start the fermentation with an initial optical density at 620 nm (OD_{620}) of 0.5 was obtained from the pre-culture. Cells were grown in 50 mL conical tubes until late exponential phase at 28ºC, 180 rpm.

3.1.4.3 Shake flask fermentation

The shake flask fermentations were performed in 12 mL of HSSL/CDM (60/40 % v/v). The vitamins and trace elements solution of CDM were added as 100 % (v/v). The biomass needed

to start the fermentation with a starting OD_{620} of 0.5 was obtained from the intermediate step culture. Cells were grown in 100 mL shake flasks at 28ºC, 180 rpm, and the best-performing clone was further studied.

3.1.5 Clone characterisation

3.1.5.1 Pre-culture

The pre-culture was performed by taking colonies of the best-performing clone from YMXH Agar plate into 10 mL of CDM. Cells were grown in 100 mL shake flaks until late exponential phase at 28ºC, 180 rpm.

3.1.5.2 Intermediate step

To obtain enough biomass for fermentations and to investigate the effect of short-term adaptation on clone performance, an extra cultivation step was required. The intermediate step was performed in two different media: (i) 100 mL of CDM, or (ii) 100 mL of HSSL/CDM (60/40 % v/v) with vitamins and trace elements solution of CDM as 100 % (v/v). The medium was inoculated with the clone pre-culture at an initial OD_{620} of 0.5, and the cells were grown in 1 L shake flasks until late exponential phase at 28ºC, 180 rpm.

3.1.5.3 Batch reactor fermentation

In order to obtain the biomass needed to start the batch fermentations, the medium was inoculated with the intermediate step-culture at an initial OD_{620} of 0.5. The fermentations were performed in a 1 L stirred tank reactor (BioLab, B. Braun, Germany) with a working volume of 800 mL. A medium consisting of 800 mL of HSSL/CDM (60/40 % v/v) with vitamins and trace elements solution of CDM as 100 % (v/v) but without buffer solution was used. The temperature was set at 28ºC, agitation at 240 rpm, aeration at 0.24 L min^{-1} and pH was maintained at 5.5 by automatic addition of 3 M KOH or 3 M H_2SO_4.

3.1.6 Analytical methods

3.1.6.1 Metabolites determination

The fermentation samples were centrifuged (2 min at 13,000 rpm) in order to remove the cells. The supernatant was then filtered through 0.20 µm membrane filters (CoStar, Cambridge, MA, USA) by centrifugation (10 min at 8,000 rpm), and stored at -20ºC.

The concentrations of glucose, xylose, xylitol, glycerol, acetate and ethanol were determined by high performance liquid chromatography (HPLC). The sample injection volume was 20 µL, and

the compounds were separated using a 10 µm Eurokat H ion-exchange column (Knauer, Germany). The separation was performed at 40ºC with 0.01 N H_2SO_4 at 0.4 mL min^{-1} as mobile phase. The HPLC equipment consisted of a Hitachi L-2130 pump, autosampler (Hitachi L-2200), column oven Gecko-2000 (Teknokroma, Spain) and Hitachi Refractive Index Detector L-2490.

3.1.6.2 Cell viability

The cell viability was evaluated as colony-forming units per milliliter (CFU mL^{-1}). CFU determination was performed by serial dilutions with 0.9 % (v/v) NaCl solution, in quadruplicate. When the desired dilution factor was achieved, 100 µL were spread on YMG Agar plates. The agar plates were then incubated at 28ºC for 48 h.

3.1.6.3 Cell dry weight

Cell dry weight was determined, in quintuplicate, by filtering 5 mL of sample with a 0.45 µm membrane filter (ME 25/21 ST, Whatman, England), and washing with 15 mL of distilled H_2O. The membranes were then dried until constant weight (at 105ºC, 72 h).

3.2 Microbial contamination study

3.2.1 Strains and maintenance

The industrial yeast strain *Saccharomyces cerevisiae* TMB3500 was used in this work [101]. A lactic acid bacteria strain identified as *Lactobacillus pentosus* was isolated from an industrial ethanol plant [7].

Yeast and bacterial strains were maintained at -80ºC in 25.0 % and 12.5 % glycerol (v/v) with YPD broth and MRS broth (Merck, Darmstadt, Germany), respectively.

For short-term storage, *S. cerevisiae* was grown at 30ºC and maintained in YPD Agar plates, and *L. pentosus* was grown at 37 ºC and maintained in MRS Agar plates. The detailed composition of the mentioned media is shown below in Table 10 (Section 3.2.2.1).

3.2.2 Cultivation media

3.2.2.1 Solid and liquid media

The composition of solid and liquid media used for yeast (YPD Agar, YNB Agar, YNB broth) and for bacteria (MRS Agar and MRS broth – Merck, Darmstadt, Germany) in this study is shown in Table 10. The media were prepared according to the manufactures' instructions.

Table 10. Composition of solid and liquid growth media. Abbreviations: YPD, Yeast Extract Peptone Dextrose; YNB, Yeast Nitrogen Base; MRS, De Man, Rogosa and Sharpe [13, 102].

	Concentration (g L^{-1})					
Components	YPD Agar	YNB Agar	MRS Agar	YPD broth	YNB broth	MRS broth
Malt extract	–	–	–	–	–	–
Meat extract	10.0	–	10.0	10.0	–	8.00
Yeast extract	20.0	–	4.00	20.0	–	4.00
Peptone from casein	–	–	10.0	–	–	10.0
YNB w/o aminoacids[a]	–	6.70	–	–	6.70	–
$KHC_8H_4O_4$	–	–	–	–	10.2	–
KOH	–	–	–	–	2.20	–
K_2HPO_4	–	–	2.00	–	–	2.00
$C_6H_{14}N_2O_7$	–	–	2.00	–	–	2.00
$C_2H_3NaO_2$	–	–	5.00	–	–	5.00
$MgSO_4$	–	–	0.20	–	–	0.20
$MnSO_4$	–	–	0.04	–	–	0.04
D-Glucose	20.0	20.0	20.0	20.0	20.0	20.0
Tween® 80	–	–	1.00	–	–	1.00
Agar	15.0	12.0	14.0	–	–	–

[a] Yeast Nitrogen Base without aminoacids.

3.2.2.2 Simulated synthetic hydrolysate

The fermentations were performed on a simulated synthetic hydrolysate (SSH) [100], whose chemical composition is shown in Table 11. SSH was formulated in order to mimic the average composition of sugars and inhibitors of SSSL formed in Scandinavia. The pH of SSH medium was adjusted to 5.0 or 6.5 with concentrated KOH. Besides, the effect of yeast extract supplementation (10 g L^{-1}) and absence of inhibitors on the fermentation performance was also investigated.

Table 11. Composition of simulated synthetic hydrolysate (SSH) medium.

Components	Conc. (g L^{-1})	Components	Conc. (mg L^{-1})
Sugars		Vitamins	
D-Mannose	25.0	Biotin	0.05
D-Glucose	10.0	Panthothenic acid calcium salt	1.00
D-Galactose	5.0	Nycotin acid	1.00
D-Xylose	10.0	Myo-inositol	25.0
Inhibitors		Thiamine·HCl	1.00
Acetic acid	6.0	Piridoxine·HCl	1.00
Furfural	0.2	ρ-Aminobenzoic acid	0.20
HMF	0.2	Trace elements	
Vanillin	0.5	$ZnSO_4 \cdot 7H_2O$	4.50
Salts		$MnCl_2 \cdot 4H_2O$	1.00
$(NH_4)_2SO_4$	5.0	$CoCl_2 \cdot 6H_2O$	0.30
$MgSO_4 \cdot 7H_2O$	0.5	$CuSO_4 \cdot 5H_2O$	0.30
KH_2PO_4	3.0	$Na_2MoO \cdot 2H_2O$	0.40
Buffer		$CaCl_2 \cdot 2H_2O$	4.50
$KHC_8H_4O_4$	10.2	$FeSO_4 \cdot 7H_2O$	3.00
		H_3BO_3	1.00
		KI	0.10

3.2.3 Cell cultivation

3.2.3.1 Pre-culture

The yeast pre-cultures were performed by taking a single colony of *S. cerevisiae* from YPD Agar plates into 5 mL of YNB broth. Cells were grown in 50 mL conical tubes until late exponential phase at 30ºC, 180 rpm.

The bacterial pre-cultures were performed by taking a single colony of *L. pentosus* from MRS plates into 25 mL of MRS broth. Cells were grown in 50 mL conical tubes until late exponential phase at 37ºC with no agitation.

3.2.3.2 Intermediate step

To obtain enough biomass for anaerobic batch fermentations, an extra cultivation step was required. The yeast intermediate step was performed in 25 mL of YNB broth/SSH medium, pH

5.0 (50/50 % v/v). The biomass needed to start the fermentation with an initial OD_{620} of 0.2 was obtained from the yeast pre-culture. Cells were grown in 250 mL baffled shake flasks until late exponential phase at 30ºC, 180 rpm.

The bacterial intermediate step was performed in 25 mL of MRS broth/SSH medium, pH 5.0 (50 % v/v). The medium was inoculated with the bacterial pre-culture at an initial OD_{620} of 0.2, and the cells were grown in 50 mL conical tubes until late exponential phase at 37ºC with no agitation.

3.2.3.3 Anaerobic batch fermentation

In order to obtain the biomass needed to start the anaerobic fermentations, cells obtained from the intermediate step were harvested by centrifugation (5 min, 4ºC at 4,000 rpm), washed with 0.9 % (v/v) NaCl and then inoculated in the medium. In order to obtain different inoculation ratios of *S. cerevisiae*/*L. pentosus* (1:0, 0:1, 1:1, 1:100), a relationship between OD_{620} and the colony-forming units per milliliter (CFU mL^{-1}) of each strain was determined.

The anaerobic batch fermentations were performed in 50 mL of SSH supplemented with ergosterol and Tween 80® at a final concentration of 0.01 g L^{-1} and 0.42 g L^{-1}, respectively. The effect of optimal pH for bacteria or yeast, yeast extract supplementation and presence/absence of chemical inhibitors on the fermentation performance and cell viability was investigated. Serum flasks sealed with a rubber stopper, and an outlet needle for carbon dioxide removal were used in all cultivations. The anaerobic conditions were maintained by using a mineral oil layer (7 mL) on the surface of SSH. The serum flasks were incubated at 30ºC in a water bath equipped with a multi-magnetic stirring plate (Variomag Telesystem, Thermo Scientific, Waltham, MA, USA) at 140 rpm. All the fermentations were performed in biological duplicates. Table 12 shows, in detail, the conditions of all the performed anaerobic fermentations.

Table 12. General scheme and parameters of anaerobic batch fermentations performed. Abbreviations: SSH, synthetic simulated hydrolysate medium; YE, yeast extract; I, inhibitors.

#	**Fermentation medium**		**Starting inocculum**				
	Substrate	pH	Ratio[a]	Yeast (CFU mL^{-1})	Yeast (OD_{620})	Bacteria (CFU mL^{-1})	Bacteria (OD_{620})
1	SSH	5.0	1:0	10^7	2.500	–	–
2	SSH + YE	5.0	1:0	10^7	2.500	–	–
3	SSH	5.0	0:1	–	–	10^7	0.150
4	SSH + YE	5.0	0:1	–	–	10^7	0.150
5	SSH	5.0	1:1	10^7	2.500	10^7	0.150
6	SSH + YE	5.0	1:1	10^7	2.500	10^7	0.150
7	SSH	5.0	1:100	10^5	0.025	10^7	0.150
8	SSH + YE	5.0	1:100	10^5	0.025	10^7	0.150
9	SSH – I	5.0	1:1	10^7	2.500	10^7	0.150
10	SSH + YE – I	5.0	1:1	10^7	2.500	10^7	0.150
11	SSH – I	6.5	1:100	10^5	0.025	10^7	0.150
12	SSH + YE – I	6.5	1:100	10^5	0.025	10^7	0.150

[a] Ratio of *S. cerevisiae*/*L. pentosus* was obtained by dividing the cell viable number per milliliter (CFU mL^{-1}) of each strain.

3.2.4 Analytical methods

3.2.4.1 Metabolites determination

The fermentation samples were centrifuged (2 min at 13,200 rpm) in order to remove the cells, and the supernatant was stored at -20ºC.

The concentrations of glycerol, xylitol, acetate, ethanol, HMF and furfural were determined by HPLC. The sample injection volume was 10 µL, and the compounds were separated using two Aminex HPX-87H resin-based columns (Bio-Rad, Hercules, CA, USA) preceded by a Micro-Guard Cation-H guard column (Bio-Rad). The separation was performed at 45ºC with 5 mM H_2SO_4 at 0.6 mL min^{-1} as mobile phase. The HPLC equipment consisted of a Waters HPLC pump, autosampler (Waters 717 plus), Waters column heater module, Shimadzu refractive index detector RID-10A and Shimadzu spectrophotometer detector UV-Vis SPD-6AV.

The concentrations of xylose, glucose, galactose and mannose were also determined by HPLC (Waters) using an Aminex HPX-87P resin-based column (Bio-Rad) preceded by a Micro-Guard Cation-H and Anion CO_3^- guard columns (Bio-Rad). Separation was performed at 60ºC, with MilliQ H_2O at 0.6 mL min^{-1} as mobile phase. The HPLC equipment consisted of a Waters HPLC pump, autosampler (Waters 717 plus), Waters column heater module, refractive index detector (Waters 2410) and absorbance detector (Waters 2487 Dual λ).

3.2.4.2 Cell viability

The cell viability was evaluated as colony-forming units per milliliter (CFU mL^{-1}). CFU determination was performed by serial dilutions in 0.9 % (v/v) NaCl, in triplicate. When the desired dilution factor was achieved, 100 µL were spread on YNB or MRS Agar plates. For *S. cerevisiae* cells counting, YNB Agar plates were incubated at 30ºC for 48 h. For *L. pentosus*, MRS Agar plates (supplied with 4 mg L^{-1} of cyclohexamide when co-cultured with yeast) were incubated anaerobically (Anaerocult A, Merck, Darmstadt, Germany) at 37ºC for 48 h.

Chapter 4

Results and discussion

4.1 Evolutionary engineering study

Currently, the evolutionary engineering is a well-established approach for the improvement of microorganisms' performance. However, most of the published studies report the use of *S. cerevisiae*, a model yeast organism, which is not able to consume pentose sugars in its wild type. Lignocellulosic feedstocks have high content in pentoses, mainly xylose, and thus the use of pentose-metabolising microorganisms combined with the evolutionary engineering methodology may be a good approach to improve simultaneously the resistance towards lignocellulosic inhibitors and fermentation performance. *S. stipitis*, a native xylose-consuming yeast, was adapted to increasing concentrations of HSSL (0-60 % v/v) during 68 days in a continuous stirred tank reactor, and an adapted population was obtained in the final of this process [98]. From that population, 10 clones were isolated, cultivated under non-selective conditions during ten sequential transfers, and their phenotypic stability was then investigated. The obtained data is presented and discussed below.

4.1.1 Screening trials

To assess the phenotypic stability of the isolated clones, their fermentation performance in 60 % (v/v) of HSSL was compared to that of the parental strain and adapted population. The results of growth rate, ethanol production and substrate uptake rates were determined and are shown in Figure 7. The parental strain *S. stipitis* Y-NRRL 7124 presented the lowest growth rate of all assays (0.116 ± 0.004 h^{-1}), and an ethanol productivity of 0.025 ± 0.003 g L^{-1} h^{-1}. The low growth rate is a consequence of *S. stipitis* cultivation in high HSSL concentrations, suggesting the major role of lignocellulosic inhibitors in yeast growth inhibition. Previous studies conducted by Xavier et al. [99] showed that cultivation in increasing HSSL concentrations led to a decrease in the growth rate, ethanol production and sugar consumption by the same yeast strain. Considering substrate consumption, the sugars (glucose, xylose) and acetate were used as carbon sources by the parental strain. The hexose sugar glucose was depleted after 41 h of fermentation, similarly to the adapted population and isolated clones (data not shown). The xylose consumption rate was considerably lower for the parental strain (0.496 ± 0.003 g L^{-1} h^{-1}), suggesting that the adaptation

approach led to an improved pentose sugars uptake. Interestingly, acetate was found to be consumed at a low rate (0.078 ± 0.004 g L^{-1} h^{-1}) if compared to the remaining trials.

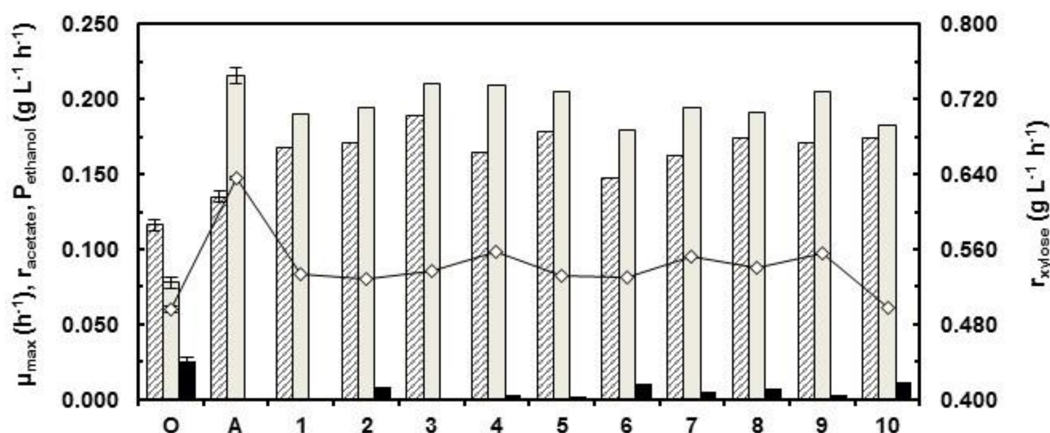

Figure 7. Fermentation performance of the parental strain *S. stipitis* Y-NRRL 7124 (O), adapted population (A) and isolated clones (1-10), named C1-10, cultivated in HSSL/CDM (60/40 % v/v). The maximum growth rate (μ_{max}) (hatched bar), acetate uptake rate ($r_{acetate}$) (white bar), ethanol productivity ($P_{ethanol}$) (black bar), and xylose uptake rate (r_{xylose}) (—◇—) were determined after 41 h of fermentation.

The adapted population showed an improved growth rate (0.135 ± 0.004 h^{-1}) and substrate consumption rate than the parental strain, which is probably due to a higher tolerance towards HSSL derived-inhibitors. While the xylose consumption was slightly faster (0.635 ± 0.00 g L^{-1} h^{-1}), the acetate uptake rate (0.216 ± 0.006 g L^{-1} h^{-1}) was increased 2-fold after the adaptation process. However, no ethanol was produced, in contrast to the parental strain. The ethanol production by *S. stipitis* is oxygen-dependent, reaching its maximum under microaerophilic conditions [13]. Since the screening trials were performed under fully aerobic conditions, this is a possible reason for the low ethanol production rates obtained.

The isolated clones showed a similar and stable fermentation profile among themselves. Besides, their fermentation performances were improved if compared to the parental strain, suggesting that the adapted population is considerably stable in the absence of selective pressure. Comparing with the adapted population, a slightly higher growth rate and similar acetate consumption rate were achieved. However, a noticeable decrease in the xylose uptake rate was displayed by the isolated clones. This finding was unexpected since the clones were cultivated in xylose-rich medium to assess their stability. As xylose is the major carbon source of HSSL, the clone showing higher xylose consumption rate (0.558 g L^{-1} h^{-1}) was named isolate C4 and selected for characterisation studies (Section 4.1.2). The ethanol yields obtained during the screening trials were calculated and are shown in Figure 8.

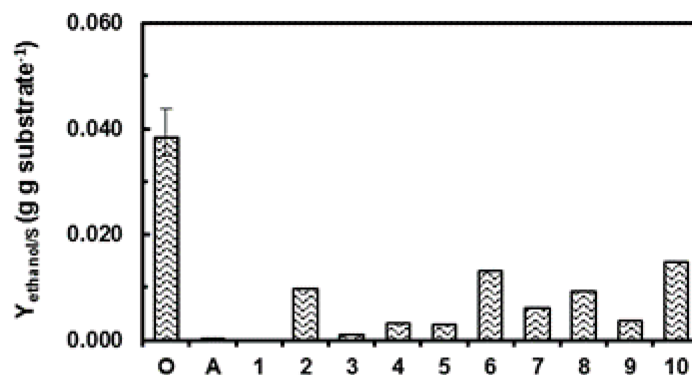

Figure 8. Ethanol yields of the parental strain *S. stipitis* Y-NRRL 7124 (O), adapted population (A) and isolated clones (1-10), named C1-10, obtained after 41 h of culture in HSSL/CDM (60/40 % v/v). Glucose, xylose and acetate were considered as substrate.

The parental strain was able to achieve the highest ethanol yield of all performed fermentations (0.038 ± 0.001 g ethanol g substrate^{-1}). The adapted population did not produce ethanol after 41 h, and thus a null ethanol yield was registered. Curiously, the isolated clones showed values of ethanol yield in between those obtained with the parental strain and adapted population. The isolate C4 showed an ethanol yield of 0.03 g g substrate^{-1}. In fact, the adapted population was expected to have the highest ethanol yield, being followed by the isolated clones and parental strain. Some factors can explain the obtained data. The evolutionary engineering approach aimed to develop an evolved *S. stipitis* strain showing higher tolerance towards lignocellulosic inhibitors, and thus aerobic conditions were used during that stage to optimise yeast growth. As a result, a microorganism with metabolism less directed to ethanol production could have been developed. To evaluate these and other factors, the highest xylose-consuming clone (isolate C4) was further characterised.

4.1.2 Characterisation of isolate C4

The isolate C4 was cultivated aerobically at bioreactor level in 60 % (v/v) of HSSL, and the effect of short-term adaptation was investigated. This effect was evaluated by pre-culturing the isolated clone in presence or absence of HSSL.

When the isolate C4 was pre-cultivated in HSSL, no lag phase was detected and a maximum growth rate of 0.083 h^{-1} was achieved (Figure 9). In the end of fermentation, a cell dry weight of 2.34 ± 0.05 g L^{-1} and a viable cell number of 2.78 x 10^8 ± 1.53 x 10^6 CFU mL^{-1} were obtained. Considering sugar consumption, the hexose glucose was depleted after 16.5 h of fermentation, while the pentose xylose only started to be consumed by the yeast afterwards. In the end of fermentation, 12.1 g L^{-1} of xylose were consumed, corresponding to an uptake rate of 0.192 g L^{-1} h^{-1}. Like in the screening trials, acetate consumption was also verified (4.4 g L^{-1} after 63 h), and a consumption rate of 0.069 g L^{-1} h^{-1} was achieved.

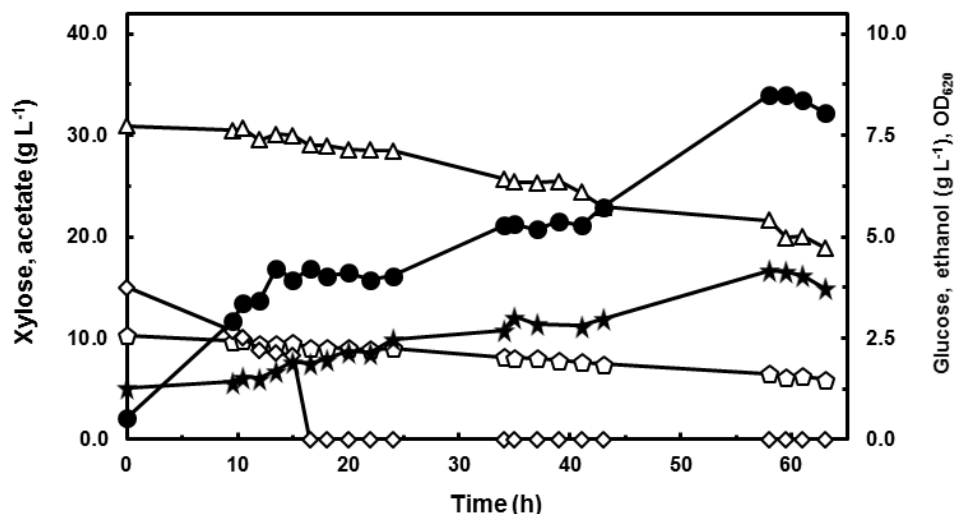

Figure 9. Fermentation performance of the pre-adapted isolate C4 cultivated in HSSL/CDM (60/40 % v/v). The intermediate step was performed in the same conditions. Symbols: OD_{620} (●); glucose (◇); xylose (△); acetate (⬠); ethanol (★).

The ethanol production reached its maximum after 58 h of fermentation (4.18 g L^{-1}), leading to a yield of 0.172 g ethanol g substrate^{-1}. In the end of fermentation, the ethanol uptake rate corresponded to 0.039 g L^{-1} h^{-1}. The low ethanol production might be due to the aerobic conditions used, which do not correspond to yeast optimal oxygen concentrations for ethanol fermentation.

The short-term adaptation is a process that is responsible by an improvement on ethanol fermentation from lignocellulose feedstocks, since it aims to improve yeast tolerance towards inhibitory compounds. To investigate the fermentation performance of isolate C4 in the absence of short-term adaptation, the clone was pre-cultivated in 100 % (v/v) of CDM and the remaining parameters were not changed (Figure 10). The isolate C4 showed a maximum growth rate of 0.117 h^{-1} and a lag phase of about 10 h, suggesting that the absence of a short-term adaptation step leads to a slower yeast growth and fermentation. At the end of the fermentation, a cell dry weight of 3.00 ± 0.06 g L^{-1} and a viable cell number of 2.84 x 10^8 ± 2.07 x 10^7 CFU mL^{-1} were achieved. These values are identical to the previous experiment, and are further discussed later. Regarding sugar consumption, glucose was depleted after 18 h of fermentation, while xylose was only partially consumed (11.3 g L^{-1} after 63 h) at a rate of 0.180 g L^{-1} h^{-1}. Besides, 6.3 g L^{-1} of the lignocellulose inhibitor acetate were consumed after 63 h of fermentation at a rate of 0.101 g L^{-1} h^{-1}. The ethanol production followed the yeast growth, reaching its maximum at 63 h (4.51 g L^{-1}). The ethanol production rate was equal to 0.42 g L^{-1} h^{-1}, while the ethanol yield was 0.125 g g substrate^{-1}.

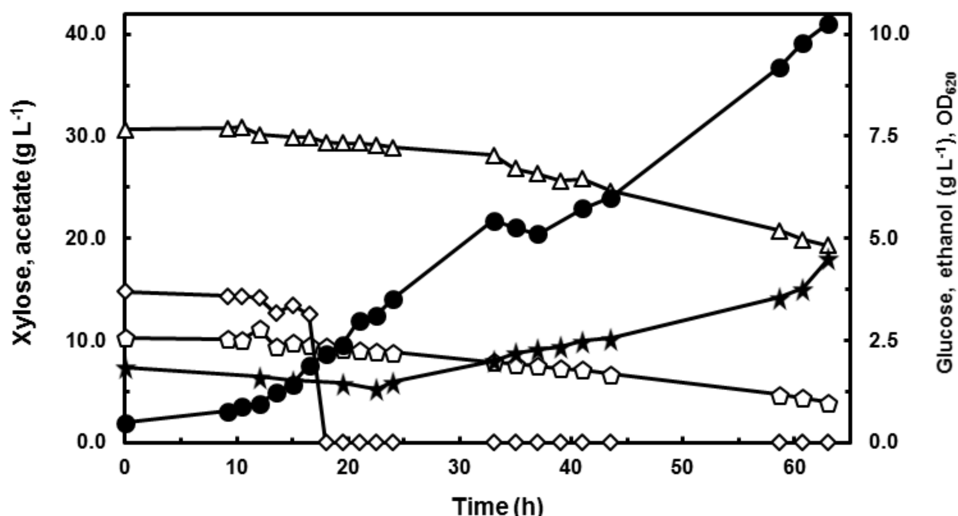

Figure 10. Fermentation performance of the non-pre-adapted isolate C4 cultivated in HSSL/CDM (60/40 % v/v). The intermediate step was performed in 100 % (v/v) of CDM. Symbols: OD_{620} (●); glucose (◇); xylose (△); acetate (⬠); ethanol (★).

4.1.2.1 Overall comparison

The summary of the results from all the experiments performed to characterise the isolate C4 in this work is shown in Table 13. In the presence of a pre-adaptation step, the isolate C4 showed a lower maximum growth rate (0.083 h^{-1}) than in its absence (0.117 h^{-1}). However, the opposite was verified for the final cell dry weight, while identical viable cell number results were achieved at the end of the fermentation. This suggests that the isolate C4, obtained from an evolutionary engineering approach, is robust towards lignocellulosic inhibitors, and thus the occurrence of a pre-adaptation step to improve the yeast fermentation performance is not mandatory.

Considering substrate consumption, the effect of pre-adaptation was not evident on sugar uptake rate in the end of fermentation. However, the consumption of acetate was substantially different and surprisingly higher in the absence of short-term adaptation (0.101 g L^{-1} h^{-1}). The ethanol maximum concentration and ethanol productivity were very similar with and without pre-adaptation step. However, when the isolate C4 was pre-adapted to HSSL, a higher ethanol yield was achieved (0.172 g g $substrate^{-1}$), suggesting that the yeast was able to produce more ethanol consuming less substrate under these conditions. Considering all the above factors, the pre-adaptation step in HSSL did not improve in general the fermentation performance of the isolate C4, suggesting that the long-term adaptation exploited the maximum capacity of the strain.

Table 13. Fermentation parameters of isolate C4 cultivation with and without a pre-adaptation step.

	Isolate C4	
Parameters	Pre-adapted	Non-pre-adapted
Yeast growth		
Maximum growth rate (h^{-1})	0.083	0.117
Final cell dry weight ($g\ L^{-1}$)	2.34 ± 0.05	3.00 ± 0.06
Final viable cell number ($CFU\ mL^{-1}$)	$2.78 \times 10^8 \pm 1.53 \times 10^6$	$2.84 \times 10^8 \pm 2.07 \times 10^7$
Substrate consumption		
$r_{glucose}$ ($g\ L^{-1}\ h^{-1}$)[a]	0.059	0.059
r_{xylose} ($g\ L^{-1}\ h^{-1}$)[a]	0.192	0.180
$r_{acetate}$ ($g\ L^{-1}\ h^{-1}$)[a]	0.069	0.101
Ethanol production		
$P_{ethanol}$ ($g\ L^{-1}\ h^{-1}$)[b]	0.039	0.042
$Ethanol_{max}$ ($g\ L^{-1}$)[c]	4.18	4.51
$Y_{ethanol/S}$ ($g\ g\ substrate^{-1}$)[d]	0.172	0.125

[a] Uptake rate of individual substrates (glucose, xylose, acetate) at the end of fermentation.
[b] Ethanol productivity at the end of fermentation.
[c] Maximum ethanol concentration during fermentation.
[d] Ethanol yield calculated for maximum ethanol concentration considering glucose, xylose and acetate as substrate.

When the parental strain was cultivated with and without a pre-adaptation step under the same conditions, completely different results emerged [98]. The cell pre-adaptation led to higher growth, substrate consumption and ethanol production. Since the parental strain was less tolerant towards the toxic compounds present in HSSL than the isolate C4, this step induced probably an adaptive response of *S. stipitis* toward inhibitors. Besides, all the results obtained with the parental strain were lower than those obtained with the isolate C4, showing the positive effect of evolutionary engineering on *S. stipitis* fermentation performance.

4.2 Microbial contamination study

4.2.1 Initial trials using SSSL

Microbial contamination poses serious economic consequences to the industrial ethanol production, and research based on the reasons triggering this occurrence has been increasing. Therefore, the predominating bacterial contaminant of an industrial ethanol production plant based on SSSL fermentation was isolated and identified as *L. pentosus* [7]. In the present study, the viabilities of *S. cerevisiae* and *L. pentosus* alone and together during an anaerobic fermentative process were investigated. Both microorganisms were not able to grow or maintain their viability in the presence of undiluted SSSL at pH 5.0 from a mixture of spruce and pine (data not shown). This fact was unexpected since previous studies showed that the same industrial yeast strain (*S. cerevisiae* TMB3500) was an efficient ethanol producer (19.5 g L^{-1} after 50 h of batch fermentation) under the same conditions as the ones tested in this work [3]. However, the feedstock was stored at 4ºC for more than 4 years, and therefore its chemical composition might have changed. SSSL analysis revealed that the content of the major sugars, acetate, HMF and furfural did not change during storage time. This result suggests that other chemical compounds with inhibitory capacity that could not be detected by HPLC (e.g. phenolics and other lignosulphonate-degradation products) may be the reason for the observed non-growth.
Due to this challenge, further fermentations were performed using a simulated synthetic hydrolysate (SSH) instead, and its content in sugars and inhibitors was adjusted to mimic SSSL average composition.

4.2.2 Viability of yeast and bacterial contaminant in SSH

The industrial yeast *S. cerevisiae* TMB3500 was cultivated anaerobically in SSH at pH 5.0 in order to reproduce the process conditions used in the ethanol plants. The effect of yeast extract addition on the yeast viability and performance was investigated (Figure 11). These results were used as a control for comparison with co-cultivation experiments.

Figure 11A shows that *S. cerevisiae* could grow in the absence of yeast extract as the CFU number was increased by 0.61 ± 0.03 log units after 48 h of fermentation. The hexose sugars (mannose, glucose and galactose) were consumed by the yeast. In contrast, xylose was not consumed since *S. cerevisiae* lacks a functional xylose assimilation pathway [103]. During all the fermentations performed in this study, no acetate was consumed (data not shown), and thus product yields were calculated considering only sugars as substrate. Besides, no lactate was produced. A maximum ethanol titer of about 19 g L^{-1} was achieved and the productivity was 0.37 ± 0.02 g L^{-1} h^{-1}, while the ethanol yield was 0.47 ± 0.04 g g consumed sugars^{-1}. Moreover, ethanol production followed yeast growth, since more than 16 g L^{-1} were produced after 10 h of fermentation.

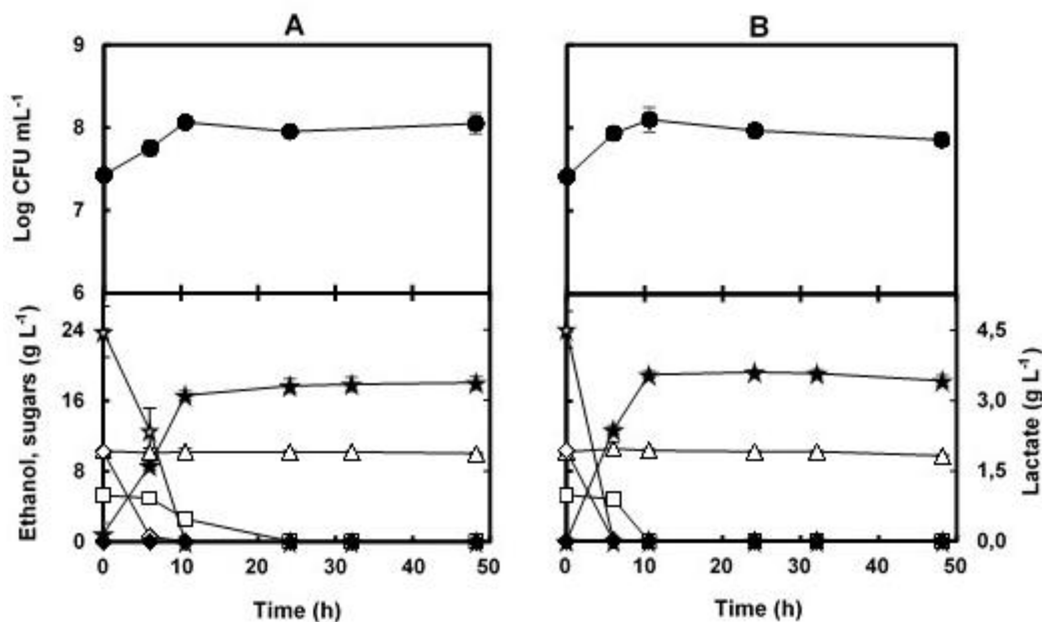

Figure 11. Cell viability (upper panels), sugars consumption and product formation (lower panels) during cultivation of *S. cerevisiae* TMB3500 in SSH, pH 5.0 without (A) and with (B) yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed. Symbols: *S. cerevisiae* CFU (●); mannose (☆); glucose (◇); xylose (△); galactose (□); ethanol (★); lactate (◆).

When yeast extract was added to the fermentation medium, a slight improvement on yeast viability, ethanol production and sugar consumption was observed during the first 10 h of fermentation (Figure 11B). The maximum CFU number was achieved at 10 h, and then the yeast maintained its viability approximately constant. After 48 h of fermentation, the CFU increased by 0.44 ± 0.00 log units. The consumption rate of the hexoses was improved during the first hours, while no xylose was metabolised. Besides, around 19 g L^{-1} of ethanol were produced on the first 10 h, and its production rate was found to be 0.39 ± 0.01 g L^{-1} h^{-1}. In the end of fermentation, the ethanol yield was 0.47 ± 0.01 g g consumed $sugars^{-1}$.

In parallel, *L. pentosus*, the bacterial contaminant of a SSSL ethanol plant [7], was also cultivated anaerobically in SSH at pH 5.0, and the effect of the presence of yeast extract on its viability and performance was also investigated (Figure 12). These results were also used as a control for comparison with co-cultivation experiments.

In the absence of yeast extract, the CFU decreased by 0.23 ± 0.06 log units after 48 h of fermentation (Figure 12A). Lactate production occurred at a slow rate (0.01 ± 0.00 g L^{-1} h^{-1}) and a maximum titer of about 0.4 g L^{-1} was achieved. After 48 h of fermentation, the lactate yield was found to be 0.25 ± 0.23 g g consumed $sugars^{-1}$. Besides, no ethanol was produced and only a residual sugar consumption was observed.

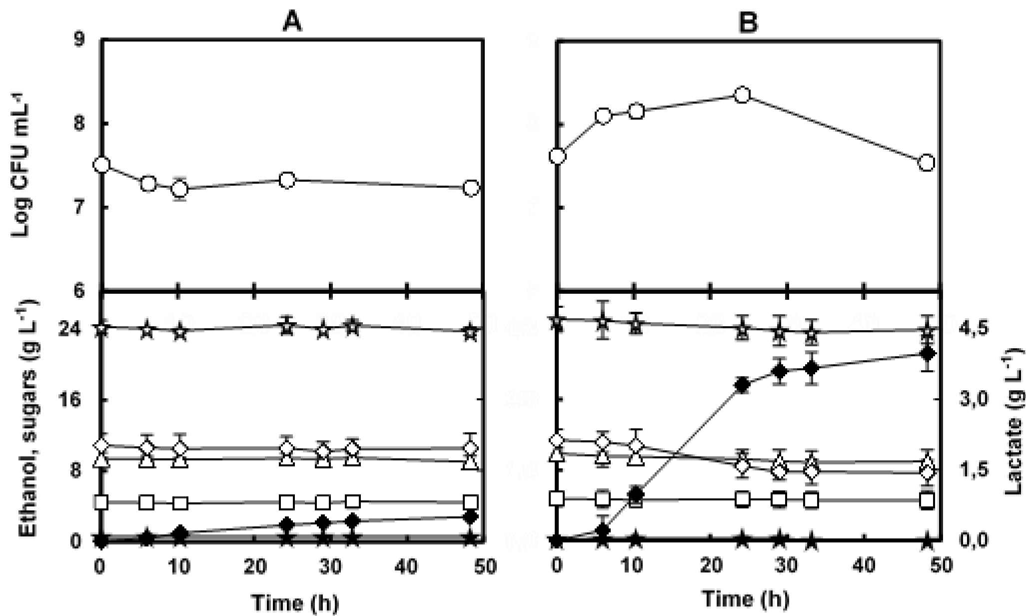

Figure 12. Cell viability (upper panels), sugars consumption and product formation (lower panels) during cultivation of *L. pentosus* in SSH, pH 5.0 without (A) and with (B) yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed. Symbols: *L. pentosus* CFU (○); mannose (☆); glucose (◇); xylose (△); galactose (□); ethanol (★); lactate (◆).

When yeast extract was added to SSH, the CFU increased during the first 24 h of fermentation, but a decrease was observed afterwards (Figure 12B). After 48 h, bacterial viability increased by 0.26 ± 0.48 log units. Xylose, mannose and galactose were not used, while glucose was only partially consumed, leading to a lactate production rate of 0.09 ± 0.01 g L^{-1} h^{-1} and a maximum concentration of about 4.5 g L^{-1}. The lactate yield was 0.78 ± 0.18 g g consumed $sugars^{-1}$ in the end of fermentation.

Therefore, the addition of yeast extract to SSH had a positive effect on bacterial viability and lactate formation. During the experiments with *L. pentosus*, the growth rate was lower than that of the yeast and no ethanol or acetate production was detected. Besides, this bacterium was also cultivated anaerobically in MRS (data not shown), a medium regularly used for *Lactobacillus* sp., and only lactate was produced (above 10 g L^{-1}). Since *L. pentosus* strain did not produce ethanol or acetate, homolactic fermentation was observed in all the referred conditions. However, *L. pentosus* is considered a facultatively heterofermentative organism with the ability to consume, among other sugars, hexoses (D-glucose, D-mannose, galactose) by the Embden-Mayerhof-Parnas pathway and pentoses (D-xylose) by the phosphoketolase pathway. As a result, only lactate is produced from hexoses (homofermentation), while lactate and ethanol or acetate are generated from pentoses (heterofermentation) [104, 105]. This could be a possible explanation for the absence of xylose consumption by *L. pentosus*, since only lactate production was detected under the studied conditions. The heterofermentative metabolism of *L. plantarum* is known to be activated by glucose depletion under anaerobicity, but nothing is known about its closely-related

organism *L. pentosus* [106]. Nevertheless, a low consumption of glucose, mannose and galactose by the bacterial strain was observed both in the presence and absence of yeast extract. As SSH composition was formulated to maximise *S. cerevisiae* performance, some essential nutrients for *L. pentosus* growth might be missing. For instance, *Lactobacillus* sp. require fair amounts of organic nitrogen and growth-promoting substances, and their absence can affect their viability and performance [107]. Alternatively, lactate production can have inhibited further sugar consumption [108].

4.2.3 Co-cultivations of yeast and bacterial contaminant

The major sources of microbial contamination in the ethanol plants are the raw-material and the water used during the process [85, 109]. Since there were no previous studies about the effective ratios of *S. cerevisiae*/*L. pentosus*, co-culture experiments were performed and different inoculation ratios of yeast/bacteria were tested in order to investigate the effect of *L. pentosus* on the *S. cerevisiae* viability and ethanol production. Firstly, SSH medium at pH 5.0 was inoculated with yeast and bacteria at a ratio of 1:1, corresponding to 10^7 CFU mL^{-1} of each microorganism at the starting point of fermentation (Figure 13).

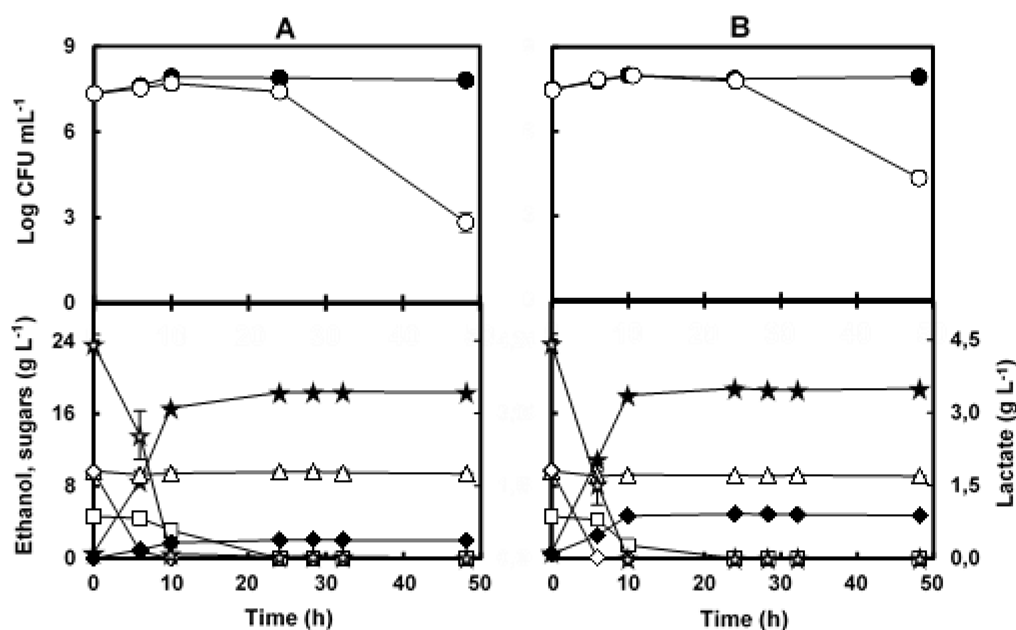

Figure 13. Cell viability (upper panels), sugars consumption and product formation (lower panels) during co-cultivation of *S. cerevisiae* TMB3500 and *L. pentosus* at a ratio of 1:1 in SSH, pH 5.0 without (A) and with (B) yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed. Symbols: *S. cerevisiae* CFU (●); *L. pentosus* CFU (○); mannose (✩); glucose (◇); xylose (△); galactose (□); ethanol (★); lactate (◆).

In the absence of yeast extract, both the viable cell number of yeast and bacteria increased during the first 10 h (Figure 13A). However, *S. cerevisiae* and *L. pentosus* showed a different behaviour

after this point. In contrast to the yeast, where the CFU were approximately constant, the bacterial viable cells substantially decreased. After 48 h of fermentation, the CFU of the yeast and bacteria increased 0.32 ± 0.21 log units and decreased 6.00 ± 2.09 log units, respectively. The sugar consumption and product formation followed the microbial growth: glucose and mannose were depleted after 10 h, being followed by galactose, which was consumed at a lower rate. On the other hand, xylose was unexpectedly not consumed after glucose and galactose depletion. Lactate and ethanol productivity was higher in the beginning of fermentation, which matches with the period when the sugar consumption occurred at a higher rate. Lactate maximum titer was reached after 30 h (approximately 0.4 g L^{-1}) and the maximum ethanol concentration was about 18.5 g L^{-1}. Lactate and ethanol productivities were 0.01 ± 0.00 g L^{-1} h^{-1} and 0.37 ± 0.00 g L^{-1} h^{-1}, respectively. Besides, the ethanol yield was found to be 0.47 ± 0.01 g g consumed $sugars^{-1}$, while the lactate yield was 0.01 ± 0.00 g g consumed $sugars^{-1}$. Gold and colleagues [110] have found that the growth of *L. pentosus* ATCC 8041 in MRS broth containing glucose or xylose as a sugar source is negatively affected by increasing concentrations of ethanol, and at concentrations of about 63 g ethanol L^{-1}, almost no growth was observed after 48 h of cultivation. Therefore, apart from other factors, the ethanol concentrations achieved during the performed fermentations could have been responsible by the high decrease of CFU number of *L. pentosus*.

Higher lactate production was obtained under the presence of yeast extract (Figure 13B). However, *L. pentosus* was unable to outcompete *S. cerevisiae*. The variation in CFU number of both yeast and bacteria was very similar to the one observed without yeast extract. In the end of fermentative process, bacterial viable cell number decreased by 5.30 ± 3.06 log units and yeast viable cell number increased by 0.33 ± 0.15 log units. The sugars were consumed faster than in the absence of yeast extract, and thus lactate titer was also increased. Whilst lactate productivity (0.02 ± 0.00 g L^{-1} h^{-1}) was positively affected by the addition of yeast extract and a lactate concentration of about 0.8 g L^{-1} was achieved, ethanol productivity (0.38 ± 0.00 g L^{-1} h^{-1}) and maximum ethanol titer (about 19 g L^{-1}) were not affected by the addition of yeast extract. After 48 h of fermentation, the ethanol and lactate yields were 0.47 ± 0.00 g g consumed $sugars^{-1}$ and 0.02 ± 0.00 g g consumed $sugars^{-1}$, respectively.

The addition of yeast extract to SSH had a positive effect on bacterial viability and lactate formation. However, both with and without the addition of yeast extract to SSH at pH 5.0 the bacteria could not compete with *S. cerevisiae*. In fact, *S. cerevisia*e growth and product formation was faster than the one of *L. pentosus*, revealing that also sugar consumption was quicker. Thus, when present in the same amounts, bacteria did not inhibit yeast, and therefore other possibilities were investigated.

The inoculation ratio of yeast/bacteria in SSH medium with and without yeast extract at pH 5.0 was increased to 1:100 in order to evaluate if *S. cerevisiae* starting from a more unfavourable condition would still outcompete *L. pentosus*. The results of this experiment are shown in Figure 14.

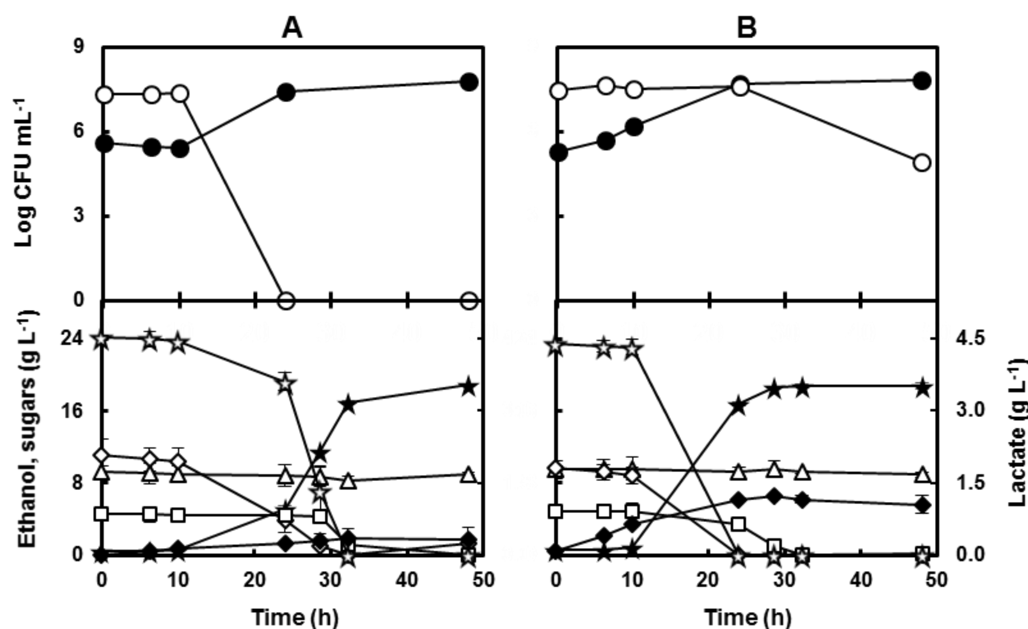

Figure 14. Cell viability (upper panels), sugars consumption and product formation (lower panels) during co-cultivation of *S. cerevisiae* TMB3500 and *L. pentosus* at a ratio of 1:100 in SSH, pH 5.0 without (A) and with (B) yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed. Symbols: *S. cerevisiae* CFU (●); *L. pentosus* CFU (○); mannose (✩); glucose (◇); xylose (△); galactose (□); ethanol (★); lactate (◆).

In the experiment without yeast extract, CFU of *S. cerevisiae* increased by 2.22 ± 0.04 log units, while CFU of *L. pentosus* decreased by 5.07 ± 3.18 log units (Figure 14A). Comparing to the previous results, the yeast growth rate was lower in the first 10 h, which can be explained by the lower amount of inoculated cells (10^5 CFU mL^{-1}). However, the lactic acid bacteria strain was still unable to take this condition as a competitive advantage during this period, since no growth and only residual levels of lactate were detected. Sugar consumption was very low in the first hours of fermentation and glucose was depleted after 30 h. At this point, yeast achieved the maximum CFU and ethanol titer that were stable until 48 h of fermentation. More than 18 g ethanol L^{-1} were produced during this experiment at a rate of 0.37 ± 0.01 g L^{-1} h^{-1}, while 0.4 g lactate L^{-1} were produced at a rate of 0.01 ± 0.00 g L^{-1} h^{-1}. In the end of the fermentative process, the ethanol and lactate yields were found to be 0.47 ± 0.01 g g consumed $sugars^{-1}$ and 0.01 ± 0.00 g g consumed $sugars^{-1}$, respectively.

When yeast extract was present, *S. cerevisiae* presented higher growth in the first stages of fermentation, and *L. pentosus* CFU number was held constant until 24 h of fermentation (Figure 14B). After 48 h, the yeast viable number increased by 2.40 ± 0.21 log units and the bacterial viable number was decreased by 5.01 ± 3.49 log units. As in the absence of yeast extract, sugar consumption was low during the first 10 h, however the maximum ethanol and lactate production was obtained earlier (around 30 h). A similar maximum ethanol concentration, production rate and yield were achieved (about 18 g ethanol L^{-1}, 0.38 ± 0.00 g L^{-1} h^{-1} and 0.49 ± 0.01 g g

consumed sugars^{-1}) as compared to the experiment without yeast extract. Nevertheless, more lactate was formed (about 1.4 g L^{-1}) at a rate of 0.04 ± 0.03 g L^{-1} h^{-1}). The lactate yield was 0.05 ± 0.04 g g consumed sugars^{-1} after 48 h.

Like in the previous experiments, a positive impact in bacterial viability and product formation was observed when yeast extract was present in SSH, but no effect on *S. cerevisiae* was detected after 48 h of fermentation. Therefore, a higher inoculation of bacteria under the presence of inhibitors and pH 5.0 did not play any negative effect on the ethanol production. The delay on the yeast growth and production of ethanol was probably due to a lower starting CFU.

Considering this, other parameters can play simultaneously a major role on bacterial viability improvement and yeast viability inhibition. The presence of lignocellulosic inhibitors affect not only *S. cerevisiae*, but also *L. pentosus* fermentation performance. Therefore, it may be possible that in the ethanol plants microbial contaminants could resist to inhibitors and only reproduce when the fermentation medium is detoxified by the ethanol-producing strain. The effect of the absence of microbial inhibitors was then investigated. For that, co-culture experiments inoculated at a ratio yeast/bacteria of 1:1 were performed using SSH without inhibitors at pH 5.0 (Figure 15A). Besides, the effect of the presence of yeast extract was also studied (Figure 15B).

In the absence of yeast extract, no viable cells of *L. pentosus* were detected after 10 h of fermentation without lignocellulose inhibitors (Figure 15A). *S. cerevisiae* demonstrated to have a faster sugar consumption and production formation than the bacteria in the previous tests. Thus, without inhibitors these parameters are expected to be even higher, which will not favour *L. pentosus* viability during the fermentation. After 48 h of fermentation, both the yeast and bacterial CFU decreased (0.99 ± 0.15 and 7.32 ± 0.20 log units, respectively) due to the quick sugar depletion. Both glucose, mannose and galactose were consumed in 10 h, while xylose was not metabolised. The ethanol production was slightly lower if compared to the previous obtained levels (around 16 g L^{-1}) at a rate of 0.33 ± 0.02 g L^{-1} h^{-1}. However, low amounts of lactate were produced (about 0.2 g L^{-1}) at a rate very close to zero, which can be explained by the fast death of *L. pentosus* cells. In the end of fermentation, the ethanol and lactate yields were 0.40 ± 0.00 g g consumed sugars^{-1} and 0.00 ± 0.01 g g consumed sugars^{-1}, respectively.

The addition of yeast extract to the medium resulted in a slower decrease of bacterial CFU (Figure 15B). However, as in the previous trial, the viability of *S. cerevisiae* and *L. pentosus* decreased by 0.28 ± 0.01 and 7.71 ± 0.61 log units, respectively. Comparing with the previous experiment, a faster sugar consumption and a slightly improved ethanol production were denoted. Ethanol was found at concentrations of about 17 g L^{-1}, and was produced at a rate of 0.35 ± 0.01 g L^{-1} h^{-1}, while lactate was found at concentrations below 0.3 g L^{-1} and produced at a very slow rate. After 48 h of fermentation, the ethanol yield was 0.43 ± 0.01 g g consumed sugars^{-1}, while the lactate yield was 0.00 ± 0.00 g g consumed sugars^{-1}.

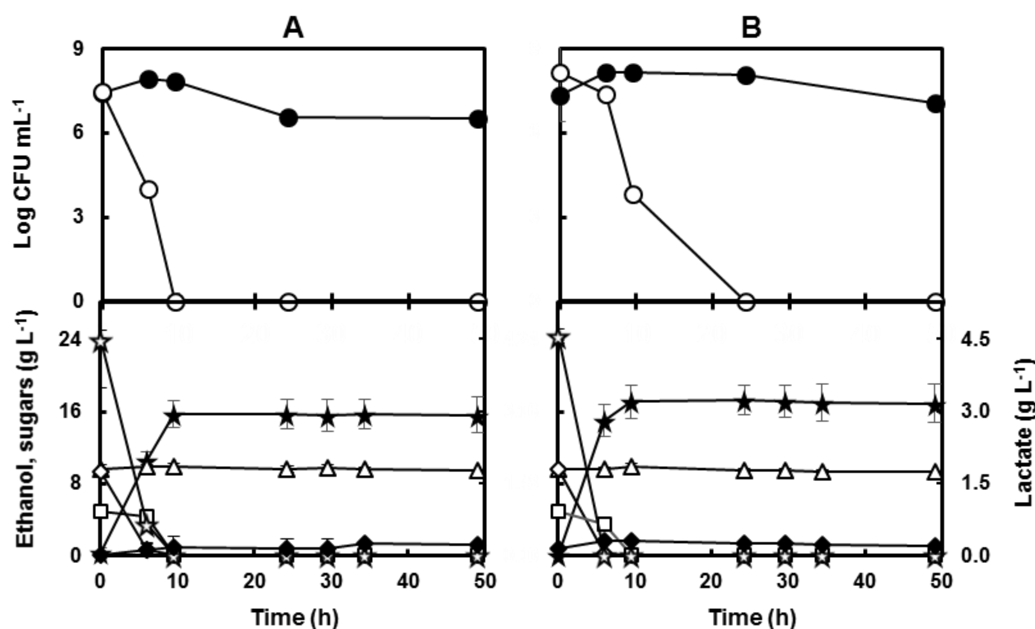

Figure 15. Cell viability (upper panels), sugars consumption and product formation (lower panels) during co-cultivation of *S. cerevisiae* TMB3500 and *L. pentosus* at a ratio of 1:1 in SSH without inhibitors, pH 5.0 in the absence (A) and presence (B) of yeast extract. Values showed are a representative profile of each fermentation, and biological duplicates were performed. Symbols: *S. cerevisiae* CFU (●); *L. pentosus* CFU (O); mannose (✩); glucose (◇); xylose (△); galactose (□); ethanol (★); lactate (◆).

The absence of inhibitors in SSH did not lead to an improvement in lactate production and *L. pentosus* viability. The addition of yeast extract improved the bacterial viability, but the amount of lactate produced was too low to produce any effect on *S. cerevisiae* viability.

The absence of inhibitors in SSH and the higher inoculation level of *L. pentosus* were not sufficient to affect the ethanol production and *S. cerevisiae* viability. Therefore, SSH can be missing some essential nutrients for the bacterial strain. Besides, the initial sugar concentration of SSH may be too high for bacteria, affecting negatively its performance. The SSH medium contains 50 g sugars L^{-1}, and thus *L. pentosus* may be inhibited by such high concentrations. Narendranath et al. [111] reported that the specific growth rates of *Lactobacillus* sp., namely *L. pentosus*, decreased with higher sugar content in liquid growth medium, and this was due to the osmotic stress exerted by the sugars on bacteria. Besides, pH and temperature are two factors that play a key role on *Lactobacillus* sp. performance. In fact, it is a possibility, since the fermentation medium and conditions (pH, temperature) were formulated to improve yeast performance and to mimic the composition of SSSL. The optimal temperature of *Lactobacillus* sp. is around 37ºC, while the optimum pH is about 6.5 [112]. However, all the fermentations were performed at 30ºC to mimic the industrial conditions.

Therefore, in order to verify if *L. pentosus* could overtake *S. cerevisiae*, more favourable conditions for bacteria were chosen. Co-culture experiments inoculated at a ratio of yeast/bacteria

1:100 were performed using SSH without inhibitors at pH 6.5. The effect of the addition of yeast extract to the fermentation medium was also investigated (Figure 16).

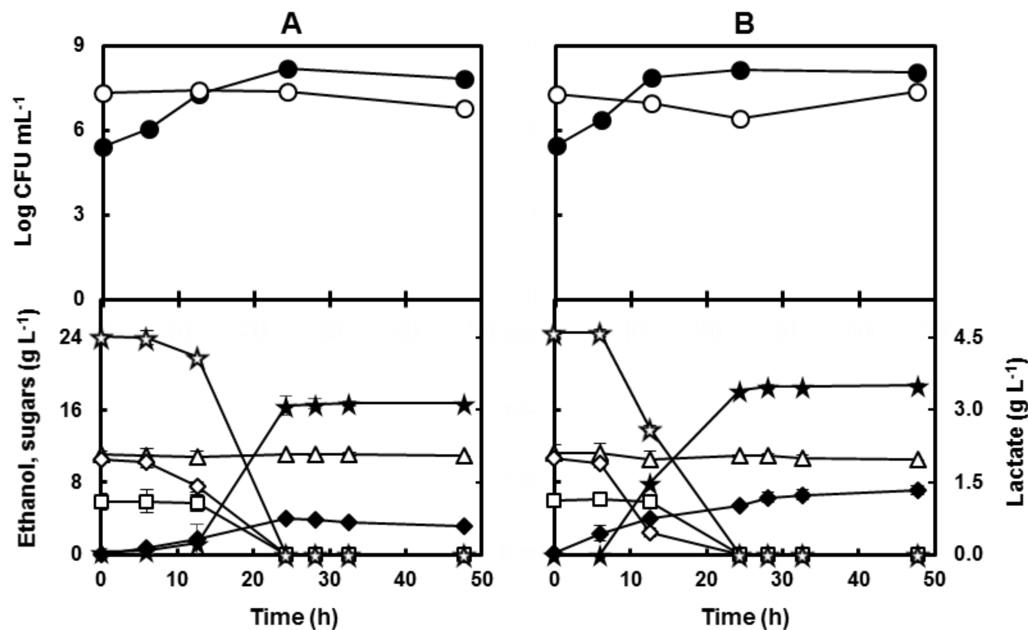

Figure 16. Cell viability during co-cultivation of *S. cerevisiae* TMB3500 and *L. pentosus* at a ratio of 1:100 in SSH without inhibitors, pH 6.5 in the absence (A) and presence (B) of yeast extract. Values showed are a representative profile of each fermentation. Symbols: *S. cerevisiae* CFU (●); *L. pentosus* CFU (○); mannose (☆); glucose (◇); xylose (△); galactose (□); ethanol (★); lactate (◆).

Interestingly, the change of pH led to an improvement of bacterial CFU, but *L. pentosus* was again unable to outcompete yeast (Figure 12). In the absence of yeast extract, *S. cerevisiae* viable cell number increased during the first 24 h, and then a slight decrease was verified. On the other hand, the bacterial CFU was kept approximately constant through the first 24 h of fermentation, decreasing afterwards. Overall, *S. cerevisiae* CFU was improved by 2.44 log units, while *L. pentosus* CFU decreased 0.53 log units. Sugar consumption was low in the first 10 h of fermentation, but after 24 h of fermentation all the hexose sugars were consumed. More than 16 g ethanol L^{-1} were produced at a rate of 0.34 g L^{-1} h^{-1}, while 0.7 g lactate L^{-1} were produced at a rate of 0.01 g L^{-1} h^{-1}. In the end of fermentation, the ethanol and lactate yields were found to be 0.40 g g consumed $sugars^{-1}$ and 0.01 g g consumed $sugars^{-1}$, respectively.

In the presence of yeast extract, *S. cerevisiae* growth was even faster than in the absence of that nutrients source, while the viable cell number of the bacterial strain decreased during the first 24 h of fermentation. However, *L. pentosus* CFU increased afterwards. In the end of fermentation, the CFU of yeast and bacterial strain was improved by 2.58 and 0.12 log units, respectively. Comparing with the previous experiment, a faster sugar consumption and an improved ethanol and lactate production were denoted. A maximum ethanol titer of about 19 g L^{-1} was achieved and the productivity was 0.39 g L^{-1} h^{-1}. The ethanol yield was found to be 0.45 g g consumed

sugars^{-1}. More than 1.3 g lactate L^{-1} were produced at a rate of 0.03 g L^{-1} h^{-1}, and the lactate yield was 0.03 g g consumed sugars^{-1}.

These results strongly suggest that the pH and cell death (through the addition of yeast extract) are of great importance to understand the reasons triggering the bacterial contamination in ethanol plants, but more research is needed. To a better comprehension of all the studied effects, all the results obtained were compared (Section 4.2.4).

4.2.4 Overall comparison – understanding the tested effects

The summary of the results from all the experiments performed in this work is shown in Table 14. As mentioned before, the experiments with yeast and bacteria alone (ratio 1:0 and 0:1, respectively) in the presence and absence of yeast extract were set as a benchmark to compare with the co-culture experiments.

During co-culture experiments, *L. pentosus* was never able to overcompete *S. cerevisiae*, since its sugar uptake was apparently slower than the one of yeast. Moreover, while the yeast was able to grow at high rates, the bacteria rapidly died in many trials. As a result, ethanol productivity was approximately constant during the experiments (~ 0.37 g L^{-1} h^{-1}) and significantly higher than lactate productivity. The values of ethanol yield were also stable during co-culture trials and very close to the ones obtained when yeast was cultivated alone (~ 0.47 g g consumed sugars^{-1}), which indicates that none of the tested conditions negatively affected alcoholic fermentation. Moreover, the lactate concentration levels reached during co-culture experiments (below 2.0 g L^{-1}) were probably too low to have a negative impact on the yeast. The maximum lactate yield and formation rate during co-cultures were obtained using SSH medium, pH 5.0, supplemented with yeast extract at a ratio 1:100 of yeast/bacteria, and equal to 0.05 ± 0.04 g g consumed sugars^{-1} and 0.04 ± 0.03 g L^{-1} h^{-1}, respectively. Since lactate was produced at a rate of 0.09 ± 0.00 g L^{-1} h^{-1} with a yield of 0.78 ± 0.18 g g consumed sugars^{-1} in the control (bacteria alone in SSH with yeast extract), *L. pentosus* was clearly affected by the presence of *S. cerevisiae*.

Table 14. General scheme and parameters of anaerobic batch fermentations performed.

Fermentation conditions				**Results**					
YE[a]	I[b]	pH	Ratio[c]	Δlog (Yeast)[d]	Δlog (Bacteria)[d]	$P_{ethanol}$[e] (g L^{-1} h^{-1})	$P_{lactate}$[e] (g L^{-1} h^{-1})	$Y_{ethanol/S}$[f] (g g^{-1})	$Y_{lactate/S}$[f] (g g^{-1})
No	Yes	5.0	1:0	0.61 ± 0.03	–	0.37 ± 0.02	–	0.47 ± 0.04	–
No	Yes	5.0	0:1	–	-0.23 ± 0.06	–	0.01 ± 0.00	–	0.25 ± 0.23
No	Yes	5.0	1:1	0.32 ± 0.21	-6.00 ± 2.09	0.37 ± 0.00	0.01 ± 0.00	0.47 ± 0.01	0.01 ± 0.00
No	Yes	5.0	1:100	2.22 ± 0.04	-5.07 ± 3.18	0.37 ± 0.01	0.01 ± 0.00	0.47 ± 0.01	0.01 ± 0.00
No	No	5.0	1:1	-0.99 ± 0.15	-7.32 ± 0.20	0.33 ± 0.02	0.00 ± 0.00	0.40 ± 0.00	0.00 ± 0.01
No	No	6.5	1:100	2.44	-0.53	0.34	0.01	0.40	0.01
Yes	Yes	5.0	1:0	0.44 ± 0.00	–	0.39 ± 0.01	–	0.47 ± 0.01	–
Yes	Yes	5.0	0:1	–	0.26 ± 0.48	–	0.09 ± 0.01	–	0.78 ± 0.18
Yes	Yes	5.0	1:1	0.33 ± 0.15	-5.30 ± 3.06	0.38 ± 0.00	0.02 ± 0.00	0.47 ± 0.00	0.02 ± 0.00
Yes	Yes	5.0	1:100	2.40 ± 0.21	-5.01 ± 3.49	0.38 ± 0.00	0.04 ± 0.03	0.49 ± 0.01	0.05 ± 0.04
Yes	No	5.0	1:1	-0.28 ± 0.01	-7.71 ± 0.61	0.35 ± 0.01	0.00 ± 0.00	0.43 ± 0.01	0.00 ± 0.00
Yes	No	6.5	1:100	2.58	0.12	0.39	0.03	0.45	0.03

[a] Presence of yeast extract.
[b] Presence of inhibitors.
[c] Ratio of *S. cerevisiae*/*L. pentosus* was obtained by dividing the cell viable number per milliliter (CFU mL^{-1}) of each strain.
[d] Difference between the viability at 48 h and at 0 h.
[e] Ethanol and lactate productivities obtained after 48 h of fermentation.
[f] Product yields calculated considering sugar consumption (mannose, glucose, galactose and xylose) after 48 h of fermentation, in g product g consumed $sugars^{-1}$.

During this work, various factors were tested in order to determine the main reasons of microbial contaminations occurring in ethanol plants. In general, the addition of yeast extract to the fermentation medium led to a better performance with both bacteria and yeast strains. This was especially denoted when *L. pentosus* was cultivated alone, since its viability was improved and the lactate production rate 9-fold higher. The yeast extract is a very rich source of nutrients, and composed by dead yeast cells, suggesting that could be a key factor for the occurrence of bacterial contamination [113]. However, this was not proved, since the bacteria was not able to outcompete the yeast during co-culture experiments.

The effect of inhibitors was also investigated during the co-cultivation experiments, however, in their absence, *S. cerevisiae* growth was even faster, resulting in bacterial death. In the end of fermentations using medium without inhibitors, the yeast viability decreased since the fermentable sugars were depleted in the first hours. Besides, the effect of ratio yeast/bacteria was also studied. However, even at a ratio of yeast/bacteria 1:100, *L. pentosus* was unable to take this advantage to outcompete *S. cerevisiae*. Since none of the referred factors had a positive effect on bacterial CFU number, the effect of pH was also studied. When yeast extract was added to SSH without inhibitors at a favourable pH to *L. pentosus* (6.5), it could survive better, but could not outcompete yeast. Of all the studied factors, pH was apparently one of the most favourable for increased bacterial viability.

Chapter 5

Conclusions

The present work aimed to tackle two of the major challenges in bioethanol production from lignocellulosic feedstocks: (i) high tolerance of microorganisms to lignocellulose inhibitors, and (ii) microbial contamination avoidance.

In the first study, a population of *S. stipitis* previously adapted to 60 % (v/v) of HSSL for 68 days in an evolutionary engineering approach was used, and its phenotypic stability in the absence of inhibitors during ten sequential transfers was investigated at single-clone level.

During the screening trials performed in shake flasks containing 60 % (v/v) of HSSL, 10 isolated clones showed higher xylose and acetate uptake rates and lower ethanol productivities than the parental strain. The results showed that evolutionary engineering was an effective strategy to improve the resistance towards microbial inhibitors. The clone exhibiting higher xylose uptake rate (0.558 g L^{-1} h^{-1}) was named isolate C4 and further characterised at bioreactor level.

The effect of short-term adaptation on the fermentation performance of the isolate C4 was evaluated by its pre-cultivation in the presence or absence of 60 % (v/v) of HSSL. The uptake rates of glucose and xylose were similar under both conditions, but a higher acetate consumption rate (0.101 g L^{-1} h^{-1}) and maximum ethanol concentration (4.51 g L^{-1}) were achieved without pre-adaptation step, suggesting that the long-term adaptation fully exploited the maximum capacity of the strain.

In the second study, the mechanism responsible by *L. pentosus* contamination on ethanol plants was investigated.

In the first phase of this project, cultivations using the yeast *S. cerevisiae* TMB3500 and *L. pentosus*, a microbial contaminant isolated from an ethanol facility, were performed in undiluted SSSL. However, none of the strains was able to grow or maintain its viability in the presence of such medium. Storage time and possible alteration of chemical compounds with inhibitory capacity were probably responsible by the observed non-growth.

To overcome this challenge, a simulated synthetic hydrolysate medium was used and its composition was adjusted to mimic the sugar and inhibitors content of SSSL average composition.

To investigate the reason of microbial contamination in ethanol plants, the impact of different factors in bacterial and yeast viability during anaerobic fermentations were studied. These factors included: (i) the inoculation ratio of yeast/bacteria at the starting point of fermentation; (ii) the presence of yeast extract; (iii) the presence of lignocellulosic inhibitors, and (iv) optimal pH for yeast and bacteria.

Different inoculation ratios of yeast/bacteria were tested, and no negative effect on yeast was detected after 48 h of fermentation. At the ratio of 1:100, despite delaying the ethanol production and yeast growth, *L. pentosus* could not outcompete yeast and its viability was completely lost, since its sugar uptake rate was apparently slow. This may indicate that *L. pentosus* will not be able to outcompete *S. cerevisiae* in conditions of excess of sugars; this can, for instance, be due to poor osmotolerance and/or limited availability of low affinity sugar transporters. It also suggests that low sugar levels may be necessary to observe contamination outbreaks, in case *L. pentosus* has more efficient high affinity sugar transporters than yeast.

The presence of yeast extract did not have any significant effect on ethanol production and yeast viability when cultured alone. However, a great improvement on lactate production (9-fold higher), yield and *L. pentosus* viability was denoted under the same conditions. This suggests that yeast cell death can be one of the factors triggering bacterial contamination during ethanol fermentation. During the co-culture experiments, the addition of yeast extract to the fermentation medium had, in general, a positive effect in the performance of both strains, however, *L. pentosus* was unable to compete with *S. cerevisiae* due to low growth rate and lactate production rate. Despite the positive effects of yeast extract, the bacterial viability was decreased in almost all the experiments.

The presence of inhibitors had, interestingly, a positive effect on bacterial viability. In fact, in their absence, yeast growth and ethanol formation was faster, resulting in a quicker bacterial death. When the pH of the medium was increased to a more favourable to bacteria, bacterial viability decreased slower, and therefore it was one of the factors leading to best results. However, as in all the experiments, *L. pentosus* did not outcompete *S. cerevisiae*.

A better understanding of other possible effects responsible by microbial contamination is necessary to avoid such occurrence in ethanol plants.

Chapter 6

Future work

Considering the evolutionary engineering study, the isolate C4 should be further characterised to investigate why its fermentation performance was improved if compared to that of parental strain *S. stipitis*. Lipidomics, proteomics, genomics and systems biology can be possible tools helping to explain such differences. From the results of bioinformatics, inverse metabolic engineering can be a useful technique to further improve the isolate C4.

Besides, *S. stipitis* fermentations should be performed under microaerophilic conditions, which correspond to the optimum for ethanol production by this yeast.

Regarding the microbial contamination study, research should be continued to understand why the spent sulphite liquor from softwoods was so toxic to *S. cerevisiae* TMB3500 and *L. pentosus*. Besides, anaerobic fermentations using new hydrolysate from the ethanol plant should be performed in order to investigate the effect of microbial contamination in real lignocellulosic hydrolysate.

Considering the investigated effects of *L. pentosus* contamination in SSH medium, the bacteria should be characterised in terms of optimum pH, temperature range and resistance to ethanol and high sugars concentration. Afterwards, new co-culture experiments should be realised to investigate more factors that can be responsible for contamination outbreak, like the addition of essential bacterial nutrients or the effect of limiting sugar availability.

Chapter 7

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