Universidade de Aveiro Departamento de Química
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Susana Raquel de Sousa Pereira

Produção de bioetanol a partir de um subproduto da indústria papeleira

Bioethanol production from a sub-product of pulping industry

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica da Doutora Ana Maria Rebelo Barreto Xavier, Professora auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Luísa Seuanes Serafim, Professora auxiliar convidada do Departamento de Química da Universidade de Aveiro.

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To all that crossed and left a mark in my path

The Jury

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Palavras-chave

Licor de cozimento ao sulfito ácido, bio-desintoxicação, *Paecilomyces variotii,* proteína microbiana, *Scheffersomyces stipitis*, fermentação de pentoses, bioetanol de segunda geração, adaptação, compostos fenólicos.

Resumo

A vida da sociedade atual é dependente dos recursos fósseis, tanto a nível de energia como de materiais. No entanto, tem-se verificado uma redução das reservas destes recursos, ao mesmo tempo que as necessidades da sociedade continuam a aumentar, tornando cada vez mais necessárias, a produção de biocombustíveis e produtos químicos. Atualmente o etanol é produzido industrialmente a partir da cana-de-açúcar e milho, matérias-primas usadas na alimentação humana e animal. Este fato desencadeou o aumento de preços dos alimentos em todo o mundo e, como consequência, provocou uma série de distúrbios sociais. Os subprodutos industriais, recursos independentes das cadeias alimentares, têm-se posicionado como fonte de matérias-primas potenciais para bioprocessamento. Neste sentido, surgem os subprodutos gerados em grande quantidade pela indústria papeleira. Os licores de cozimento da madeira ao sulfito ácido (SSLs) são uma matéria-prima promissora, uma vez que durante este processo os polissacarídeos da madeira são hidrolisados originando açúcares fermentáveis. A composição dos SSLs varia consoante o tipo de madeira usada no processo de cozimento (de árvores resinosas, folhosas ou a mistura de ambas).

O bioprocessamento do SSL proveniente de folhosas (HSSL) é uma metodologia ainda pouco explorada. O HSSL contém elevadas concentrações de açúcares (35-45 $g.L^{-1}$), na sua maioria pentoses. A fermentação destes açúcares a bioetanol é ainda um desafio, uma vez que nem todos os microrganismos são capazes de fermentar as pentoses a etanol. De entre as leveduras capazes de fermentar naturalmente as pentoses, destaca-se a *Scheffersomyces stipitis*, que apresenta uma elevada eficiência de fermentação. No entanto, o HSSL contém também compostos conhecidos por inibirem o crescimento de microrganismos, dificultando assim o seu bioprocessamento. Neste sentido, o principal objetivo deste trabalho foi a produção de bioetanol pela levedura *S. stipitis* a partir de HSSL, resultante do cozimento ao sulfito ácido da madeira de *Eucalyptus globulus*. Para alcançar este objetivo, estudaram-se duas estratégias de operação diferentes.

Em primeiro lugar estudou-se a bio-desintoxicação do HSSL com o fungo filamentoso *Paecilomyces variotii*, conhecido por crescer em resíduos industriais. Estudaram-se duas tecnologias fermentativas diferentes para a bio-desintoxicação do HSSL: um reator descontínuo e um reator descontínuo sequencial (SBR). A remoção biológica de inibidores do HSSL foi mais eficaz quando se usou o SBR. *P. variotii* assimilou alguns inibidores microbianos como o ácido acético, o ácido gálico e o pirogalol, entre outros. Após esta desintoxicação, o HSSL foi submetido à fermentação com *S. stipitis*, na qual foi atingida a concentração máxima de etanol de 2.36 $g.L^{-1}$ com um rendimento de 0.17 $g.g^{-1}$.

Resumo (continuação)

P. variotti, além de desintoxicar o HSSL, também é útil na produção de proteína microbiana (SCP) para a alimentação animal pois, a sua biomassa é rica em proteína. O estudo da produção de SCP por *P. variotii* foi efetuado num SBR com HSSL sem suplementos e suplementado com sais. A melhor produção de biomassa foi obtida no HSSL sem adição de sais, tendo-se obtido um teor de proteína elevado (82,8%), com uma baixa concentração de DNA (1,1%). A proteína continha 6 aminoácidos essenciais, mostrando potencial para o uso desta SCP na alimentação animal e, eventualmente, em nutrição humana. Assim, a indústria papeleira poderá integrar a produção de bioetanol após a produção SCP e melhorar a sustentabilidade da indústria de pastas.

A segunda estratégia consistiu em adaptar a levedura *S. stipitis* ao HSSL de modo a que esta levedura conseguisse crescer e fermentar o HSSL sem remoção de inibidores. Operou-se um reator contínuo (CSTR) com concentrações crescentes de HSSL, entre 20 % e 60 % (v/v) durante 382 gerações em HSSL, com uma taxa de diluição de 0.20 h^{-1}. A população adaptada, recolhida no final do CSTR (POP), apresentou uma melhoria na fermentação do HSSL (60 %), quando comparada com a estirpe original (PAR). Após esta adaptação, a concentração máxima de etanol obtida foi de 6.93 $g.L^{-1}$, com um rendimento de 0.26 $g.g^{-1}$. POP possuía também a capacidade de metabolizar, possivelmente por ativação de vias oxidativas, compostos derivados da lenhina e taninos dissolvidos no HSSL, conhecidos inibidores microbianos. Por fim, verificou-se também que a pré-cultura da levedura em 60 % de HSSL fez com que a estirpe PAR melhorasse o processo fermentativo em HSSL, em comparação com o ensaio sem pré-cultura em HSSL. No entanto, no caso da estirpe POP, o seu metabolismo foi redirecionado para a metabolização dos inibidores sendo que a produção de etanol decresceu.

Keywords

Hardwood spent sulphite liquor , biodetoxification, *Paecilomyces variotii,* single-cell protein, *Scheffersomyces stipitis*, pentoses fermentation, second generation bioethanol, adaptation, phenolic compounds.

Abstract

The fossil resources are declining while the requirements of modern lifestyle for energy and materials are growing. Hence, the search for sustainable alternatives to produce fuels and chemicals from non-fossil feedstocks is increasing. Among all biofuels, ethanol is currently being industrially produced from sugar-containing biomass such as sugarcane and corn. The use of these raw-materials, belonging to human and animal feeding, resulted in the rise of prices of food all over the world and, consequently, in social disturbance.

The use of industrial by-products, raw-materials outside the food chain, with polysaccharides hydrolysed to fermentable sugars, is an attractive prospect for future biotechnologies. In this context, spent sulphite liquors (SSLs), by-products from the pulp and paper industry, are promising feedstocks for bioprocessing. The composition of SSLs depends on the type of wood used by the pulp and paper industry (softwoods, hardwoods or mixture of both).

Hardwood spent sulphite liquor (HSSL) is a by-product from the pulp and paper industry, rich in pentoses, which is not fully exploited for bioprocessing. The sustainable fermentation of pentoses into bioethanol is a challenge to overcome since not all the microorganisms are able to use these sugars. *Scheffersomyces stipitis* is one of the most efficient yeast to naturally ferment pentoses to ethanol. However, besides sugars (35-45 $g.L^{-1}$), HSSL contains microbial inhibitors that limit the possibility of its bioprocessing. Therefore, the main purpose of this work was the production of bioethanol by *S. stipitis* from HSSL of *Eucalypt globulus*. To accomplish this objective two different strategies were studied.

The first one was the bio-detoxification of HSSL with the filamentous fungus *Paecilomyces variotii*, known for growing in polluted residues. Two fermentative approaches were compared, a single batch and a sequential batch reactor (SBR). Biological treatment of HSSL to remove microbial inhibitors was more efficient in the SBR. *P. variotti* was able to assimilate acetic acid as well as low molecular weight phenolics such as, gallic acid and pyrogallol, recognized yeast inhibitors. This bio-detoxified HSSL was subjected to a successful fermentation by *S. stipitis*, attaining a maximum ethanol concentration of 2.36 $g.L^{-1}$ with a yield of 0.17 $g.g^{-1}$. Moreover, the biomass produced by *P. variotii* is a potential source of protein and other nutrients for animal feeding. Hence, SCP production by *P. variotii* from HSSL was studied using a SBR with and without mixed salts supplementation. The best approach for SCP production was the SBR without salts addition. The biomass produced presented 82.8 % of protein with 6 essential amino acids and 1.1 % of DNA.

Abstract (continuation)

Therefore the produced SCP could be considered a good candidate for animal feeding and, eventually, human nutrition. This is a major advantage for a biorefinary approach, since this bio-detoxification process and the SCP production can be integrated with bioethanol production by *S. stipitis*.

The second strategy to produce bioethanol was to improve the tolerance of *S. stipitis* in order to utilize the xylose present in HSSL without the removal of inhibitory compounds. A continuous reactor with increasing HSSL concentrations, between 20 % and 60 % (v/v) was operated during 382 generations of HSSL, at a dilution rate of 0.20 h^{-1}. The resulting adapted population (POP) showed improved fermentation behaviour in 60 % HSSL when compared with the parental strain (PAR). POP achieved a maximum ethanol concentration of 6.93 $g.L^{-1}$, with a maximum ethanol yield of 0.26 $g.g^{-1}$. It was also showed that POP could assimilate dissolved lignin oligomers and tannins probably through activating oxidative pathways. Moreover, preculturing PAR in HSSL improved its tolerance towards the HSSL inhibitors and also the yeast fermentation ability. Nevertheless, preculturing POP in HSSL, redirected its metabolism to the assimilation of inhibitors, reducing the ethanol production.

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Abbreviations/Acronyms

$[EtOH]_{max}$	Maximum ethanol concentration
μ_{max}	Maximum specific growth rate
AA	Amino acid
Ac	Acetyl group
ADP	Adenosine diphosphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B	Batch assay
BSA	Bovine serum albumin
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
ca.	From the Latin meaning "about"
CBS	Fungal Biodiversity Centre
CDM	Chemically defined medium
CFU	Colony-forming unit
COD	Chemical oxygen demand
CRBF	Cell recycle batch fermentation
CSTR	Continuous stirred tank reactor
D	Dilution rate
DNA	Deoxyribonucleic acid
DNS	3,5-Dinitrosalicylic acid
e.g.	From the Latin meaning "for example"
EFs	Elementary Fibrils
FAO	Food and agriculture organization
G	Guaiacyl
GC-FID	Gas chromatography with a flame ionization detector
GC-MS	Gas chromatography with a mass spectrometry detector
GMO	Genetically modified organism
H	*p-hydroxyphenyl*
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
HSSL	Hardwood Spent Sulphite Liquor
i.e.	From the Latin meaning "that is"
LCB	Lignocellulosic Biomass
LS	Lignosulphonates
M	Pulping base (Na, Ca, Mg or NH3)
ME	Malt Extract medium
MF	Microfibrils

Abbreviations/Acronyms (continuation)

NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NRRL	Northern Regional Research Laboratory
OD	Optical density
OD_{620nm}	Optical density at 620nm
OTR	Oxygen transference rate
PAR	Referent to *Scheffersomyces stipitis* NRRL Y-7124
POP	Referent to the final population obtained from the adaptation to HSSL of *S. stipitis* NRRL Y-7124 in a CSTR during 382 generations.
$Q_{ethanol}$	Ethanol productivity
Quorn™	Brand of mycoprotein from *Fusarium venenatum*
$r_{acetic\ acid}$	Acetic acid consumption rate
$r_{glucose}$	Glucose consumption rate
RI	Refractive index
rpm	Rotations per minute
r_{sugars}	Sugars consumption rate
r_{xylose}	Xylose consumption rate
S	Syringyl
SDS	Sodium dodecyl sulfate
SBR	Sequential batch reactor
SCP	Single-cell Protein
SM	Supplementary medium
SSL	Spent Sulphite Liquor
SSSL	Softwood Spent Sulphite Liquor
TCA	Tricarboxylic acid cycle
THSL	Sulphite Thick Liquor
TMS	Trimethylsilane
TMSC	Trimethylchlorosilane
USDA	United States Department of Agriculture
UV	Ultraviolet radiation
v	Volume
w	Weight
X5P	Xylulose-5-phosphate

Abbreviations/Acronyms (continuation)

XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylokinase
XOS	Xylooligosaccharides
XR	Xylose reductase
$Y_{ethanol}$	Ethanol yield at the maximum ethanol concentration point
YM	Yeast medium
(-)PAR	Batch assay with S. stipitis parental strain in HSSL performed with a precultivation in CDM
(+)PAR	Batch assay with S. stipitis adapted population in HSSL performed with a precultivation in CDM
(-)POP	Batch assay with S. stipitis parental strain performed with a precultivation in HSSL
(+)POP	Batch assay with S. stipitis adapted population performed with a inoculum in HSSL
***detox*(-)POP**	Batch assay with S. stipitis adapted population in bio-detoxified HSSL performed with a precultivation in bio-detoxified HSSL
***detox*(+)POP**	Batch assay with S. stipitis adapted population in bio-detoxified HSSL performed with a inoculum in bio-detoxified HSSL

Chapter I

Introduction
Objectives

1.1 Introduction

The world is facing a decline of fossil fuels resources, while modern lifestyle requirements for energy and materials are growing. In order to face the needs of energy supply and to response to climate change issues the search for sustainable alternatives to produce fuels and chemicals from non-fossil feedstocks is attracting more interest worldwide [1, 2].

Many countries in Europe, North and South America and Asia are replacing fossil fuels by biomass-based fuels according to international regulations, as a result of economic incentives implemented by governments [1]. One of the directives of European Union imposed a quota of 20 % of bioenergy in all traffic fuel until 2020 [3, 4, 5]. Currently, bioethanol is produced mainly from sugar-containing biomass such as sugarcane and corn. The growth of biofuels industry raised questions regarding the sustainability of "first generation" biofuels [1, 3, 6]. The use of feedstocks for human and animal feeding for bioethanol production resulted in the rise of prices of food all over the world and, consequently, in social disturbance [1, 3]. For these reasons, researchers attention was focused in the generation of energy and sources of energy from forestry wastes, residual agricultural biomass and industrial by-products, the so-called "second generation" biofuels [3, 7].

Portugal has not sugar cane or other agriculture raw-materials for an extensive production of first generation biofuels. Sugars present in industrial by-products and wastes could be a good resource for the second generation biofuels, namely bioethanol. Also, environmental concerns about the removal of industrial by-products and wastes are increasing the search for biotechnological integrated processes with the objective of obtaining their valorisation and economical profit [2, 7].

Around 1/3 of the total land area of Portugal is occupied by forests and a significant part of it is used by pulp and paper industry, a sector with significant impact on national economy. The pulp and paper industry has a very important role in this country and according to the Portuguese Pulp and Paper Industry Association, Portugal was in 2009 the 4th largest European producer of pulp, with a share of 7.1 % of the total production [8].

Spent sulphite liquor (SSL) is the main effluent resulting from the acidic sulphite pulping process. In 1993 it was reported that its production corresponded to 90 billion litres worldwide, annually [9]. SSL contains significant amounts of dissolved organic matter (COD > 100000 $mg_{O2}.L^{-1}$). Hence, it cannot be discharged into natural basins due to environmental concerns and must be processed [10]. SSL is normally concentrated by evaporation producing Sulphite thick liquor (THSL) which is then burned for the energy and base recovering [10, 11]. The application and processing of sulphite liquors depend significantly on their chemical composition, which is basically determined by the origin of wood transformed in the pulping process [10, 12]. Although, the utilization of SSL is recognised for long time to produce value-added products, only softwood SSL (SSSL) potential was studied so far [9, 12]. Hardwood SSL or HSSL potential for value-added products has never been evaluated as in SSSL.

1.2 Objectives

The main purpose of this work was the production of bioethanol by the yeast *Scheffersomyces stipitis* from *Eucalyptus globulus* HSSL. For fulfilling this objective two different strategies were studied. The first one was focused in the bio-detoxification of HSSL with *Paecilomyces variotii*. This would allow the yeast *S. stipitis* to grow in HSSL. At the same time the fungal biomass was analysed for single-cell protein (SCP) production. The second approach consisted in the adaptation of *S. stipitis* to the HSSL without the detoxification step. The obtained adapted population was then studied and compared with the parental strain. Thus this work took in consideration the following specific objectives:

a) To remove the major inhibitors in HSSL with the filamentous fungus *P. variotii*.
b) To analyse the chemical composition of HSSL before and after the detoxification step.
c) To evaluate the bioethanol production potential by *S. stipitis* in the bio-detoxified HSSL.
d) To study the fermentation of HSSL by *P. variotii* in order to produce single-cell protein.
e) To analyse the composition of the SCP produced.
f) To adapt *S. stipitis* to ferment xylose to bioethanol in the HSSL with all the inhibitors.
g) To characterize the fermentative behaviour of the final adapted population obtained and compare it with the parental strain.

This thesis is the result of the scientific work developed to attain these objectives and this work has already given raise to some published articles and to other manuscripts submitted or in preparation to submit for publication SCIENTIFIC PEER REVIEW JOURNALS. In the Chapter II the state of the art for the ethanol production using HSSL is exposed. This Chapter presents the review article already published in Process Biochemistry (Pereira *et al.* (2013)), under the title: "Advances in ethanol production from hardwood spent sulphite liquors".

In Chapter III, the work performed to attain the objectives a), b) and c) is presented. This study, entitled "Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol", was already published in Bioresource Technology (Pereira *et al.*(2012)).

The objectives d) and e) gave rise to Chapter IV and one publication was prepared and just submitted, with the title: "Production of Single-cell protein from eucalypt sulphite spent liquor by *Paecilomyces variotii "*.

In the Chapter V, the work made to fulfil objective f) is exposed. This study was made with the collaboration of Prof. Marie Gorwa-Grauslund at the Division of Applied Microbiology in Lund University. The work developed to achieve the objective f), was object of a purposed article entitled: "Adaptation of *Scheffersomyces stipitis* to hardwood spent sulphite liquor".

The next Chapter (VI) is a continuation from Chapter V and is related to objective g). An article entitled: "Influence of the precultivation medium on *Scheffersomyces stipitis* growth and fermentation performances in hardwood spent sulphite liquor" is already prepared based on this study.

In the final Chapter (VII), the conclusions of this work are stated and some future research perspectives are proposed.

1.3 References

1. Mussatto, S.I. and I.C. Roberto, *Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review*. Bioresource Technology, 2004. **93**(1): p. 1-10.

2. Sannigrahi, P., Y. Pu, and A. Ragauskas, *Cellulosic biorefineries-unleashing lignin opportunities*. Current Opinion in Environmental Sustainability, 2010. **2**(5): p. 383-393.
3. Xavier, A.M.R.B., M.F. Correia, S.R. Pereira, and D.V. Evtuguin, *Second-generation bioethanol from eucalypt sulphite spent liquor*. Bioresource Technology, 2010. **101**(8): p. 2755-2761.
4. Zhang, Y.H.P., *Reviving the carbohydrate economy via multi-product lignocellulose biorefineries*. Journal of Industrial Microbiology & Biotechnology, 2008. **35**(5): p. 367-375.
5. European Commission and Directorate-General for Energy, *Renewables make the difference*.2011, Luxemburg.
6. Balat, M., H. Balat, and C. Oz, *Progress in bioethanol processing*. Progress in Energy and Combustion Science, 2008. **34**(5): p. 551-573.
7. Bacovsky, D., W. Mabee, and M. Worgetter, *How close are second-generation biofuels?* Biofuels Bioproducts & Biorefining, 2010. **4**(3): p. 249-252.
8. CELPA. Associação da Indústria Papeleira. 2009 [accessed: 2015, 5th of July]; Available from: http://www.celpa.pt/images/pdf/art209_pt_be_2009.pdf.
9. Lawford, H.G. and J.D. Rousseau, *Production of ethanol from pulp-mill hardwood and softwood spent sulfite liquors by genetically-engineered Escherichia coli*. Applied Biochemistry and Biotechnology, 1993. **39**(1): p. 667-685.
10. Evtuguin, D.V., M.R.B. Xavier, C.M. Silva, and A. Prates. *Towards comprehensive utilization of products from sulphite pulp production: A biorefinary approach*. in XXI Encontro Nacional da TECNICELPA / VI CIADICYL. 2010. Lisbon, Portugal.
11. Sjöström, E., *Wood Chemistry. Fundamentals and Applications*. 2nd Ed., 1993, New York, USA: Academic Press.
12. Marques, A.P., D.V. Evtuguin, S. Magina, F.M.L. Amado, and A. Prates, *Chemical Composition of Spent Liquors from Acidic Magnesium-Based Sulphite Pulping of Eucalyptus globulus*. Journal of Wood Chemistry and Technology, 2009. **29**(4): p. 322-336.
13. Björling, T. and B. Lindman, *Evaluation of xylose-fermenting yeasts for ethanol production from spent sulfite liquor*. Enzyme and Microbial Technology, 1989. **11**(4): p. 240-246.
14. Holmgren, M. and A. Sellstedt, *Identification of white-rot and soft-rot fungi increasing ethanol production from spent sulfite liquor in co-culture with Saccharomyces cerevisiae.* Journal of Applied Microbiology, 2008. **105**(1): p. 134-140.

CHAPTER II

Bibliographic Review:

Advances in ethanol production from hardwood spent sulphite liquors

Hardwood spent sulphite liquors (HSSLs) are by-products from the pulping industry rich in pentoses, which are not yet fully exploited for bioprocessing, namely for the production of bioethanol. The sustainable fermentation of pentoses into bioethanol is a challenge to overcome. Besides sugars, HSSLs contains inhibitors that decrease the possibility of bioprocessing of these by-products. Nevertheless, recent studies have brought new insights in using HSSLs for bioethanol production. This chapter reviews the results of relevant studies carried out with HSSLs towards bioprocessing to bioethanol. The composition of SSLs was compared and related with the wood origin stressing specificity of microbial inhibitors from HSSL and their anti-microbial effect. The different fermentative processes, the microorganisms used, and the strategies to improve yield and productivity used so far were also reviewed. This review allowed concluding that research is still needed in several areas, including optimization of detoxification processes, fermentation strategies and selection of suitable microbial strains in order to achieve the integration of the different steps needed for HSSLs bioconversion into ethanol thus contributing for sustainability of pulping mills within a biorefinery concept.

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2.1 Introduction

The world is facing a decline of fossil fuels resources, petroleum, natural gas, and charcoal, while energy requirements are progressively growing up. Moreover, the finding of new fossil sources is decelerating and the costs of extraction are increasing. Accordingly, the search for sustainable alternatives to produce fuels and chemicals from non-fossil feedstocks, especially biomass, is attracting a considerable interest worldwide [1, 2].

Among all biofuels, ethanol is currently being produced on a large scale, and can be easily mixed with gasoline and used in internal combustion engine vehicles [3, 4]. Alternatively bioethanol may be converted to ethylene, ethylene glycol or butadiene to produce bio-based poly(ethylene), poly(ethylene terephthalate) and butadiene rubber/styrene, respectively [5]. Currently, bioethanol is produced mainly from sugar-containing biomass such as sugarcane and corn [4]. However, the growth of bioethanol industry and the consequent increase in the usage of cereals food crops for ethanol production raised questions regarding the sustainability of the so-called “first generation” biofuels. The use of raw materials belonging to the human and animal food chains for biofuels production resulted in the rise of prices of food all over the world and, consequently, in social disturbance [1, 4, 6]. For these reasons, the research efforts are focused on the use of forestry, agricultural and industrial wastes, rich in biomass to generate the so-called “second generation” biofuels [4, 7].

The recalcitrance of lignocellulosic biomass (LCB) hinders its conversion to monomeric sugars and consequently to ethanol [1, 8]. Hence the use of industrial wastes, which contain fermentable sugars, is an attractive prospect. In this context, spent sulphite liquors (SSLs), by-products from the acidic sulphite pulping process of wood in pulp and paper industry, can be considered as promising raw materials for the production of bioethanol [6, 9, 10] since, 90 billion litres of SSLs are produced annually [11]. The main objective of the wood pulping process is the removal of lignin from wood, maintaining the cellulose and hemicelluloses integrity, thus providing the cellulosic fibres with desired composition (percentage of cellulose and hemicelluloses) and appropriate yield (depending on the degree of the delignification) [12, 13]. Various wood species like softwoods, hardwoods or mixture of both, can be used by the pulp and paper industry [12, 14]. The

extreme conditions, that include high temperature (125‑145 ^{o}C) and the medium acidity (pH 1‑2), applied during acidic sulphite pulping cause the partial hydrolysis of hemicelluloses releasing monomeric sugars and oligosaccharides, which could be easily fermented into ethanol [12, 13]. This fact is an advantage of SSLs over LCB raw materials and their complex hydrolytic processes needed for the production of 2nd generation bioethanol and other bio-based products [6, 15].

Not all the liquors generated by pulping processes are suitable as substrates for fermentative processes. The spent liquors that result from alkaline Kraft pulping, responsible for more than 90 % of cellulosic pulp production worldwide, have a chemical composition unsuitable for ethanol production [16]. Namely, most of sugars/oligosaccharides released from wood in Kraft spent liquor are degraded to C_2-C_6 hydroxy- and dicarboxylic acids [13, 17].

The composition of acidic SSLs depends strongly on the type of wood used in the pulping process [12, 13], and will be discussed in Section 2.2. Thus, SSLs obtained from pulping of softwoods (coniferous) contain a high proportion of hexoses (> 70 %), while those obtained from pulping of hardwoods (deciduous) contain mainly pentoses (> 70 %) [12]. This difference in composition will be pointed out in Section 2.3. The fermentative conversion of hexoses to ethanol by robust yeast *Saccharomyces cerevisiae* is a well-known industrially implemented process [18-20]. However, *S. cerevisiae* cannot naturally ferment pentoses [20-22]. Moreover, pentose-utilizing yeasts showed difficulties in fermenting pentoses from HSSL due to a lack of appropriate resistance to toxic contaminants [6, 10, 23]. Pentose rich hemicelluloses are predominant in vegetable biomass but still a non-used raw material [4, 20]. Research and development of sustainable fermentation processes for bioethanol production from raw materials rich in pentoses is an important challenge for the future.

In this work, the problems associated with bioethanol production from hardwood spent sulphite liquors will be discussed after a brief introduction. The chemical composition of hardwoods and softwoods is compared in Section 2.2. The generation of hardwood spent sulphite liquors (HSSLs) is presented in Section 2.3 followed by the composition of HSSLs in Section 2.4. Section 2.5 presents the HSSL microbial inhibitors and their removal

strategies are reviewed in Section 2.6. The following sections are dedicated to bioethanol production using wild (Section 2.7.1) and engineered or adapted (Section 2.7.2) strains. This review ends with a list of challenges that must be solved in order to develop a sustainable technology for bioethanol production from HSSL.

2.2 Chemical composition of hardwoods versus softwoods

Botanically, trees can be classified as softwoods or hardwoods. Softwoods are gymnosperms (mostly conifers) like pine, spruce, larch, etc. Hardwoods are angiosperms (deciduous) like poplar, beech, eucalyptus, etc. [13]. These two types of wood differ from each other in fibre morphology and chemical composition. Although fibres from both types of wood are composed by the same components (cellulose, hemicelluloses, lignin and minor amounts of extractives and inorganics), their proportions and structural features are remarkably different (**Table 2.1**) [12, 13]. The general chemical composition of the most common industrially processed hardwoods is presented in **Table 2.1**.

Table 2.1. Chemical composition and dimensions of wood fibers (adapted from [12, 13]).

Fibre type	Cell dimensions		Chemical composition		
	Average length mm	Average diameter μm	Cellulose %	Hemicellulose %	Lignin %
Softwood	3.3	33	40-44	23-28	25-31
Hardwood	1.0	20	45-50	25-35	18-24

Table 2.2. Chemical composition (%, w/w) of hardwoods.

Wood Species		Cellulose [12,14,17,41]	Hemicelluloses		Lignin [9,13,14,41]	Extractives [13,14,41]	Ash [9,13,14,41]
Common name	Scientific Name		Glucuronoxylan [9,14,41,57]	Glucomannan [9,14,57]			
Red maple	*Acer rubrum*	40.7 - 45.0	22.1 - 25.0	3.1 - 4.0	22.8 - 25.4	2.5 - 5.3	0.30 - 5.2
Yellow birch	*Betula alleghaniensis*	45.0-47.0	15.0 - 30.0	2.0 - 5.0	21.2	2.6	1.7 - 2.9
White birch	*Betula papyrifera*	39.4 - 45.0	29.7 - 35.0	1.4	18.9 – 21.4	2.6	-
Mockernut hickory	*Carya tomentosa*	43.5 - 48.0	15.0 - 30.0	1.5	23.6	5.0	0.40
Blue gum	*Eucalyptus globulus*	50.0-53.0	18.0-24.0	2.0-4.0	19.0 – 22.0	1.0 -2.7	0.30-0.50
Beech	*Fagus sylvatica*	39.4-43.0	26.0-29.0	1.5-3.0	23.0– 24.8	1.5-2.5	0.40
American beech	*Fagus gradifolia*	36.0 - 49.0	25.0-28.0	2.7-3.0	22.0 - 30.9	3.0-4.0	0.40
White ash	*Fraxinus americana*	39.5 - 41.0	20.0 - 26.0	3.8	24.8	6.3	0.30
Sweet gum	*Liquidambar styraciflua*	40.8 - 46.0	17.0 - 24.0	3.2	22.4	5.9	0.20
Yellow poplar	*Liriodendron tulipifera*	39.1 - 45.0	15.0 - 20.0	4.9	25.3 - 30.3	2.4 - 3.6	0.30 - 2.8
Sweet bay	*Magnolia virginiana*	44.2	15.0 - 25.0	4.3	24.1	3.9	0.20
Water tupelo	*Nyssa aquatica*	45.0 - 45.9	15.0 - 25.0	3.5	25.1	4.7	0.40
Black tupelo	*Nyssa sylvatica*	42.6 - 45.0	15.0 - 21.0	3.6	26.6	2.9	0.60
Eastern cottonwood	*Populus deletoids*	46.5 - 47.0	16.0 - 20.0	4.4	25.9	2.4	0.60
Quaking aspen	*Populus tremoides*	48.0-50.0	18.0 - 21.0	2.0 - 5.0	18.2	2.4	4.0
Aspen	*Populus tremulus*	47.0-50.0	16.0 – 20.0	2.0 - 5.0	21.0-23.0	1.0-2.0	0.30-0.40
White oak	*Quercus alba*	41.7 - 47.0	15.0 - 25.0	3.1	24.6	5.3	0.20
Scarlet oak	*Quercus coccinea*	43.2 - 46.0	16.0 - 25.0	2.3	20.9	6.6	0.10
Southern red oak	*Quercus falcata*	40.5 - 42.0	17.0 -25.0	1.7	23.6	9.6	0.50
Blackjack oak	*Quercus marylandica*	33.8 - 44.0	15.0 - 23.0	2.0	30.1	6.6	1.3
Chestnut oak	*Quercus prinus*	40.8 - 46.0	17.0 - 26.0	2.9	22.3	6.6	0.40
Northern red oak	*Quercus rubra*	42.2 - 46.0	17.0 - 22.0	3.3	20.2	4.4	0.20
Black oak	*Quercus velutina*	39.6 - 48.0	15.0 - 25.0	1.9	25.3	6.3	0.50
American elm	*Ulmus americana*	42.6 - 51.0	18.0-22.0	4.0 - 4.6	24.0 - 27.8	1.9 - 2.0	0.80

Cellulose is a linear homopolysaccharide composed of β-D-glucopyranose units, which are linked by β-(1 $\rightarrow$ 4) glycosidic bonds (**Figure 2.1**). The repeating stereo regular unit is a cellobiose residue (1.03 nm) formed by two molecules of glucose forming an 180^o angle. Wood cellulose can consist of about 5000-6000 glucose units [13, 17]. The glucose units form intramolecular and intermolecular hydrogen bonds, which pre-determine its fibrillar structure. Cellulose molecules are primary assembled into elementary fibrils (EFs) with highly crystalline domains alternated with less ordered amorphous regions. EFs are aggregated sequentially into microfibrils (MF), which constitute the skeleton of the cell embedded in the lignin matrix [12, 14]. Because of this structural hierarchy chemical or enzymatic hydrolysis of cellulose chains becomes very difficult [12]. Softwoods contain slightly less cellulose than hardwoods (**Table 2.1**).

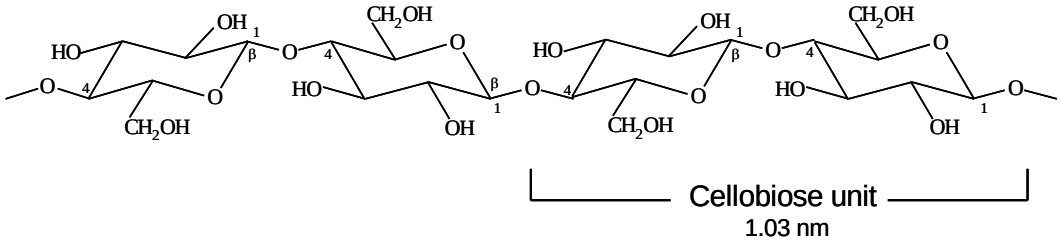
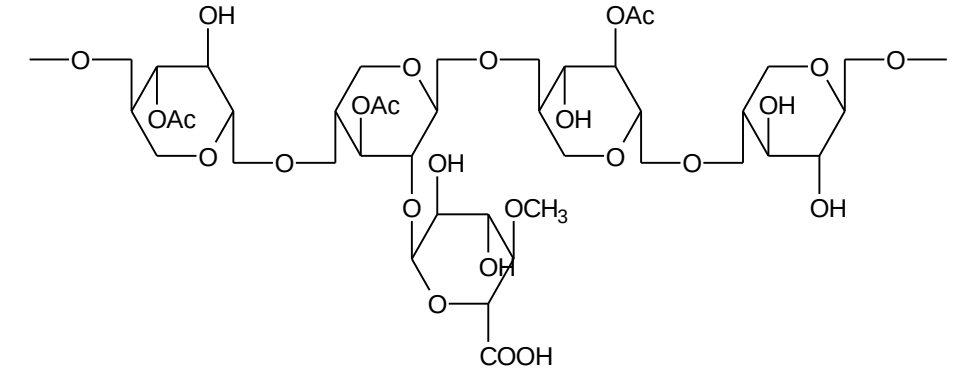


Figure 2.1. Simplified representation of celulose structure

Hemicelluloses are structural polysaccharides of non-cellulosic type, which have a polymerization degree between 100 and 200. Like cellulose, hemicelluloses contribute to the support of the cell wall [12, 14]. The major hemicellulose (15‑35%) of hardwoods is a partially acetylated 4-*O*-methyl-glucurono-β-D-xylan (glucuronoxylan) although a small amount of non-acetylated glucomannan (2‑5%) can be also detected (**Table 2.2** and **Figure 2.2**). In hemicellulose chains the ratio glucose to mannose molecules depends on the wood species but usually is between 1:2 and 1:1 [12]. In contrast to hardwoods, the most abundant hemicellulose of softwoods is a partially acetylated galactoglucomannan (15‑20%), whereas non-acetylated arabinoglucuronoxylan (5‑10%) is much less abundant [12, 14]. Hence the majority of hardwood hemicelluloses are pentosans (mainly

glucuronoxylan) and the softwood hemicelluloses are essentially hexosans (mainly galactoglucomannans) (**Figure 2.2**) [12, 13, 17].



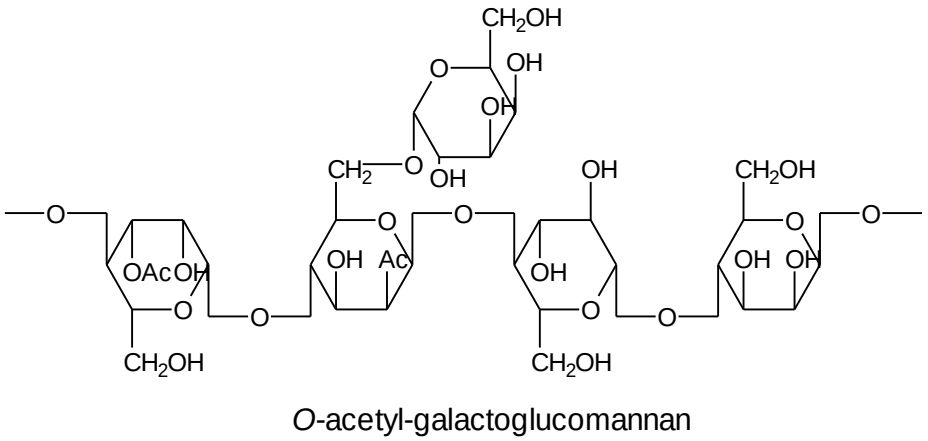


Figure 2.2. Schematic representation of the most abundant hemiceluloses in hardwoods (*O*-acetyl-4-*O*-methyl-glucuronoxylan) and in softwoods (*O*-acetyl-galactoglucomannan).

Lignin is an amorphous polymer constituted by phenyl propane units linked by ether and/or carbon‑carbon bonds [12]. This polymer is an integral part of the cell wall of plants and contributes to the stiffness, antiseptic and hydrophobic properties of plant tissues [12, 17]. Lignin corresponds to 15‑30% of plant biomass and is the principal non-hydrolysable residue of LCB [12, 14, 17, 18]. The lignin precursors (monolignols) are *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol giving rise to the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin substructures [12, 14, 17]. **Figure 2.3** presents a schematic structure of lignin and its structural units. Lignins from softwoods, hardwoods and grasses possess different proportion of S, G and H units and can be divided into three groups. The “guaiacyl

lignin" (G-type lignin), occurring in softwoods, is a polymerization product of coniferyl alcohol. The "guaiacyl-syringyl lignin" (GS-type lignin), typical in hardwoods, is a copolymer of coniferyl and sinapyl alcohols, with the S/G ratio varying from 4:1 to 1:2 [12, 14]. Typical lignins of herbaceous plants also contain significant amounts of H units and these are referred to as the GSH-type. The most abundant inter-unit linkage in lignin is β-*O*-4, which may reach up to 60 % of all existing linkages between phenylpropane units in the hardwood lignin (**Figure 2.3**).

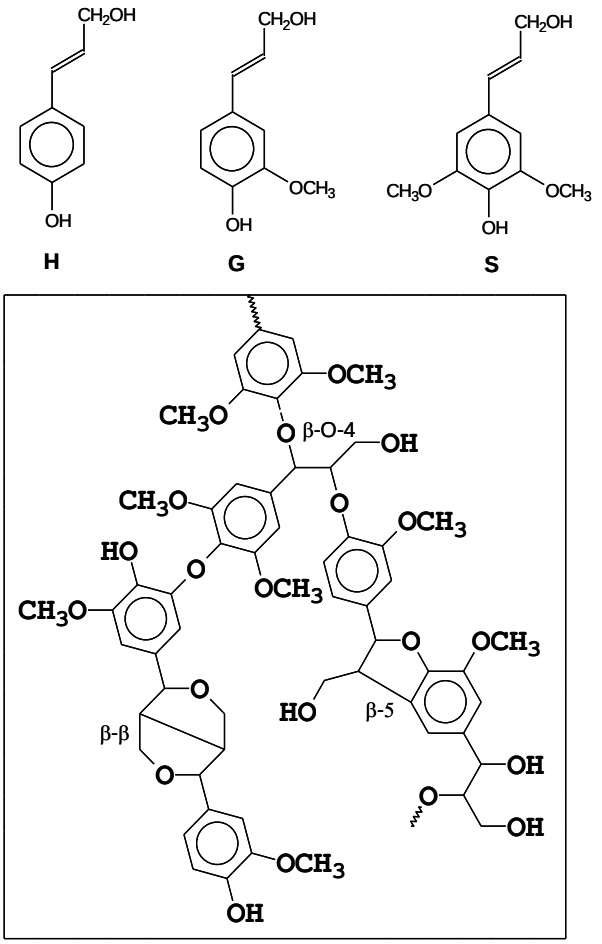


Figure 2.3. Lignin structural units, *p*-hydroxyphenyl (H), guiacyl (G) and syringyl (S), and the schematic representation of hardwood lignin (G and S units are linked by ether and carbon-carbon bonds).

The other relevant linkages found in the lignin are the following: α-*O*-4, β-5, β-1, β-β, 4-*O*-5 and 5-5 biphenyl. In addition, lignin itself is also linked to polysaccharides thus forming a unique lignin-carbohydrate network. The amount of ether linkages (β-*O*-4, α-

O-4 and 4-*O*-5) in hardwood lignins is 15-20% higher than those in softwoods [12]. This fact together with 50 % lower total lignin content, in hardwoods than in softwoods (**Table 2.1**), results in a much easier delignification of hardwoods during pulping [12, 13, 17].

2.3 Generation of hardwood spent sulphite liquors

In pulp and paper industry, lignin is removed from the wood during the pulping process thus liberating the fibre material (pulp) that is used further for the papermaking or as a chemical feedstock [9]. Chemical pulping can proceed either under strong acid or strong basic conditions [12, 13]. Currently, sulphate pulping (Kraft) is the dominant process responsible for more than 90 % of pulp production [13]. In Kraft pulping, wood chips are impregnated with the pulping liquor (a mixture of NaOH and Na_2S at pH=13‑14). The soaked chips are heated up to 160‑180 ^oC, for 1‑3 h, in batch or continuous digesters [12]. Alternatively, acid sulphite chemical pulping is carried out under acidic conditions (pH 1‑2) at 135‑145 ^oC for 8‑12 h in batch digesters using $SO_2/MHSO_3/MSO_3$ aqueous solution (M is a pulping base, Na, Ca, Mg or NH_3) [12, 13]. About 6 % of all chemical pulps are produced by sulphite pulping acid, essentially by acidic sulphite process. The most sustainable pulping processes are those using regenerable pulping bases (*i.e.* Na^+ and Mg^{2+}) [12, 13]. The use of hardwoods, mainly beech, eucalypt and birch, in the production of acid sulphite pulps is gaining an increased attention in the last years. Besides papermaking sulphite pulps are also applied in the production of viscose and cellulose esters [9], valorising these cellulosic pulps.

During the sulphite pulping, lignin is sulphonated and removed from wood as salts of lignosulphonates (LS) (**Figure 2.4**). A significant part of hemicelluloses are hydrolysed and removed from wood to spent sulphite liquor. Cellulose is maintained almost intact during acidic sulphite pulping [12,13]. The block scheme of acid sulphite pulp production is presented in **Figure 2.5**. After the pulping process, unbleached pulp is washed and HSSL containing dissolved LS and degraded carbohydrates are concentrated by evaporation [11].

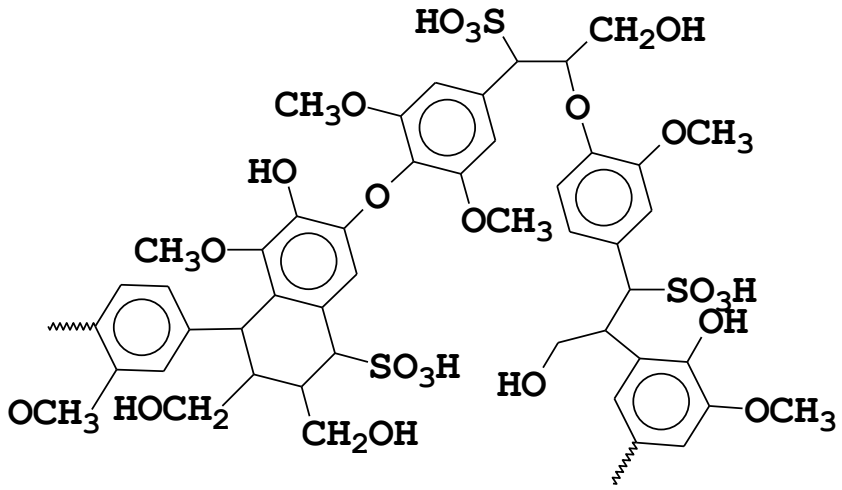


Figure 2.4. Schematic representation of hardwood lignosulphonic acid (lignosulphonate). Sulphonic groups are linked essentially at benzylic carbon of lignin structural units.

Concentrated HSSLs may be burned for the energy and reagents recovery (case of Na^+ and Mg^{2+} bases) or commercialised. Alternatively, HSSLs can be bioprocessed for ethanol production. The remaining fermentation broth after ethanol extraction can be further concentrated and used either for burning or as a chemical feedstock [11].

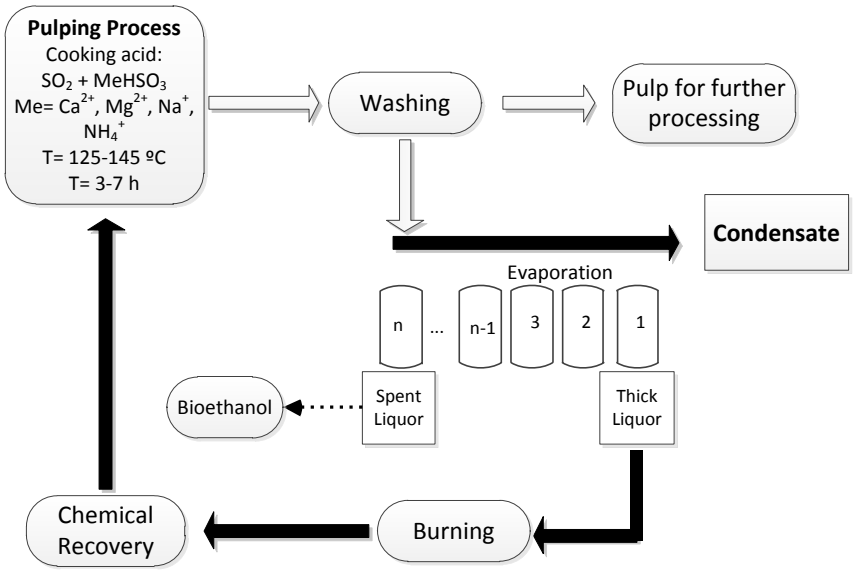


Figure 2.5. General block-scheme of acidic sulphite wood pulping process with the SSL release.

The fermentation of softwood SSLs (SSSLs) into bioethanol is a conventional industrial practice in North America and Europe [18]. Besides bioethanol production, LS from SSSL are widely used as concrete additives, binding agents and additive to animal

feed, among other applications [24]. On the contrary, the fermentation of HSSL into bioethanol is not yet implemented in practice, because of the more complex metabolism of pentoses requiring different biological and technological solutions. One of the main challenges for industrial implementation of this process is the search of a suitable ethanol-producing organism able to ferment pentoses and simultaneously resistant to HSSL inhibitors [6, 10, 23]. More than 50 % of the SSLs produced annually are HSSLs, what means that at least 45 billion litres are produced per year [15].

2.4 Composition of hardwood spent sulphite liquors

The application and processing of HSSLs depend significantly on their chemical composition, which is basically determined by the origin of wood involved in the pulping process [9, 24]. Since hemicelluloses of softwoods are mainly hexosanes and hemicelluloses of hardwood are essentially pentosans, the corresponding SSLs after acidic sulphite pulping contain mainly hexoses and pentoses [12], respectively (**Table 2.3**). HSSL contains significant amounts of dissolved organic matter corresponding to a high chemical oxygen demand (COD > 100000 $mg_{O^2}.L^{-1}$). Hence, it cannot be discharged into natural basins due to environmental concerns and must be treated before its disposal [11, 24]. This is especially important for HSSL from sulphite pulping involving non-regenerable pulping base (Ca^{2+} or NH_4^+).

Table 2.3. General chemical composition of SSSL and HSSL.

	SSSLs ($g.L^{-1}$) [12, 13]	HSSLs ($g.L^{-1}$) [6,9]
Lignosulphonates	110.0-120.0	77.6 – 78.8
Mannose	21.0 – 27.0	7.6 – 9.4
Glucose	7.0 – 9.7	2.2 – 2.4
Galactose	4.7 – 6.0	4.4 - 4.6
Xylose	9.0 – 11.0	24.1 – 25.1
Arabinose	0.69 – 2.0	7.5 – 8.1
Acetic Acid	3.0	9.5 – 9.7
Furfural	0.20	Traces
SO_2	0.50	-[a]
Extractives	Traces	Traces

[a]Data not available

As mentioned above, during the sulphite pulping process, a significant part of hemicelluloses are solubilised enriching HSSLs in monomeric sugars [6, 9, 25]. The composition of sugars in HSSL depends on the composition of wood processed (**Table 2.2**). A direct correlation between the chemical composition of wood used in pulping and the chemical composition of HSSL is possible when single species are involved (*e.g. Eucalyptus globulus*) (**Table 2.2** and **Table 2.4**). Furthermore the composition of HSSLs from pulping of mixed hardwoods is less predictable due to the undefined composition of incoming raw materials. In general, the major components of HSSLs are LS followed by the monomers from hydrolysed hemicelluloses (**Table 2.4**). Pentoses are the dominant sugars in HSSLs, namely xylose (16–43 $g.L^{-1}$) and arabinose (1.0–21.0 $g.L^{-1}$). Hexoses are present at lower levels namely glucose (2.3–9.0 $g.L^{-1}$) mannose (1.0–9.0 $g.L^{-1}$) and galactose (1.6–5.0 $g.L^{-1}$). Worth noting, a remarkable part of dissolved carbohydrates (up to 25%) is present in HSSL in the form of oligosaccharides [11, 13]. Among the volatile compounds, acetic acid is the most abundant although furfural is also present but at low concentrations (**Table 2.4**) [9, 12]. The significant amounts of acetic acid and xylose in HSSL (**Table 2.4**) are the result of extensive degradation of acetylated glucuronoxylan, which is the predominant hemicellulose in hardwoods (**Table 2.2**) [9].

Table 2.4. Chemical composition ($g.L^{-1}$) of different HSSLs.

	E. globulus			Mixed HSSL[a]			Red Oak [23]		Oak
	[12]	[6,9]	[54]	[55]	[56]	[25]	Untreated	Pretreated	[42]
pH	2.9	3.4	2.0	5.5	5.4 - 6.5	7.0	1.7	6.5	-**
Dry solids	128.0	148.0	-	-	240.0	-	220.0	-	-
Ash	28.0	19.8	-	-	-	-	-	-	-
HMF	-[b]	-	-	1.1	-	-	-	-	0.90
Furfural	2.0	≤ 0.10	-	1.8	0.50	-	0.20	0.060	0.30
Acetic acid	8.0	8.2	-	10.0	9.0	8.5	9.3	4.2	10.9
Lignosulphonates	59.0	78.2	-	-	170.0	-	-	-	-
Glucose	3.0	2.3	3.9	2.4	4.5	2.4	3.0	5.4	9.0
Mannose	1.0	8.5	7.8	4.3	8.0	6.8	6.5	9.0	3.4
Xylose	21.0	24.6	21.7	20.0	16.0	22.4	26.7	40.2	43.5
Arabinose	1.0	7.8	-	0.80	1.0	-	1.5	21	2.9
Galactose	5.0	4.5	2.4	1.6	2.0	-	-	-	3.3

[a] Mixture of hardwood and softwood SSL

[b] Data not available

The presence of acetic acid is one of the problems of HSSL utilization for bioethanol production, since it can act as an inhibitor for many ethanol-producing organisms. HSSLs also contain other possible fermentation inhibitors and a preliminary detoxification step should be considered [6, 10, 25]. The presence and type of such inhibitors will be discussed in more detail below (Section 2.5).

2.5 Microbial inhibitors present in HSSL

When compared to the fermentation of pure sugars, the use of industrial by-products like HSSL as substrate provides slower kinetics with lower yields and productivities due to the presence of microbial inhibitors [26]. In some cases, a complete inhibition of cell growth and/or ethanol production can be observed. Hence, the major challenge for commercial production of bioethanol from HSSL is to overcome the presence of inhibitory compounds that are generated during the wood pulping. These may affect not only cell growth but also the metabolic pathways related to ethanol production [1, 8, 27]. The maximum concentration allowed for each inhibitor, without losing fermentation efficiency, depends on several factors: type of compound and its inhibition mechanism, the microbial strain and the physiological state. The fermentative process technology and the dissolved oxygen concentration in the medium as well as the pH are also important features to consider [1, 8, 28]. Moreover, a synergistic effect between the different inhibitors can occur frequently [1]. Fermentation inhibitors are conventionally classified into four different groups according to their origin: sugar degradation products, lignin degradation products, compounds derived from extractives and heavy metal ions [1, 27]. This classification does not include free SO_2 in HSSL, because it is normally eliminated by vapour distillation or by liquor heating under vacuum (*e.g.* during the first evaporation stage of spent liquor, (**Figure 2.5**).

Sugar degradation products are formed after polysaccharide hydrolysis by dehydration [29]. Pentoses produce furfural and hexoses are converted in hidroxymethylfurfural (HMF) and levulinic acid [29]. Furfural can inhibit cell growth, affecting the specific growth rate and yield [27]. However, at low concentrations (< 1 $g.L^{-1}$), some ethanol-producing microorganisms like *Scheffersomyces stipitis* are not affected by

furfural [30]. Previously, Nigam (2001) also had shown that the yield and productivity of ethanol by this yeast were not affected by 0.25 $g.L^{-1}$ of furfural. Nevertheless, concentrations above 1.5 $g.L^{-1}$ were found to interfere with microbial respiration and inhibit cell growth almost completely, decreasing ethanol yield by 90 % and productivity in 85 % [23]. HMF presents an inhibitory effect similar to furfural, but to lower extent. In the acid sulphite pulping processes reduced amounts of hexoses are degraded originating low concentrations of HMF in HSSL. Due to the high reactivity of HMF under acidic conditions, this compound is readily converted to levulinic acid. Hence, HMF is present in lower concentrations than furfural. HMF was found to increase the lag phase extension and decrease the growth rate of microorganisms [26, 27]. The extension of inhibition is usually increased when various compounds are mixed together. Mussatto and Roberto (2004) reported a synergistic effect when furfural and HMF are combined with compounds originated from lignin degradation thus forming aromatic, polyaromatic, phenolic, and aldehydic molecules [9].

Lignin derivatives constitute another group of microbial inhibitors. In particular, it was found that LS from sulphite pulping of *E. globulus* correspond to partially sulphonated lignin oligomers including monomeric phenolic compounds with one or two the sulphonic groups [9, 10]. The amounts of sulphonic groups in those LS were rather high (up to 20% of SO_3H groups) and were constituted predominantly by syringyl units, with a similar S:G proportion as in wood [31].

Lignin degradation products are considered even more toxic to microorganisms than furfural and HMF. All phenolic compounds, resulting from delignification can be associated to a certain toxicity. Low weight phenolics are the most toxic, even in low concentrations [8, 21, 32, 33]. Xavier *et al.*(2010) and Pereira *et al.*(2012) highlighted the toxicity of HSSL from *E. globulus* as biological substrate. With 60% (v/v) of HSSL, no growth of *S. stipitis* was observed [6]. Biological removal of acetic acid was carried out with a deacidificant yeast (*S. cerevisiae*) but ineffective fermentation to bioethanol was obtained due to the presence of phenolic inhibitors [6]. In recent work by Pereira *et al.*(2012), *S. stipitis* was able to produce ethanol after a previous step of bio-detoxification with a filamentous fungus, *Paecilomyces variotti* [10]. Palmqvist and Hahn-Hagerdal (2010)

described that phenolic compounds could cause a loss of integrity of biological membranes, thus, affecting their ability as selective barriers and as enzyme matrices. These authors also detected a decrease in cell growth and sugar assimilation rates in the presence of these compounds [27, 34]. Syringaldehyde and vanillic acid, two phenolic compounds of low molecular weight, also affected cell growth [35] and the ethanolic fermentative metabolism of several microorganisms, including *S. stipitis* [26].

Another group of inhibitors includes extractives (acidic resins, tanninic acid and terpenic acids) and acetic acid. In general, the extractives are considered less toxic to microbial growth than lignin derivatives [1]. However, some hardwoods (*e.g.* eucalypt and oak) contain high amount of hydrolysable tannins giving rise to gallic acid and pyrogallol during the pulping [9]. These phenolic compounds are known inhibitors of microbial growth, possessing strong anti-fungal properties [36, 37].

Acetic acid can also act as an inhibitor for some microorganisms and its inhibition mechanism is well-understood [27, 33, 38]. As acetic acid is a weak acid, there is always a fraction of this acid that is protonated, according to respective pK_a. According to Russel (1992), this form of protonated acid is lipophilic and travels across the cell membrane releasing one proton in response to the higher intracellular pH [38]. This dynamic equilibrium regulated by the pH gradient is certainly responsible for intracellular anion accumulation giving rise to cell inhibition [1, 22, 25, 33, 38]. The inhibition degree of acetic acid was reported to depend not only on its concentration, but also on the dissolved oxygen concentration and on the external pH [39].

Besides the aforementioned compounds, heavy metals, released from equipment corrosion during acidic pulping processes, can be found in HSSL and have a negative effect on the metabolism of microorganisms [11].

2.6 Detoxification methods

Several technologies have been employed to remove inhibitory compounds from HSSLs. These include overliming (mainly with $Ca(OH)_2$), treatment with activated charcoal, separation by ion exchange resins, and extraction with solvents [1, 19, 28]. Activated charcoal can be used as an adsorbent to eliminate high quantities of phenolic compounds.

Nevertheless, the use of activated charcoal can reduce the amount of sugars in the raw material and it is an expensive method since the used charcoal cannot be easily regenerated [1]. The overliming is considered as an effective tool for the elimination of colloids [40, 41]. However, this purification practice, carried out with $Ca(OH)_2$, may cause some damage to the equipment due to deposits with calcium salts (usual calcifications in the piping industrial systems).

Treatment with anionic exchange resins effectively removes inhibitors originating a feedstock that allows for a fermentation performance similar as pure sugars [1, 6, 15]. Thus ion-exchange resins (cationic and anionic) were successfully used to separate sugars present in HSSL from acetic acid and polyphenolic inhibitors. A cation-exchange resin was used to remove Mg^{2+}, the pulping base, and other cations and an anion-exchange resin to remove free carboxylic acids and polyphenolics including LS [6, 15]. The purified fraction contained essentially monomeric sugars, although a very small proportion of neutral polyphenolics was also detected, *ca.* 4 $g.L^{-1}$ [6]. The procedure was described employing ion-exchange resins for the purification of sugars present in HSSL as a way to improve the biological production of value-added products from HSSL [6, 15]. However, ion-exchange resins are expensive and difficult to implement and operate in large scale industries [11].

Recently the researcher's attention is increasing towards biological detoxification methods, using fungi whole cultures or their extracellular enzymes [33]. Biological removal of acetic acid by using deacidificant yeasts was tested with *Saccharomyces cerevisae*, *Candida tropicalis*, *Candida utilis* and *Pichia anomala* but it did not allow bioethanol production from HSSL since other inhibitors were present [6, 42]. Wood-degrading white-rot fungi are attractive microorganisms to detoxify industrial by-products, such as HSSLs, since they effectively degrade low molecular weight phenolics. This kind of fungi excretes extracellular enzymes able to degrade lignin and LS [1, 11, 27]. The most important lignolytic enzymes are laccase, manganese peroxidase and lignin peroxidase, but the mechanism of phenolics degradation is not yet completely understood [20, 21, 33]. *Trametes versicolor* is the most used fungus for lignolytic enzymes production to degrade a broad variety of pollutants [1].

Recently, filamentous fungus *P. variotti* was used to detoxify HSSL. *P. variotti* was able to assimilate acetic acid and low molecular weight phenolics namely gallic acid and pyrogallol [10]. These compounds were already reported as exhibiting anti-fungal properties and as inhibiting *S. stipitis* pentoses fermentation into ethanol [6, 10]. *P. variotti* is usually found in air and soils of tropical countries and has been used for the production of single cell protein (SCP) from SSL, vinasse, rayon hydrolysate and wood hydrolysate, among other raw materials [10]. Several studies showed the ability of *P. variotii* to degrade efficiently toxic compounds, namely acetic acid, toluene, ethyl benzene and partially assimilate benzene, *m*- and *p*-xylenes in liquid cultures [43]. *P. variotii* also has the ability to degrade *p*-coumaric acid, a phenolic compound widely present in the cell wall of graminaceous plants [44]. Wang *et al.*(2010) have studied the degradation of phenol by *P. variotii* and the obtained results suggested that this strain was able to consume phenol as sole carbon source [45]. These studies also showed that phenolics can be converted into compounds with lower toxicity, increasing the possibility of using the industrial by-products as substrates for other microorganisms [44-46]. *P. variotti* treatment, in preliminary results, is a very promising detoxification method despite needing some specific controlled conditions (aeration, temperature and pH can increase the process cost). Furthermore, its biomass has been used as SCP for animal feeding [47] and currently is considered a high nutritional protein source. Transforming HSSL into high value added products fits the biorefinary concept and improves the economic and environmental sustainability of pulping industries.

2.7 Bioethanol production from HSSL

2.7.1 Wild strains

In order to obtain an economically viable conversion process of any raw material, it is imperative that the microorganisms chosen should be able to efficiently convert the sugars present into the desired end-product [1, 21, 22]. The ideal yeast for bioethanol

production from LCB should consume sugars present and resist to inhibitory effects from other components providing high production yields and specific productivities [21].

One of the most effective and well-known ethanol-producing microorganism from hexoses is *S. cerevisiae.* This yeast is successfully employed at industrial scale, allows for high ethanol productivity, namely from SSSL, and bears high tolerance to ethanol and to inhibitors present in LCB [6]. Therefore, the use of *S. cerevisiae* in fermentation of SSSL has been studied since 1907, when it started to be used in Sweden for bioethanol production, and also during the World War II, for yeast production as a nutritional source of protein and vitamins [18].

Fermentative processes by *S. cerevisiae* are regulated by sensing the presence of glucose [20]. Cellular transport of glucose can be made by different transporters, with higher or lower glucose affinity, and its expression depends on glucose concentration on the media [48]. Efficient glucose fermentation to ethanol must involve anaerobic conditions in order to minimize the formation of biomass and glycerol. The metabolism of glucose and also mannose and galactose by *S. cerevisiae* is also well understood at large scale fermentations [7, 18, 21]. In contrast, *S. cerevisiae* is unable to efficiently utilize xylose as a sole carbon source and cannot ferment it to ethanol [21-22]. Since hexoses are directly metabolized through glycolysis, pentoses metabolization is initiated by pentose phosphate pathway before entering in glycolysis [11, 21, 22]. *S. cerevisiae* does not possess specific xylose transporters and xylose enters the cell through glucose transporters. When xylose arrives the *S. cerevisiae* cytosol all the subsequent enzymes needed for the complete metabolic pathway are present but the expression levels are very low [18, 21, 22]. Consequently, production rates of ethanol from xylose are ten times lower than those obtained using glucose as substrate [8, 21, 22]. Therefore to use *S. cerevisiae* in order to bioethanol production from HSSL further strain optimization must be done to achieve efficient pentoses metabolization.

Other yeasts were also tested to convert efficiently the xylose present in HSSL to ethanol, such as, *S. stipitis*, *Candida shehatae* and *Pachysolen tannophilus*. Among all of

them, *S. stipitis* showed the best potential for industrial application due to the high ethanol yield obtained [11, 49].

The majority of the D-xylose-fermenting yeasts of interest belongs to the genus *Scheffersomyces* [50]. *S. stipitis* (the new taxonomic classification for the yeast *Pichia stipitis* [50]) is one of the few known yeast species that have the capacity to efficiently ferment pentoses, mainly D-xylose. Moreover it was described as the native yeast with the highest capacity for xylose fermentation of any known microbes, which is essential for application at industrial level [21, 49]. This yeast is able to metabolize all of the major monosaccharides found in wood with no other significant by-products. Moreover, since this microorganism is closely related to yeast endosymbionts that inhabit and degrade white-rotted hardwood, it can transform low-molecular weight lignin moieties [49].

In addition to its extensively studied capacity for xylose fermentation, some studies found that *S. stipitis* is also able to metabolize glucose, mannose, galactose and cellobiose along with mannan and xylan oligomers [49]. Recently, Jeffries *et al.*(2007) sequenced and assembled the complete genome of *S. stipitis* and the sequence data revealed numerous genes for the utilization of LCB components. These authors gave a preliminary insight into the regulation of main metabolic pathways and several examples of co-localized genes with related functions [49]. Moreover, *S. stipitis* is highly sensitive to compounds normally found in HSSLs, namely formic, acetic and levulinic acids, phenolics, and furfural. For this reason, HSSL needs a preliminary detoxification step [6, 10].

However, ethanol production by *S. stipitis* is not industrially implemented yet, since the fermentation process is not optimized. The optimum oxygen concentration in the bioreactor, which is the main process bottleneck, still needs to be determined [23, 49]. Since this yeast produces ethanol in response to oxygen limitation, the optimal activity can be reached by applying micro-aerophilic conditions [23]. The oxygen supply should be limited in order to prevent both xylose uptake only for cell growth and oxidation of the produced ethanol. Nevertheless, the oxygen should be enough to cellular maintenance and to avoid slow xylose utilization. This means that specific micro-aeration conditions should

be carefully chosen to obtain ethanol successful production [6, 23]. Besides, the mechanism of pentose fermentation of *S. stipitis* is repressed during the presence of hexoses [51]. Hence, it is necessary to improve the fermentative strategy in the presence of a sugars mixture in order to maximize ethanol yields from pentoses [51, 52]. Also *S. stipitis* exhibit lower ethanol tolerance than *S. cerevisae* showing that strain improvement must consider product inhibition mechanism.

In *S. stipitis* metabolism, xylose is converted through pentose-phosphate pathway (**Figure 2.6**), which includes NADH- or NADPH-dependent xylose reductase (XR) and NAD-dependent xylitol dehydrogenase (XDH), as seen in **Figure 2.6** [11, 21]. Xylose conversion may also occur by xylose isomerase (XI) activity. In pentose-fermenting yeasts, xylose catabolism begins with its reduction to xylitol by XR, which is oxidized to xylulose by NAD-dependent XDH. Xylulose is phosphorylated by the enzyme xylulokinase (XK) to produce xylulose-5-phosphate (X5P), entering then in the pentose phosphate pathway and after in the glycolytic pathway. The formed intermediates are converted to pyruvate in the Embden‑Meyerhof‑Parnas pathway. Pyruvate fermentation occurs by decarboxylation promoted by pyruvate decarboxylase to acetaldehyde which is then reduced to ethanol by alcohol dehydrogenase [11, 21].

There are few studies about bioethanol production from HSSL [11], which are summarized in the **Table 2.5**. As mentioned before, Xavier *et al.*(2010) used adsorption in ion-exchange resins to remove toxic compounds from HSSL for a preliminary sugar purification followed by ethanol fermentation with *S. stipitis.* This process provided a fraction containing essentially neutral monomeric sugars with traces of neutral polyphenols. This sugar fraction was fermented by *S. stipitis* with a high fermentation substrate conversion, 96%, productivity, 1.22 $g.L^{-1}.h^{-1}$, and yield, 0.49 $g.g^{-1}$ (**Table 2.5**) [6]. For the industrial implementation this type of technology for sugar purification needs to be optimized since the associated costs are high [11, 15, 24].

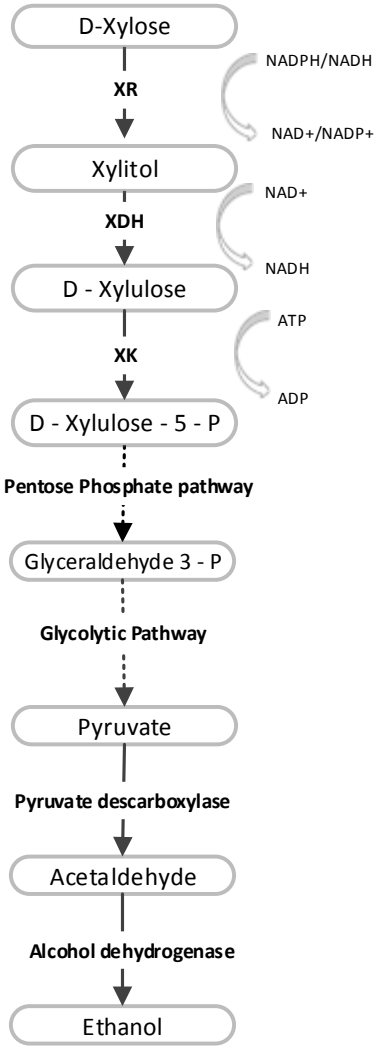


Figure 2.6. General D-xylose metabolic pathway in yeasts and fungi.

Pereira *et al.*(2012) studied a preliminary biological treatment of HSSL with *P. variotti* as an alternative methodology of purification. A successful fermentation of the detoxified HSSL by *S. stipitis* was performed, attaining an ethanol yield of 0.24 $g.g^{-1}$ (**Table 2.5**). However, more research is required in order to improve and optimize the ethanol fermentation yields and productivities [10].

More detailed information about genomics, proteomics and metabolism regulation in *S. stipitis* is required in order to improve bioethanol production from xylose. A further knowledge about *S. stipitis* will allow for a targeted genetic engineering to improve fermentative activities [49].

Table 2.5. Fermentation parameters of HSSLs using different microorganisms.

HSSL	Detoxification method	Microorganism	$[EtOH]_{max}$ ($g.L^{-1}$)	$Y_{ethanol}$ ($g.g^{-1}$)	$Q_{ethanol}$ ($g.L^{-1}.h^{-1}$)	Conversion efficiency (%)	Reference
E. globulus	Ion exchange resins	*S. stipitis* NRRL Y-7124	8.1	0.49	1.22	96.1	[6]
E. globulus	Biological treatment w/ *P. variotii*	*S. stipitis* NRRL Y-7124	2.4	0.24	0.09	47.1	[10]
Red Oak	Overliming	*S. stipitis* (HSSL-adapted)	20.2	0.41	0.44	82.4	[23]
		S. stipitis NRRL Y-7124	9.7	0.30	0.11	59.0	
		S. cerevisiae RLJY - 019 (HSSL - adapted)	6.8	0.47	0.45	92.4	
Red Oak	Untreated	*S. stipitis*(HSSL - adapted)	6.7	0.28	0.070	55.0	[23]
		S. stipitis NRRL Y-7124	1.2	0.16	0.010	31.4	
Mixture[a] (Tembeq)	-[b]	*S. cerevisiae* R57	-[b]	0.41	-[b]	80.0	[55]
Mixture (Tembeq)	Untreated	*S. cerevisiae* 259ST (G.M.O.)[c]	12.0	0.32 - 0.42	0.10	62.7 - 82.4	[54,56]
		S. cerevisiae 259 A	3.5	0.15 - 0.32	0.030	29.4 - 62.7	
		S. cerevisiae T2 (SSL-adapted)	3.5	0.15 - 0.32	0.030	29.4 - 62.7	
Mixture (Tembeq)	Untreated	*E. coli* (G.M.O.)[c]	12.0	0.40	0.16	78.4	[25]
	Overliming		8.1	0.44	0.49	86.3	
	Diluted/untreated		7.3	0.43	0.61	84.3	

[a]Mixture of hardwood and softwood SSL
[b]Data nor available
[c]Genetically modified organism

2.7.2 Engineered and adapted strains

During recent years, a number of different approaches were used to modify *S. cerevisiae* for xylose, xylan or cellulose metabolism, including genetic engineering with specific genes from *S. stipitis* (XR or XDH related genes) [20-22, 25]. According to Jeffries *et al.*(2007) *S. cerevisiae* lacks sufficient levels of the assimilatory genes, sugar transporters and mechanisms for balancing cofactor levels under oxygen-limiting conditions [53]. Hence, *S. cerevisiae* transformations were only partially successful because inappropriate regulatory responses were obtained [49, 53].

Helle *et al.*(2004) compared the ability to ferment SSSL and HSSL of a genetically modified *S. cerevisiae* 259ST (with introduction of XR and XDH genes from *S. stipitis* and overexpression of XK) with robust industrial strains. Ethanol yields were the lowest for HSSL (**Table 2.5**). However, with micro-aerophilic conditions a significant improvement in the ethanol yield was observed. Under these conditions, over 99% of the mannose and glucose,

and 95% of galactose were consumed during fermentation with the genetically modified strain. For HSSL, the highest ethanol concentration (28 $g.L^{-1}$) was attained when the liquor was supplemented with sugars (glucose and xylose), yeast extract and peptone [54]. However, this was not an appropriate approach for ethanol production by genetically modified *S. cerevisiae* 259ST in HSSL, since both sugars and supplements were added. This could not be applied in an industrial scale and could not be considered as HSSL fermentation.

More recently, Pinel *et al.*(2011) tried to improve the HSSL tolerance of an *industrial S. cerevisiae* strain by genome shuffling. Genome shuffling is a way to rapidly propagate beneficial mutations in a directed evolution experiment. In this study, large populations of mutants were crossed, with a selection step between crossing events, to increase the genetic diversity within the parent populations. Three strains were isolated, after five rounds of genome shuffling, which were able to grow on undiluted HSSL and to produce ethanol efficiently from the sugars present in HSSL. It was used as a system mimicking the CRBF (cell recycle batch fermentation) used in industry, where the culture was recycled repeatedly into fresh HSSL, to assess the effect of prolonged exposure to HSSL on ethanol productivity at a high cell density. *S. cerevisiae* R57 was the most productive strain isolated in this study, reaching theoretical ethanol yields of approximately 80 % (after 5 passes of HSSL). This finding suggests that this strain would be able to maintain ethanol productivity during prolonged exposure to HSSL inhibitors in an industrial setting [55].

Not only *S. cerevisae* was improved by genetic engineering but other microorganisms like *S. stipitis* or *Escherichia coli* were also modified for ethanol production from HSSL. Nigam (2001) studied ethanol production from HSSL obtained from red oak cooking with an adapted strain of *S. stipitis*, obtained by sequentially transferring and growing in media containing increased concentrations of HSSL. The liquor was boiled and also pre-treated with calcium hydroxide achieving a final xylose concentration in the range of (4–5%, w/v) (**Table 2.5**). As described before, Nigam (2001) obtained a better fermentation performance when the HSSL was treated. The ethanol yield and productivity

increased from 0.16 to 0.30 $g.g^{-1}$ and 0.01–0.11 $g.L^{-1}.h^{-1}$ (**Table 2.5**). As expected, the adapted strain produced ethanol at rates, yields and concentrations higher than the parent strain. Ethanol yield and productivity were increased to 0.41 $g.g^{-1}$ and 0.44 $g.L^{-1}.h^{-1}$, respectively [23]. Nigam (2001) also analysed the optimum oxygen transference rate (OTR) value for the adapted strain, which was 2.0 $mmol_{O_2}\ L^{-1}.h^{-1}$.

Lawford and Rousseau (1993) analysed the fermentative performance of a recombinant *E. coli* B (ATCC 11303 pLOI297) using anaerobic batch fermentations of both nutrient-supplemented soft and hardwood SSL (30–35 $g.L^{-1}$ total reducing sugars). *E. coli* took 150 h to ferment only 3 % of the sugars present in the HSSL, and the ethanol conversion efficiency was 70 % [25]. The introduction of a preliminary treatment of HSSL, with calcium hydroxide (overliming pretreatment), resulted in a decrease of fermentation time from 150 to 24 h and in a slight improvement of yield, from 0.36 to 0.40 $g.g^{-1}$ (**Table 2.5**).

2.8 Challenges

The technologies to produce ethanol from sugar or starch are already well established, while the technologies to produce bioethanol from by-products or wastes are far from being optimised. In the specific case of industrial by-products enriched in pentoses, like HSSL there are still some challenges to overcome, such as:

- The detoxification step still needs improvements since this stage needs to obey two presumptions. First, the detoxification should efficiently eliminate the inhibitors from the liquor. The second presumption relates to the fact that this step has to be implemented in an industrial scale and the associated costs must be considered;
- The ethanol conversion rate and yield is also a feature that needs to be improved in order to produce higher amounts of ethanol. For this reason, major research efforts are still needed especially in the optimization of strategies for finding efficient microorganisms. The selected microbial strain should efficiently metabolize all pentose and hexose sugars but also present high inhibitors tolerance in order to achieve a commercial production of ethanol through HSSL.

The bioethanol production from HSSL must be further optimized. More specific research is still needed in order to define new strategies towards a comprehensive utilization production to fulfil environmental concerns and improve the sustainability of pulp plants.

2.9 References

1. Mussatto, S.I. and I.C. Roberto, *Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review.* Bioresource Technology, 2004. **93**(1): p. 1-10.
2. Sannigrahi, P., Y. Pu, and A. Ragauskas, *Cellulosic biorefineries-unleashing lignin opportunities.* Current Opinion in Environmental Sustainability, 2010. **2**(5): p. 383-393.
3. Hamelinck, C.N., G.v. Hooijdonk, and A.P.C. Faaij, *Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term.* Biomass and Bioenergy, 2005. **28**(4): p. 384-410.
4. Limayem, A. and S.C. Ricke, *Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects.* Progress in Energy and Combustion Science, 2012. **38**(4): p. 449-467.
5. Haveren, J.v., E.L. Scott, and J. Sanders, *Bulk chemicals from biomass.* Biofuels, Bioproducts and Biorefining, 2008. **2**(1): p. 41-57.
6. Xavier, A.M.R.B., M.F. Correia, S.R. Pereira, and D.V. Evtuguin, *Second-generation bioethanol from eucalypt sulphite spent liquor.* Bioresource Technology, 2010. **101**(8): p. 2755-2761.
7. Bacovsky, D., W. Mabee, and M. Worgetter, *How close are second-generation biofuels?* Biofuels Bioproducts & Biorefining, 2010. **4**(3): p. 249-252.
8. Balat, M., *Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review.* Energy Conversion and Management, 2011. **52**(2): p. 858-875.
9. Marques, A.P., D.V. Evtuguin, S. Magina, F.M.L. Amado, and A. Prates, *Chemical Composition of Spent Liquors from Acidic Magnesium-Based Sulphite Pulping of Eucalyptus globulus.* Journal of Wood Chemistry and Technology, 2009. **29**(4): p. 322-336.
10. Pereira, S.R., Š. Ivanuša, D.V. Evtuguin, L.S. Serafim, and A.M.R.B. Xavier, *Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol.* Bioresource Technology, 2012. **103**(1): p. 131-135.
11. Fernandes, D.L.A., S.R. Pereira, L.S. Serafim, D. Evtuguin, and A. Xavier, *Second Generation Bioethanol from Lignocellulosics: Processing of Hardwood Sulphite Spent Liquor*, in *Bioethanol*, M.A.P. Lima, Editor 2011, Intech
12. Sjöström, E., *Wood Chemistry. Fundamentals and Applications*. 2nd Ed., 1993, New York, USA: Academic Press. 293.
13. Sixta, H., A. Potthast, and A.W. Krotschek, Editors. *Handbook of Pulp*., H. Sixta. Editor, Vol. 1. 2006, Wiley-VCH: Weinheim. 109-427.

14. Hon, D.N.-S. and N. Shiraishi, Editors. *Wood And Cellulosic Chemistry*. 2nd Ed., 2001, Marcel Dekker: New York.
15. Fernandes, D.L.A., C.M. Silva, A.M.R.B. Xavier, and D.V. Evtyugin, *Fractionation of sulphite spent liquor for biochemical processing using ion exchange resins.* Journal of Biotechnology, 2012 **162**(4): p. 415-421.
16. Chirat, C., D. Lachenal, and M. Sanglard, *Extraction of xylans from hardwood chips prior to kraft cooking.* Process Biochemistry, 2012. **47**(3): p. 381-385.
17. Rowell, R.M., R. Pettersen, J.S. Han, J.S. Rowell, and M.A. Tshabalala, *Structure and Chemistry: Cell wall chemistry* in *Handbook of wood chemistry and wood composites* R.M. Rowell, Editor 2005, CRC Press: Boca Raton. p. 43-79.
18. Kosaric, N., H.J. Pieper, T. Senn, and F. Vardar-Sukan, *The Biotechnology of Ethanol: Classical and Future Applications*, M. Roehr Editor, 2001, Weinheim: WILEY-VCH.
19. Rutz, D. and R. Janssen, *Biofuel Technology Handbook*, 2nd Ed., 2008, München: WIP Renewable Energies.
20. Margeot, A., B. Hahn-Hägerdal, M. Edlund, R. Slade, and F. Monot, *New improvements for lignocellulosic ethanol.* Current Opinion in Biotechnology, 2009. **20**(3): p. 372-280.
21. Hahn-Hagerdal, B., K. Karhumaa, C. Fonseca, I. Spencer-Martins, and M.F. Gorwa-Grauslund, *Towards industrial pentose-fermenting yeast strains.* Applied Microbiology and Biotechnology, 2007. **74**(5): p. 937-953.
22. Hahn-Hägerdal, B., K. Karhumaa, M. Jeppsson, and M. Gorwa-Grauslund, *Metabolic Engineering for Pentose Utilization in Saccharomyces cerevisiae*, in *Biofuels*, L. Olsson, Editor 2007, Springer Berlin / Heidelberg. p. 147-177.
23. Nigam, J.N., *Ethanol production from hardwood spent sulfite liquor using an adapted strain of Pichia stipitis.* Journal of Industrial Microbiology & Biotechnology, 2001. **26**(3): p. 145-150.
24. Evtuguin, D.V., M.R.B. Xavier, C.M. Silva, and A. Prates. *Towards comprehensive utilization of products from sulphite pulp production: A biorefinary approach*. in *XXI Encontro Nacional da TECNICELPA / VI CIADICYL*. 2010. Lisbon, Portugal.
25. Lawford, H.G. and J.D. Rousseau, *Production of ethanol from pulp-mill hardwood and softwood spent sulfite liquors by genetically-engineered Escherichia coli.* Applied Biochemistry and Biotechnology, 1993. **39**(1): p. 667-685.
26. Delgenes, J.P., R. Moletta, and J.M. Navarro, *Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae.* Enzyme and Microbial Technology, 1996. **19**(3): p. 220-225.
27. Palmqvist, E. and B. Hahn-Hagerdal, *Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition.* Bioresource Technology, 2000. **74**(1): p. 25-33.
28. Palmqvist, E. and B. Hahn-Hägerdal, *Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification.* Bioresource Technology, 2000. **74**(1): p. 17-24.
29. Rosatella, A.A., S.P. Simeonov, R.F.M. Frade, and C.A.M. Afonso, *ChemInform Abstract: 5-Hydroxymethylfurfural (HMF) as a Building Block Platform: Biological Properties, Synthesis and Synthetic Applications.* ChemInform, 2011. **42**(32): p. 754-793.

30. Liu, Z.L., P.J. Slininger, B.S. Dien, M.A. Berhow, C.P. Kurtzman, and S.W. Gorsich, *Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran.* Journal of Industrial Microbiology & Biotechnology, 2004. **31**(8): p. 345-352.
31. Marques, A.P., D.V. Evtuguin, S. Magina, F.M.L. Amado, and A. Prates, *Structure of Lignosulphonates from Acidic Magnesium-Based Sulphite Pulping of Eucalyptus globulus.* Journal of Wood Chemistry and Technology, 2009. **29**(4): p. 337-357.
32. Bajwa, P.K., T. Shireen, F. D'Aoust, D. Pinel, V.J.J. Martin, J.T. Trevors, and H. Lee, *Mutants of the Pentose-Fermenting Yeast Pichia stipitis With Improved Tolerance to Inhibitors in Hardwood Spent Sulfite Liquor.* Biotechnology and Bioengineering, 2009. **104**(5): p. 892-900.
33. Chandel, A., S. da Silva, and O. Singh, *Detoxification of Lignocellulose Hydrolysates: Biochemical and Metabolic Engineering Toward White Biotechnology*. BioEnergy Research, 2012. **6**(1): p. 1-14.
34. Parajó, J.C., H. Domínguez, and J. Domínguez, *Biotechnological production of xylitol. Part 3: Operation in culture media made from lignocellulose hydrolysates.* Bioresource Technology, 1998. **66**(1): p. 25-40.
35. Cortez, D.V. and I.C. Roberto, *Individual and interaction effects of vanillin and syringaldehyde on the xylitol formation by Candida guilliermondii.* Bioresource Technology, 2010. **101**(6): p. 1858-1865.
36. Panizzi, L., C. Caponi, S. Catalano, P.L. Cioni, and I. Morelli, *In vitro antimicrobial activity of extracts and isolated constituents of Rubus ulmifolius.* Journal of Ethnopharmacology, 2002. **79**(2): p. 165-168.
37. Upadhyay, G., S.P. Gupta, O. Prakash, and M.P. Singh, *Pyrogallol-mediated toxicity and natural antioxidants: Triumphs and pitfalls of preclinical findings and their translational limitations.* Chemico-Biological Interactions, 2010. **183**(3): p. 333-340.
38. Russell, J.B., *Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling.* Journal of Applied Microbiology, 1992. **73**(5): p. 363-370.
39. Van Zyl, C., B.A. Prior, and J.C. du Preez, *Acetic acid inhibition of d-xylose fermentation by Pichia stipitis.* Enzyme and Microbial Technology, 1991. **13**(1): p. 82-86.
40. Millati, R., C. Niklasson, and M.J. Taherzadeh, *Effect of pH, time and temperature of overliming on detoxification of dilute-acid hydrolyzates for fermentation by Saccharomyces cerevisiae.* Process Biochemistry, 2002. **38**(4): p. 515-522.
41. Conde-Mejía, C., A. Jiménez-Gutiérrez, and M. El-Halwagi, *A comparison of pretreatment methods for bioethanol production from lignocellulosic materials.* Process Safety and Environmental Protection, 2012. **90**(3): p. 189-202.
42. Perego, P., A. Converti, E. Palazzi, M. Del Borghi, and G. Ferraiolo, *Fermentation of hardwood hemicellulose hydrolysate by Pachysolen tannophilus, Candida shehatae, and Pichia stipitis.* Journal of Industrial Microbiology & Biotechnology, 1990. **6**(3): p. 157-164.
43. Garcia-Pena, I., I. Ortiz, S. Hernandez, and S. Revah, *Biofiltration of BTEX by the fungus Paecilomyces variotii.* International Biodeterioration & Biodegradation, 2008. **62**(4): p. 442-447.

44. Sachan, A., S. Ghosh, and A. Mitra, *Biotransformation of p-coumaric acid by Paecilomyces variotii.* Letters in Applied Microbiology, 2006. **42**(1): p. 35-41.
45. Wang, L., Y. Li, P. Yu, Z. Xie, Y. Luo, and Y. Lin, *Biodegradation of phenol at high concentration by a novel fungal strain Paecilomyces variotii JH6.* Journal of Hazardous Materials, 2010. **183**(1-3): p. 366-371.
46. Prenafeta-Boldú, F.X., R. Summerbell, and G. Sybren de Hoog, *Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard?* FEMS Microbiology Reviews, 2006. **30**(1): p. 109-130.
47. Holmgren, M. and A. Sellstedt, *Identification of white-rot and soft-rot fungi increasing ethanol production from spent sulfite liquor in co-culture with Saccharomyces cerevisiae.* Journal of Applied Microbiology, 2008. **105**(1): p. 134-140.
48. Kruckeberg, A.L., *The hexose transporter family of Saccharomyces cerevisiae.* Archives of Microbiology, 1996. **166**(5): p. 283-292.
49. Jeffries, T.W., I.V. Grigoriev, J. Grimwood, J.M. Laplaza, A. Aerts, A. Salamov, J. Schmutz, E. Lindquist, P. Dehal, H. Shapiro, Y. Jin, V. Passoth, and P.M. Richardson, *Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast Pichia stipitis.* Nature Biotechnology, 2007. **25**(3): p. 319-326.
50. Kurtzman, C. and M. Suzuki, *Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera Babjeviella, Meyerozyma, Millerozyma, Priceomyces, and Scheffersomyces.* Mycoscience, 2010. **51**(1): p. 2-14.
51. Agbogbo, F.K., G. Coward-Kelly, M. Torry-Smith, and K.S. Wenger, *Fermentation of glucose/xylose mixtures using Pichia stipitis.* Process Biochemistry, 2006. **41**(11): p. 2333-2336.
52. Webb, S.R. and H. Lee, *Regulation of d-xylose utilization by hexoses in pentose-fermenting yeasts.* Biotechnology Advances, 1990. **8**(4): p. 685-697.
53. Jeffries, T.W. and J.R.H. Van Vleet, *Pichia stipitis genomics, transcriptomics, and gene clusters.* FEMS Yeast Research, 2009. **9**(6): p. 793-807.
54. Helle, S.S., A. Murray, J. Lam, D.R. Cameron, and S.J.B. Duff, *Xylose fermentation by genetically modified Saccharomyces cerevisiae 259ST in spent sulfite liquor.* Bioresource Technology, 2004. **92**(2): p. 163-171.
55. Pinel, D., F. D'Aoust, S.B. del Cardayre, P.K. Bajwa, H. Lee, and V.J.J. Martin, *Saccharomyces cerevisiae Genome Shuffling through Recursive Population Mating Leads to Improved Tolerance to Spent Sulfite Liquor.* Applied and Environmental Microbiology, 2011. **77**(14): p. 4736-4743.
56. Helle, S. and S.J.B. Duff, *Supplementing spent sulfite liquor with a lignocellulosic hydrolysate to increase pentose/hexose co-fermentation efficiency and ethanol yield*. Report submitted to Tembec Industries Inc. Chemical Products Group: Vancouver. 2004, p. 177.
57. Gírio, F.M., C. Fonseca, F. Carvalheiro, L.C. Duarte, S. Marques, and R. Bogel-Łukasik, *Hemicelluloses for fuel ethanol: A review.* Bioresource Technology, 2010. **101**(13): p. 4775-4800.
58. Björling, T. and B. Lindman, *Evaluation of xylose-fermenting yeasts for ethanol production from spent sulfite liquor.* Enzyme and Microbial Technology, 1989. **11**(4): p. 240-246.

CHAPTER III

Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol

The fermentation of reducing sugars from hardwood (eucalypt) spent sulphite liquor (HSSL) into ethanol by *Pichia* (*Scheffersomyces*) *stipitis* is hindered by concomitant inhibitors of microbial metabolism. The conditions for the HSSL biological treatment step by *Paecilomyces variotii* were evaluated and optimised. Two different strategies of reactor operation were compared using single batch (B) and sequential batch reactor (SBR).

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3.1 Introduction

Lignocellulosic biomass is the most abundant renewable biological resource and, since is outside the human food chain is also an attractive and relatively inexpensive raw-material. In 2008 approximately 200 $\times$ 10^9 tons of lignocellulosics (mainly wood) were harvested and only 3% of them were used in pulp and paper industries [1]. Lignocellulosics are the main source of renewable biomass possessing a potential target for fuel to heat and power production and also for chemicals or materials feedstocks. These chemicals can serve as precursors for many products replacing fossil-based resources [1, 2].

The majority of lignocellulosic raw materials requires a complex chemical or enzymatic hydrolysis in order to obtain sugars before their bioprocessing. The hydrolysis is frequently one of the most problematic steps due to the difficult degradability of cellulose, caused by physical, chemical, structural and composition factors [3, 4].

Hardwood (eucalypt) spent sulphite liquor (HSSL), the side product from acidic sulphite pulping of wood, is normally burned to produce energy. The annual production of bleached sulphite eucalypt pulp is around one million tones per year contributing to the economic profits of South Africa, Portugal and Spain [5]. Acidic sulphite wood pulping is carried out at high temperature (135–140 ^oC) under acidic conditions (pH < 1.8) in order to delignify wood and obtain bleachable pulps for papermaking. The main advantage of SSLs over other lignocellulosics is that after wood pulping process, sugars are already in a monomeric fermentable form [5, 6].

The practical application of SSLs depends significantly on their chemical composition, which is basically determined by the wood origin involved in the pulping process [7, 8]. Typical HSSL from *Eucalyptus globulus* contains lignosulphonates (60–80 $g.L^{-1}$) and sugars (35–45 $g.L^{-1}$) from hydrolysed hemicelluloses, mainly xylose. Hence, HSSL is a prospective substrate for bioprocessing once it has a high concentration of monomeric sugars and some proportion of oligomeric saccharides [5]. However, the presence of high amounts of acetic acid (8–9 $g.L^{-1}$), furfural, polyphenols and low molecular weight lignosulphonates inhibits the microbial metabolism which is the main drawback for HSSL bioprocessing [3, 6]. These products of hemicellulose and lignin degradation negatively affect fermentation efficiency, due to their toxicity towards fermentative microorganisms,

inhibiting both growth and alcoholic metabolisms [3]. The removal of inhibitors from HSSL is a difficult task and not resolved at a practical level yet.

Paecilomyces variotii is a fungus usually found in air and soils of tropical countries. This fungus has been used for the production of single cell protein (SCP) due to its optimal performance in degrading polluted residues like SSL, vinasse, rayon hydrolyzate and wood hydrolyzate, amongst others [9]. Several studies showed the ability of *P. variotii* to consume toxic compounds. García-Peña *et al.*(2008) have shown that this fungus was able to degrade efficiently toluene, ethyl benzene and partially assimilate benzene, *m*- and *p*-xylenes in liquid culture [10]. *P. variotii* has also the ability to degrade a phenolic compound widely present in the cell wall of graminaceous plants, *p*-coumaric acid [11]. Wang *et al.*(2010) have studied the degradation of phenol by *P. variotii.* The reported results suggested that the strain consumed phenol as sole carbon source [12]. For the reasons stated *P. variotti* can be used due to its huge capacity of specific detoxification, making toxic industrial by-products into suitable substrates for further bioprocessing by more sensitive microorganisms.

Scheffersomyces stipitis, a pentose-utilising yeast, is considered a good candidate for ethanol production from HSSL since presents high conversion rates [6, 13]. Bioethanol is one of the products which can be obtained via biorefinery and one of the most attractive biofuels, since it can be easily produced in large amounts and blended with gasoline or used pure as a "green" fuel. Its bioproduction from renewable lignocellulosic feedstocks contributes to the replacement of fossil fuels and the reduction of CO_2 build-up.

The main objective of this study was to analyse and optimise the HSSL detoxification step with the filamentous fungus *P. variotii.* Two different fermentative approaches were performed and compared in order to study the best operational strategy: (i) single batch reactor (B), and (ii) sequential batch reactor (SBR). The fermentations were monitored essentially by HPLC though the profiles of inhibitory phenolic compounds in the fermentation media were followed by GC‑MS. The potential of HSSL in the bioethanol production by *S. stipitis* was also evaluated.

3.2 Materials and methods

3.2.1 HSSL supply and pretreatment

Industrial HSSL from magnesium based acidic sulphite pulping of *E. globulus* was supplied by Caima-Indústria de Celulose SA (Constância, Portugal). The pretreatment of HSSL consisted in pH adjustment to 7.0 with 6 M KOH solution, followed by aeration with compressed air for 2 h per litre of HSSL processed. The precipitated colloids were centrifuged at 2000 rpm (1500×*g*) during 1 h and then the supernatant was filtered using a 1.0 µm glass microfiber filter (Ahlstrom, grade 131).

3.2.2 Microorganisms and media

P. variotii NRRL-1115 and *S. stipitis* NRRL Y-7124 [14], were gently supplied by Agricultural Research Service Culture Collection at National Center for Agricultural Utilization Research, USDA.

The stock culture of *P. variotii* was grown at 28 ± 0.5 ^{o}C on Petri dishes with ME agar medium (30 g malt extract, 5 g peptone and 15 g agar per litre of distilled water), stored at 4 ^{o}C and transferred monthly.

S. stipitis culture was grown at 28 ± 0.5 ^{o}C and maintained at 4 ^{o}C on YM agar slants (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 20 g agar per litre of distilled water). All media and material were autoclaved at 121 ^{o}C for 20 min.

3.2.3 Preparation of inocula

P. variotii was grown in Petri dishes during 7 days at 28 ^{o}C. Inocula were prepared in order to achieve an initial cell concentration of 70.0 mg.L^{-1} according to Tavares *et al.*(2005) procedure [15]. The necessary volume of inoculum was transferred to 500 mL Erlenmeyer flasks, with 250 mL of working volume.

The inocula of *S. stipitis* were prepared with YM liquid broth (3.0 g yeast extract, 3.0 g malt extract, 5.0 g peptone and 10.0 g glucose per litre of distilled water) and incubated during 20 h before being transferred to fermentations in a proportion of 10 % (v/v).

3.2.4 Biological treatment of HSSL

The single batch (B) assays were carried out in duplicate in 500 mL Erlenmeyer flasks containing 250 mL of HSSL. Each shake flask was inoculated with an initial cell concentration of 70.0 $mg.L^{-1}$ and cultivated at 28 ^{o}C in an orbital shaker at 180 rpm for 16 days.

Regarding the SBR tests, the initial cycle started as a single batch. Each cycle was ended when the acetic acid reached a non-inhibitory concentration. Fungal biomass was sterile filtrated and transferred into the new flask with fresh HSSL, starting the next cycle. This procedure was repeated for three cycles.

At the end of the process, the bio-treated HSSL was centrifuged at 2000 rpm (1500 $\times$ *g)* during 1 h, filtered using a 1.0 µm glass microfiber filter (Ahlstrom, grade 131) and kept at 4 ^{o}C until being utilised for bioethanol production.

3.2.5 Fermentation of bio-treated HSSL to bioethanol

The fermentation media were prepared with 60% (v/v) of bio-treated HSSL, 30% (v/v) of a supplementary medium (SM, $g.L^{-1}$): yeast extract 2.5; $(NH_4)_2HPO_4$ 2.0; $(NH_4)_2SO_4$ 1.0 and $MgSO_4$. $7H_2O$ 0.50) and 10% (v/v) of inoculum. Fermentations were carried out in 500 mL Erlenmeyer flasks containing 250 mL of working volume in an incubator operating at 28 ^{o}C and 180 rpm. All assays were performed in duplicate.

3.2.6 Analytical methods

P. variotii concentration was determined by dry weight of fungal mycelium. *S. stipitis* biomass was monitored measuring optical density (OD) at 650 nm and converted into concentration using a calibration curve of OD versus biomass dry weight.

Acetic acid and ethanol were analysed by HPLC using a 10 µm Eurokat H (Knauer) ion-exchange column, 300 $\times$ 7.5 mm with an oven (ParaLab) set at 40 ^{o}C, and refraction index (RI) detector Gilson 131. The eluent was sulphuric acid 0.01 N, with a flow rate of 0.4 mL min^{-1}. The injected volume was 20 µL. All samples were centrifuged and filtered off with 0.20 µm filters (VectaSpin Micro, Whatman) before the analysis. An absolute calibration was applied for all analysed compounds.

Reducing sugars were analysed by the DNS (dinitrosalicylic acid) method described by Miller (1959) [16].

For the phenolic compounds analysis, 20.0 mL of HSSL in different stages of fermentation (before starting fermentation, after 8 days and 16 days of *P. variotii* fermentation) were acidified until pH 2.0 with concentrated HCl. Three liquid‑liquid extractions (1:1, v/v) were made to each sample using diethyl ether. The organic phase was collected and passed through anhydrous sodium sulphate to remove traces of aqueous phase. Then, the collected organic phase was concentrated on a vacuum rotor evaporator at 40 ^{o}C until complete dryness. The remaining solids were dissolved in 0.500 mL solution of pyridine with the internal standard, tetracosane (2.0 mg.mL^{-1}). Then, 200 µL BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide) and 50 µL TMSC (trimethylchlorosilane) were added and the mixture was left to react at room temperature during 24 h. The samples were analysed by GC‑MS, as trimethylsilane (TMS) derivatives according to Marques et al. (2009) and results obtained are related to the amount of internal standard added to the samples.

3.3 Results and discussion

3.3.1 HSSL biological treatment

3.3.1.1Single batch reactor

Direct fermentation of HSSL by *P. variotti* in a single batch (B) reactor has been studied. The batch assay lasted for 16 days in order to study the fungus consumption of sugars, acetic acid and other compounds from this complex substrate. *P. variotii* fermentative behaviour is showed in **Figure 3.1**. At the beginning, HSSL contained 65.8 g.L^{-1} of reducing sugars and 11.4 g.L^{-1} of acetic acid. On the 11th day, acetic acid was depleted by *P. variotii* and sugars were also consumed attaining a concentration of 9.0 g.L^{-1} . Comparing the sugars uptake rate, until 11th day, (5.2 g.L^{-1}.day^{-1}) with the acetic acid uptake rate (1.0 g.L^{-1}.day^{-1}) the preference in sugars as carbon sources by *P. variotti* has been evidenced.

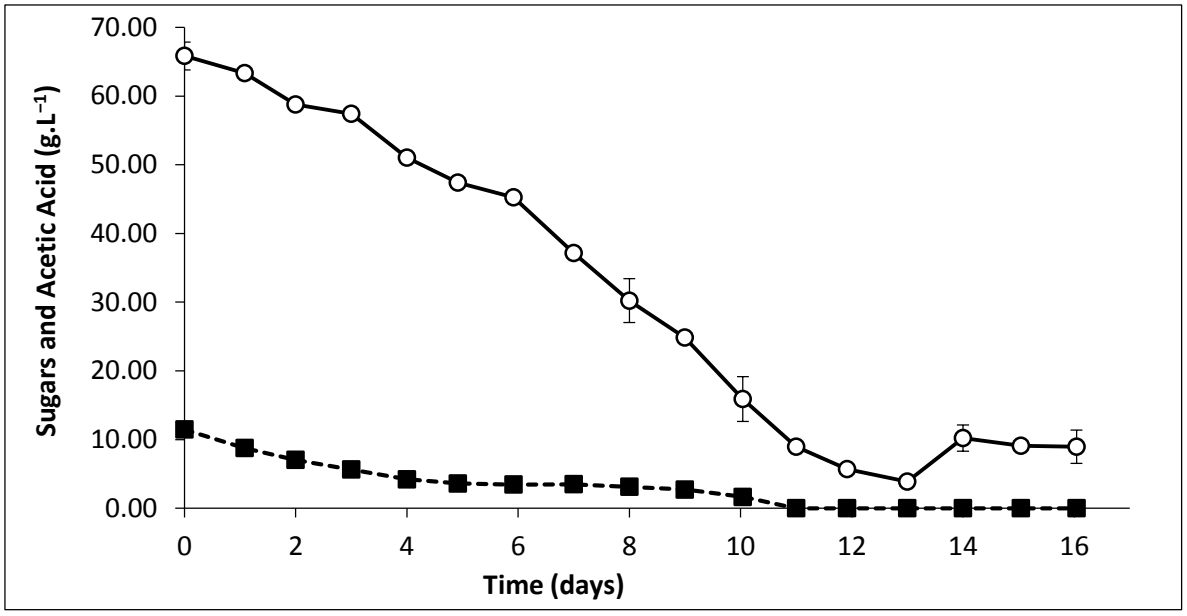

Figure 3.1. Fermentation of HSSL by *P. variotii* during 16 days: reducing sugars (○) and acetic acid (■).

Observing the time course, after the 6th day of fermentation, the acetic acid concentration reached a value (3.5 $g.L^{-1}$) that was lower than the inhibitory levels for *S. stipitis* growth and fermentation. According to Van Zyl *et al.*(1991), *S. stipitis* can ferment pentoses to ethanol under oxygen-limited conditions and acetic acid concentration lower than 9.0 $g.L^{-1}$ [17]. The concentration of sugars on the 6th was high enough (45.0 $g.L^{-1}$) for a successful bioethanol fermentation by *S. stipitis.*

Sugars consumption continued until the 13th day, after which a small increase in sugars concentration was observed. This fact certainly resulted from the hydrolytic action of fungal enzymes on xylo-oligosaccharides (XOS) still present in the HSSL. Marques *et al.*(2009) reported that 30 % of xylose in HSSL is usually in the form of XOS, which resulted from polysaccharides hydrolysis during the industrial pulping. *P. variotti* is a eukaryotic microorganism able to develop different metabolic mechanisms under specific conditions, namely in presence of different carbon sources. It was also, reported that *P. variotti* is able to produce enzymes such as, β-glucosidase and xylanase [18, 19]. These enzymes are capable to hydrolyse the oligomers still present in HSSL.

After 16 days of operation, despite the depletion of acetic acid, *P. variotti* was still active, for this reason the following experiments were planned with the objective of testing the possibility to reutilize the fungus in consecutive batches.

3.3.1.2 Sequential batch reactor process

After the promising results obtained in the B reactor, a set of trials in sequential batch reactor (SBR) was planned in order to test the possibility of reutilization of *P. variotti* in the HSSL biological treatment. A SBR with three cycles was planned and each cycle ended when the acetic acid reached a non-inhibitory concentration. The evolution of sugars and acetic acid concentration along the three SBR cycles are displayed in **Figure 3.2**.

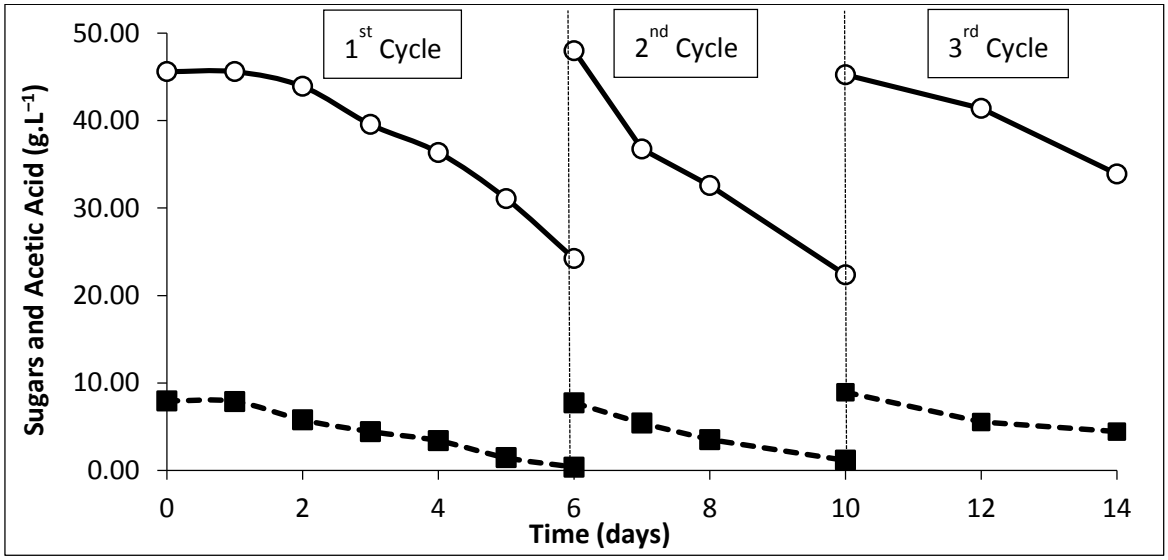

Figure 3.2. Fermentation of HSSL by *P. variotii in s*equential batch reactor with three cycles in HSSL: reducing sugars (○) and acetic acid (■).

As in the B reactor assay, the 1st cycle of the SBR has started with similar sugar and acetic acid concentrations. *P. variotti* consumed sugars and acetic acid with the depletion of the latter after 6 days. The acetic acid was consumed slower than sugars, with uptake rate values of 1.3 and 3.6 $g.L^{-1}.day^{-1}$, respectively (**Table 3.1**). These differences in the uptake rates showed that the fungus, probably, assimilated preferentially sugars as the main carbon source. The 2nd cycle ended after 4 days since the decrease in acetic acid concentration was faster than in the previous cycle with the uptake rate of 1.6 $g.L^{-1}.day^{-1}$.

The values of acetic acid uptake rate as well as the sugars uptake rate (6.4 $g.L^{-1}.day^{-1}$) were higher than the values obtained in the 1st cycle (**Table 3.1**). These facts indicate that the fungus was already adapted to the substrate (HSSL) since no lag phase has been observed (**Table 3.1**).

Table 3.1. Sugars and acetic acid uptakes rates for the single batch and SBR assays.

	Sequential Batch Reactor			Single Batch Reactor
	1st cycle	2nd cycle	3rd cycle	
r_{sugars} ($g.L^{-1}.day^{-1}$)	3.6	6.4	2.8	3.5
$r_{acetic\ acid}$ ($g.L^{-1}$)	1.3	1.6	1.1	1.0

In the 3rd cycle, *P. variotti* was unable to deplete acetic acid during the same time as in the previous cycle. Not only acetic acid but also sugars were consumed slowly since lower values of acetic acid and sugars uptake rates were obtained, 1.1 and 2.8 $g.L^{-1}.day^{-1}$, respectively. These results showed that *P. variotti* was probably affected in its metabolism being unable to keep the performance observed during the 2nd cycle. The results showed that two SBR cycles were the best option or, at least, no more than three SBR cycles should be performed, because the acetic acid and sugars uptake rates obtained were lower than those of 1st cycle. This inhibition in sugars and acetic acid uptaking certainly resulted from the fact that HSSL is a complex mixture of many compounds, namely lignosulphonates and low molecular phenolic compounds that can also have been consumed by *P. variotti* and resulted in metabolic damage.

Although the B assay lasted 16 days, after the 6th day the acetic acid in HSSL was low enough for ending the process. In the SBR approach, a higher amount of HSSL was processed (1.2 L in three cycles contrast 0.40 L in a B) in a shorter period of time than in the B assay. SBR takes advantage over B in the fermentation of bio-detoxified HSSL to bioethanol. Another advantage results from the fact that for three SBR cycles only one inoculum was needed, thus reducing the labour and the overall operation time. Phenolic compounds can cause loss of integrity of biological membranes, thereby affecting their ability to act as selective barriers and enzyme matrices. For this reason physiological functions of *P. variotti,* as cell growth and sugar assimilation, occurred at lower rates.

3.3.2 Phenolics evolution of HSSL during *P. variotii* fermentation

As it was noted in section 3.3.1.2, the performance of *P. variotti* decreased with time. The reasons for the loss of efficiency in consumption of acetic acid and sugars were investigated by analysing the profile of phenolic compounds along the operational period of reactor B. The type and amount of low molecular weight extractive compounds extracted with diethyl ether were determined in initial HSSL before inoculation, on day 8 and on day 16 of the batch assay and results are shown in **Table 3.2**.

Table 3.2. HSSL composition through different times of fermentation.

	Compound	% m/m[a]		
		Day 0	8 days fermentation	16 days fermentation
Organic acids	Lactide acid	0.55	1.02	0
	Hydroxyacetic acid	1.24	0	29.46
	Levulinic Acid	0	1.01	0
	Furoic Acid	9.67	11.84	15.75
	Propanoic acid	0	0	1.86
	3-methyl-butanoic acid	0	0	1.09
	Succinic acid	0	18.60	0
	Malic Acid	1.85	4.45	3.33
Syringil and guaycil derivatives	Pyrogallol	3.18	0	0
	2,6-dimethoxy-4-(1-propenyl)-phenol acid	7.51	2.37	0
	Syringaldehyde	1.22	0	0
	Vanillic alchool	0	0.80	0
	5-methylbenzene-tricarboxylic acid	3.72	0	0
	Vanillic acid	1.78	0	0
	Syringic acid	0	1.66	0
	Gallic acid	18.07	0	0

[a] % is related to the internal standard used

Amongst the phenolic compounds identified in the HSSL before the fermentation, gallic acid was the most abundant (**Table 3.2**). This acid is normally formed from hydrolysable tannins during the sulphite pulping [5] and it bears strong antimicrobial properties. Gallic acid, usually present in leaves and soil, has been reported as inhibitory to fungi [20]. Panizzi *et al.*(2002) reported the inhibition of *Candida albicans* growth by this acid [21]. Another abundant polyphenol detected in HSSL, pyrogallol (**Table 3.2**), occurs

naturally in eucalyptus as a decomposition product of hydrolysable tannins and exhibits anti-fungal properties [22]. Syringaldhyde and vanillic acid found in HSSL are of lower abundance than gallic acid and pyrogallol and also can display inhibitory effects on microbial growth [3, 23] and on their ethanolic fermentative metabolism. Delgenes *et al.*(1996) reported similar effect for *S. stipitis* [24]. This type of compounds due to their associated toxicity is the major factor that limits the bioconversion processes of HSSL.

On 8th day of biotreatment the composition of HSSL suffered marked changes. The initially recognised toxic compounds (gallic acid, pyrogallol, syringaldehyde and vanillic acid) were not detected and, probably were degraded by *P. variotti.* In opposite, increased amounts of levulinic, succinic, furoic, lactic and malic acids were observed up on biotreatment of HSSL (**Table 3.2**). Simultaneously, a set of new phenolic compounds was identified, such as syringic acid and vanillic alcohol. The low molecular organic acids probably resulted from the ring cleavage of phenolics found in the initial HSSL sample and from the degradation of furfural and hydroxymethyl furfural into furoic and levulinic acid, respectively. The unexpected appearance of several phenolics during bio-purification of HSSL by *P. variotii* can be explained by its metabolism. Vanillic acid is a known metabolite of lignin degradation derivatives, such as ferulic acid [5, 25]. Rahouti *et al.*(1989) described that *P. variotii* can metabolize ferulic acid to vanillic acid, which can be further metabolized to pyrogallol [25]. Syringaldehyde can be formed from the conversion of sinapic acid type structures present in HSSL and can be converted to syringic acid, which was actually detected on the HSSL sample from the 8th day [26, 27].

In the last sample, after 16 days of fermentation, all phenolic compounds were completely degraded by *P. variotti* and the main compounds present were low molecular weight organic acids, essentially furoic and glycolic acids.

Hence *P. variotii* consumed the major toxic compounds present in HSSL and, despite the excretion of other metabolites, the growth of *P. variotii* in HSSL is a suitable bio-detoxification method for transforming this industrial by-product into a substrate for other microorganisms.

3.3.3 Bioethanol production by *S. stipitis* with bio-detoxified HSSL

In previous studies *S. stipitis* has showed a good capacity to produce bioethanol from HSSL when ion-exchange resins were applied to remove toxic compounds, namely acetic acid and phenolic compounds [6]. After its biological detoxification via SBR, HSSL was supplied to *S. stipitis* for the fermentation of sugars to ethanol. **Figure 3.3** shows that *S. stipitis* was able to ferment the reducing sugars from HSSL to ethanol. During the fermentation, three main stages were observed. The first stage that corresponded to the lag phase lasted from the beginning of fermentation until time 4 h. During this time, as expected, no sugars consumption or ethanol production occurred and no biomass growth was observed. The second stage started at 4 h and ended at 28 h and an increase in the sugars consumption was verified, reaching a concentration of 8.04 $g.L^{-1}$. The maximum amount of ethanol produced (2.36 $g.L^{-1}$) was obtained at 28 h. During the last stage (from 28 h until 32 h) sugars concentration remained almost constant, and the ethanol concentration decreased to 1.80 $g.L^{-1}$. This decrease in ethanol concentration was observed possibly due to the metabolic ethanol oxidation.

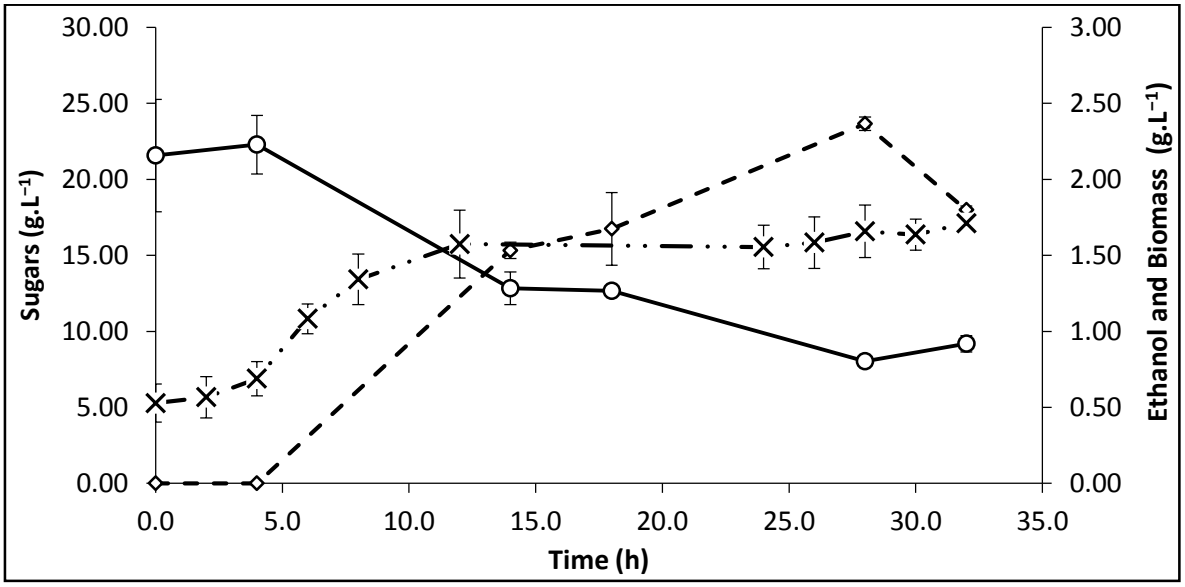

Figure 3.3. Fermentation of bio-detoxified HSSL with *S. stipitis* on HSSL treated by *P. varioti*: reducing sugars (○), biomass (×) and ethanol (◇).

During this experiment, an ethanol yield of 0.17 $g.g^{-1}$ was achieved. The maximum specific growth rate was 0.17 h^{-1}, which is of the same order of magnitude as reported in literature for *S. stipitis* [6, 13].

The results of this study showed that biological detoxification of HSSL could be successfully performed by *P. variotti* thus removing the inhibitory compounds and allowing the ethanol production by *S. stipitis*. However, the ethanol yield and the sugars conversion efficiency by *S. stipitis* are still far from optimised. The optimisation of bioethanol production by *S. stipitis* process should be accomplished in future work.

3.4 Conclusions

The results of this study revealed that the biological detoxification of eucalypt HSSL with *P. variotti* is an effective way to ennoble this industrial sub-product. *P. variotti* was able to assimilate acetic acid as well as low molecular weight phenolics (gallic acid and pyrogallol amongst others). These are recognised inhibitors of pentoses fermentation into ethanol by *S. stipitis*. Bio-processing of HSSL in SBR was considered as a promising strategy for a fast and efficient HSSL detoxification. With this technology a higher amount of detoxified liquor could be obtained when compared to the processing in B reactor. The bio-detoxification by *P. variotti* allowed for a successful ethanolic fermentation of HSSL by *S. stipitis*. The maximum concentration attained was 2.36 $g.L^{-1}$ with a yield of 0.17 $g.g^{-1}$.

3.5 References

1. Zhang, Y.H.P., *Reviving the carbohydrate economy via multi-product lignocellulose biorefineries.* Journal of Industrial Microbiology & Biotechnology, 2008. **35**(5): p. 367-375.
2. Sánchez, Ó.J. and C.A. Cardona, *Trends in biotechnological production of fuel ethanol from different feedstocks.* Bioresource Technology, 2008. **99**(13): p. 5270-5295.
3. Mussatto, S.I. and I.C. Roberto, *Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review.* Bioresource Technology, 2004. **93**(1): p. 1-10.

4. Alvira, P., E. Tomas-Pejo, M. Ballesteros, and M.J. Negro, *Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review.* Bioresource Technology, 2010. **101**(13): p. 4851-4861.
5. Marques, A.P., D.V. Evtuguin, S. Magina, F.M.L. Amado, and A. Prates, *Chemical Composition of Spent Liquors from Acidic Magnesium-Based Sulphite Pulping of Eucalyptus globulus.* Journal of Wood Chemistry and Technology, 2009. **29**(4): p. 322-336.
6. Xavier, A.M.R.B., M.F. Correia, S.R. Pereira, and D.V. Evtuguin, *Second-generation bioethanol from eucalypt sulphite spent liquor.* Bioresource Technology, 2010. **101**(8): p. 2755-2761.
7. Sjöström, E., *Wood Chemistry. Fundamentals and Applications*. 2nd Ed., 1993, New York, USA: Academic Press.
8. Smook, G.A., *Handbook for pulp & paper technologists*. 3rd Ed., 2002, Canada: Angus Wilde Publications.
9. Almeida e Silva, J.B., U.A. Lima, M.E.S. Taqueda, and F.G. Guaragna, *Use of response surface methodology for selection of nutrient levels for culturing Paecilomyces variotii in eucalyptus hemicellulosic hydrolyzate.* Bioresource Technology, 2003. **87**(1): p. 45-50.
10. Garcia-Pena, I., I. Ortiz, S. Hernandez, and S. Revah, *Biofiltration of BTEX by the fungus Paecilomyces variotii.* International Biodeterioration & Biodegradation, 2008. **62**(4): p. 442-447.
11. Sachan, A., S. Ghosh, and A. Mitra, *Biotransformation of p-coumaric acid by Paecilomyces variotii.* Letters in Applied Microbiology, 2006. **42**(1): p. 35-41.
12. Wang, L., Y. Li, P. Yu, Z. Xie, Y. Luo, and Y. Lin, *Biodegradation of phenol at high concentration by a novel fungal strain Paecilomyces variotii JH6.* Journal of Hazardous Materials, 2010. **183**(1-3): p. 366-371.
13. Nigam, J.N., *Ethanol production from hardwood spent sulfite liquor using an adapted strain of Pichia stipitis.* Journal of Industrial Microbiology & Biotechnology, 2001. **26**(3): p. 145-150.
14. Kurtzman, C. and M. Suzuki, *Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera Babjeviella, Meyerozyma, Millerozyma, Priceomyces, and Scheffersomyces.* Mycoscience, 2010. **51**(1): p. 2-14.
15. Tavares, A.P.A., M.S.M. Agapito, M.A.M. Coelho, J.A. Lopes da Silva, A. Barros-Timmons, J.A.J. Coutinho, and A.M.R.B. Xavier, *Selection and Optimization of Culture Medium for Exopolysaccharide Production by Coriolus (Trametes) Versicolor.* World Journal of Microbiology and Biotechnology, 2005. **21**(8-9): p. 1499-1507.
16. Miller, G.L., *Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar.* Analytical Chemistry, 1959. **31**(3): p. 426-428.
17. van Zyl, C., B.A. Prior, and J.C. du Preez, *Acetic acid inhibition of d-xylose fermentation by Pichia stipitis.* Enzyme and Microbial Technology, 1991. **13**(1): p. 82-86.
18. Guimaraes, L.H.S., S.C. Peixoto-Nogueira, M. Michelin, A.C.S. Rizzatti, V.C. Sandrim, F.F. Zanoelo, A.C.M.M. Aquino, A.B. Junior, and M.d.L.T.M. Polizeli, *Screening of*

filamentous fungi for production of enzymes of biotechnological interest. Brazilian Journal of Microbiology, 2006. **37**(4): p. 474-480.

19. Job, J., R. Sukumaran, and K. Jayachandran, *Production of a highly glucose tolerant β-glucosidase by Paecilomyces variotii MG3: optimization of fermentation conditions using Plackett–Burman and Box–Behnken experimental designs.* World Journal of Microbiology and Biotechnology, 2010. **26**(8): p. 1385-1391.
20. Dix, N.J., *Inhibition of fungi by gallic acid in relation to growth on leaves and litter.* Transactions of the British Mycological Society, 1979. **73**(2): p. 329-336.
21. Panizzi, L., C. Caponi, S. Catalano, P.L. Cioni, and I. Morelli, *In vitro antimicrobial activity of extracts and isolated constituents of Rubus ulmifolius.* Journal of Ethnopharmacology, 2002. **79**(2): p. 165-168.
22. Upadhyay, G., S.P. Gupta, O. Prakash, and M.P. Singh, *Pyrogallol-mediated toxicity and natural antioxidants: Triumphs and pitfalls of preclinical findings and their translational limitations.* Chemico-Biological Interactions, 2010. **183**(3): p. 333-340.
23. Cortez, D.V. and I.C. Roberto, *Individual and interaction effects of vanillin and syringaldehyde on the xylitol formation by Candida guilliermondii.* Bioresource Technology, 2010. **101**(6): p. 1858-1865.
24. Delgenes, J.P., R. Moletta, and J.M. Navarro, *Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae.* Enzyme and Microbial Technology, 1996. **19**(3): p. 220-225.
25. Rahouti, M., F. Seigle-Murandi, R. Steiman, and K.-E. Eriksson, *Metabolism of Ferulic Acid by Paecilomyces variotii and Pestalotia palmarum.* Applied and Environmental Microbiology, 1989. **55**(9): p. 2391-2398.
26. Bergbauer, M., *Degradation and oligomerization of syringic acid by distinctive ecological groups of fungi.* Microbial Ecology, 1991. **21**(1): p. 73-84.
27. Mukherjee, G., A. Sachan, S. Ghosh, and A. Mitra, *Conversion of sinapic acid to syringic acid by a filamentous fungus Paecilomyces variotii.* The Journal of General and Applied Microbiology, 2006. **52**(2): p. 131-135.

CHAPTER IV

Production of Single Cell Protein from Eucalypt sulphite spent liquor by *Paecilomyces variotii*

Spent Sulphite Liquors (SSLs) are by-products from the acidic sulphite pulping process, rich in monomeric sugars that can be converted to bioethanol. However SSLs contain high amounts of acetic acid and lignosulphonates at inhibitory levels for microbial growth. *Paecilomyces variotii* is able to grow in SSLs using this inhibitors. Its biomass can be used as single cell protein (SCP). Hence, pulp industries could integrate bioethanol production following SCP production improving their sustainability. The aim of this work was to study the growth of *P. variotii* using eucalypt SSL in order to produce SCP. Therefore, two approaches were followed using a sequential batch reactor (SBR): with and without mixed salts supplementation.

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4.1 Introduction

The preservation and management of the different resources are fundamental to achieve a sustainable development in the future [1]. The conversion of the global economy into a sustainable bio-based economy with bioenergy, biofuels, and bio-based products has been the focus of many researchers recently [1, 2]. In this way, the search for biotechnological integrated processes with the objective of wastes valorisation and economical profit has become object of research and development worldwide. In the last decades the use of industrial by-products and wastes as feedstocks to produce value-added products has been implemented at industrial scale and fits well the biorefinery concept [1-3]. Spent sulphite liquors (SSLs) are by-products of pulping industry that result from the acidic sulphite pulping process [2-4]. These by-products are produced in large amounts, about 90 billion litres annually worldwide [3, 5]. SSLs are usually burned, for chemical and energy recovery after their concentration by evaporation [2-4]. The main objective of acidic sulphite pulping process is the wood delignification in order to remove lignin and hemicelluloses, maintaining cellulose integrity as much as possible [4]. During this process lignin and hemicelluloses are hydrolysed and released to the aqueous phase [2-4]. The practical application of SSLs depends significantly on their chemical composition, which is determined by the type of wood involved in the pulping process [3, 4]. Typically SSL from hardwoods (HSSL), namely *Eucalyptus globulus*, contains lignosulphonates (60-80 $g.L^{-1}$) and hydrolysed sugars, essentially monosaccharides (40-45 $g.L^{-1}$), mainly xylose [3, 6]. Hence, HSSL is a prospective substrate for bioethanol production by yeasts [2, 3, 6]. However, the presence of high amounts of acetic acid (9-10 $g.L^{-1}$), polyphenols and low molecular weight lignosulphonates inhibits the yeast growth, which is the main drawback for HSSL bioprocessing [2, 3, 7].

Paecilomyces variotii is a fungus usually found in air and soils of tropical countries [8]. *P. variotii* has been used for the production of single cell protein (SCP) due to its optimal performance using as substrate several by-products/wastes like SSL, vinasse, rayon hydrolyzate and wood hydrolyzate, among others [7, 8]. SCP corresponds to the non-viable dried cells of microorganisms such as yeasts, mycelial fungi, bacteria and microalgae that can be used in human nutrition or animal feeding [9, 10]. SCP is not only composed by

proteins but also by other important compounds for feeding, such as lipids, carbohydrates, vitamins and minerals and several substrates can be used for its production [11, 12]. The use of industrial by-products as substrates for SCP production could provide an economically feasible protein source [12, 13]. The development of microbial systems to be used in human food or animal nutrition showed already some advantages [11, 12, 14]. The growth of microorganisms is faster than the development of animals or crops, does not depend on the weather and climacteric conditions being not seasonable, and needs a significant lower area for growing [12]. Presently, SCP can be produced using several species of microorganisms such as algae, fungi and bacteria. Depending on the substrate used all of them have benefits and drawbacks, as reviewed by Anupama and Ravindra (2000).

P. variotii is able to grow in HSSLs and has the ability to metabolise and degrade some phenolic and lignocellulosic compounds, making HSSL a more suitable resource for bioprocessing [2, 7]. Hence, acidic sulphite pulping industries could integrate the production of bioethanol after the SCP production and improve their sustainability. SSLs were used as a substrate for SCP since 1909 in Sweden. The first organism to be used was *Saccharomyces cerevisiae*, although this yeast was unable to consume pentoses which are a major component in this type of by-products. Later, other yeasts able to assimilate pentoses were chosen, namely *Candida tropicalis* and *Candida utilis* [12]. Nevertheless, in 1975, in Finland, the Pekilo process started to be used as a method to produce SCP from SSL [12, 15]. This method consisted in a continuous fermentation of pulp mill effluent (softwood SSL) using the fungus *P.variotii*. The protein content obtained exceeded 55% (w/w), and was officially approved in Finland for animal feeding [12, 15]. In 1983, the biomass production was estimated to be around 7000 tons per year [12]. It is our knowledge that the present work describes for the first time the use of HSSL from *Eucalyptus globulus* for production of SCP. Accordingly, the aim of this work was to study the fermentation of HSSL, from *E. globulus* by *P. variotii* in order to produce SCP. Direct fermentation of HSSL by *P. variotii* in a single batch reactor has already been studied in a previous work by Pereira *et al.*(2012). The obtained results showed that the sequential batch reactor (SBR) strategy could be considered promising for a fast and efficient HSSL

fermentation [7]. Therefore, in the present work two fermentation methods were followed using a SBR strategy: with and without mixed salts supplementation. The HSSL was supplemented with a mixture of salts, described in literature as being necessary for *P. variotii* growth [16]. The aim of the salts supplementation was to improve *P.variotii* growth in HSSL, in order to increase the number of SBR cycles and final amount of biomass obtained. These two approaches were compared in order to study the best operational strategy for SCP production from HSSL. The fermentations were monitored by HPLC and the DNA and protein contents biomass were quantified. The amino acids (AA) composition of SCP obtained was determined by GC-FID.

4.2 Materials and Methods

4.2.1 HSSL supply and pretreatment

Industrial HSSL from magnesium based acidic sulphite pulping of *E. globulus* was supplied by Caima-Indústria de Celulose SA (Constância, Portugal). Pre-evaporated HSSL was collected. The pretreatment of HSSL consisted in pH adjustment to 7.0 with KOH, followed by aeration with compressed air for 2 h per litre of HSSL processed. The precipitated colloids were centrifuged at 5000 rpm during 20 min at 4 ^{o}C and then the supernatant was filtered using a 1.0 μm glass microfiber filter (Fioroni).

4.2.2 Microorganism and media

P. variotii NRRL-1115 was gently supplied by Agricultural Research Service Culture Collection at National Centre for Agricultural Utilisation Research, USDA. The stock culture of *P. variotii* was grown at 28 ± 0.5 ^{o}C on Petri dishes with ME agar medium (30.0 g malt extract, 5.0 g peptone and 15.0 g agar per litre of distilled water), stored at 4 ^{o}C and transferred monthly.

4.2.3 Preparation of inocula

P. variotii was grown in Petri dishes during 7 days at 28 ^{o}C. Inocula were prepared in order to achieve an initial cell concentration of 70.0 mg.L^{-1} according to Tavares *et*

al.(2005) procedure. The calculated volume of inoculum suspension was transferred to 500 mL Erlenmeyer flasks, with 250 mL of working volume.

4.2.4 SCP production

The SBR tests started as single batch and duplicates were carried out in 500 mL Erlenmeyer flasks containing 250 mL of working volume. Two sets of experiments were performed: one with HSSL supplemented with salts and the other, without. The mixture of salts supplemented was composed by $NaNO_3$, 2.0 $g.L^{-1}$; KCl, 0.50 $g.L^{-1}$; $MgSO_4$, 0.50 $g.L^{-1}$; $FeSO_4.4H_2O$, 0.010 $g.L^{-1}$; K_2SO_4, 0.35 $g.L^{-1}$. Each shake flask was inoculated and incubated at 28 ^oC in an orbital shaker at 180 rpm. Each cycle was ended when the acetic acid reached a concentration lower than 2.0 $g.L^{-1}$. Fungal biomass was sterile filtrated and transferred into the new flask with fresh HSSL, starting the next cycle. This procedure was repeated for three cycles. In the final cycle the biomass was washed with distilled water to remove the medium and kept at – 20 ^oC for further analyses.

4.2.5 HPLC analysis

Glucose, xylose and acetic acid, were analysed by HPLC using a 10 µm Eurokat H (Knauer) ion-exchange column, 300 × 7.5 mm with an oven (Gecko 2000) set at 40 ^oC, a pump (Gilson) and refraction index (RI) detector Gilson 131. The eluent was sulphuric acid 0.01 N, with a flow rate of 0.4 mL min^{-1}. The injected volume was 20 µl. All samples were centrifuged and filtered off with 0.20 µm filters (Spin-X, Costar) before the analysis. An absolute calibration was applied for all analysed compounds.

4.2.6 Fungal biomass analysis

4.2.6.1 Cell lysis

In order to analyse the protein and nucleic acids composition of *P. variotii* biomass cell lysis was carried out by using an ultrasonic probe (Sonics – Vibra Cell Ultrasonic Processor, 50 watts output) in order to release the cellular content. 70.0 mg of dry biomass were solubilized in 10.0 ml of Tris-HCl buffer at pH 7 with 100 mM NaCl and 1% (w/v) of sodium dodecyl sulfate (SDS).The cellular lysis was performed in an ice bath (to avoid

protein denaturation due to heat dissipation caused by ultrasound waves). The biomass was exposed to the ultrasonic probe for 2 min followed by 1 minute interval. This procedure was repeated 3 times, performing a total of 6 min of ultrasound exposure. In the end the samples were heated to 60 ^{o}C in a water bath for 15 min and then left to cool down in ice. The samples were centrifuged at 12 000 rpm (12 557 × *g*) for 20 min and the supernatant recovered for further analysis.

4.2.6.2 DNA quantification

The DNA present in the samples was purified by adding 2.0 mL of a isoamyl alcohol : chloroform mixture (1:24 v/v) to 2.0 mL of the supernatant obtained in section 4.2.6.1. Then, samples were centrifuged at 12 000 rpm (12 557 × *g*) for 10 min. To 2.0 mL of supernatant were added 200 µL of 3M sodium acetate and 5.5 mL of cold absolute ethanol. The samples were left overnight at -20 ^{o}C for nucleic acids precipitation. A centrifugation was performed (12 000 rpm for 10 min) to remove the supernatant and the precipitated DNA was washed with a 70 % ethanol solution (v/v). The remaining ethanol was evaporated in a 29 ^{o}C chamber.

The DNA pellet was solubilised in 2.0 mL of sterile water and the absorbances at 260/280 nm were measured. A correlation of 1 unit of optical density with 50 $\mu g.mL^{-1}$ of nucleic acid was used to quantify the nucleic acids [18].

4.2.6.3 Protein quantification

Biuret method described by Walker (2000) was used to quantify protein in biomass [19]. A calibration curve build using bovine serum albumin (BSA) standards with a concentration range of 1.0- 6.0 $g.L^{-1}$ was used. Samples were diluted to fit the calibration curve.

4.2.6.4 Aminoacids analysis

The aminoacids (AAs) analysis was performed using GC-FID. The biomass hydrolysis was made following the Zumwalt *et al.*(1987) method [20]. The derivatization process was

based on MacKenzie and Tenaschuk(1974) and MacKenzie *et al.*(1987) [21, 22]. The derivatized AAs were dissolved in 50 µL of ethyl acetate and analysed by GC-FID.

4.3 Results and Discussion

4.3.1 HSSL supplemented with salts

A SBR configuration and salts supplementation of HSSL were attempted in order to increase the amount of SCP produced and reutilize *P. variotii* for a higher number of cycles. **Figure 4.1** shows the *P. variotii* performance in this assay over time.

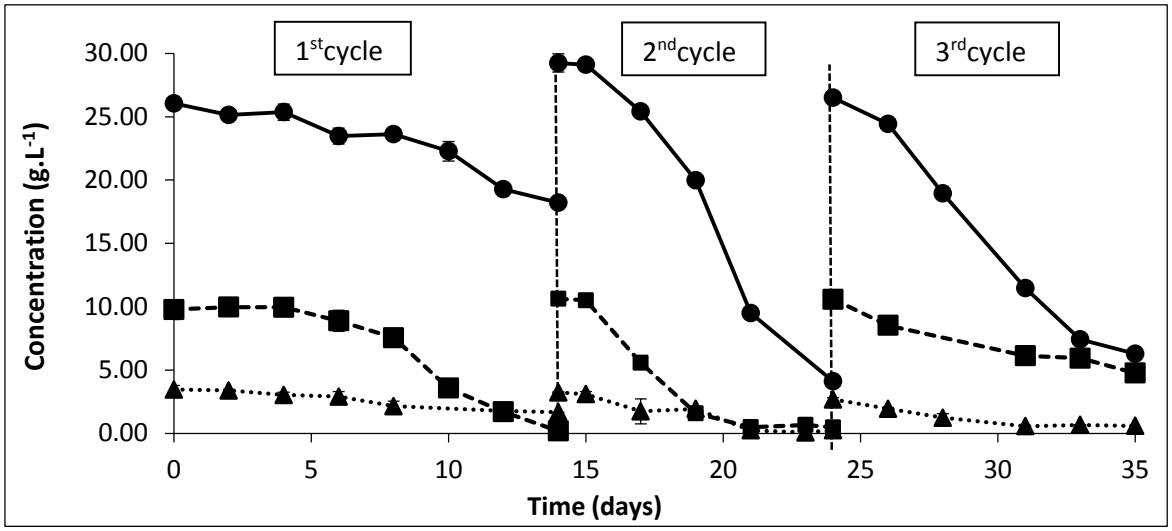

Figure 4.1. Fermentation profile of *P. variotii* in a SBR fed with HSSL with salts supplementation: (O)Xylose , (■) Acetic acid, (▲) Glucose.

In the 1st cycle while sugars (mainly xylose) were consumed since the beginning, acetic acid started to be consumed by *P. variotii* only after the 4th day, being depleted on the 14th day, showing a preference of *P. variotii* for sugars. The uptake rates of sugars and acetic acid were similar, 0.6 $g.L^{-1}.day^{-1}$ and 0.7 $g.L^{-1}.day^{-1}$, respectively (**Table 4.1**).

During the 2nd cycle, xylose and acetic acid uptake rates increased (**Table 4.1**) and this cycle lasted only 10 days. Comparing sugars and acetic acid uptake rates (2.6 and 1.0 $g.L^{-1}.day^{-1}$, respectively) it seemed that, again, *P. variotii* preferred sugars over acetic acid. The glucose uptake rate was constant during the 1st and 2nd cycles (0.1 $g.L^{-1}.day^{-1}$) and still lower than the xylose uptake rate (2.5 $g.L^{-1}.day^{-1}$) indicating that, among sugars, *P. variotii* favoured xylose.

In the 3rd cycle, acetic acid was not fully consumed, after 11 days, its concentration remained relatively high (4.8 $g.L^{-1}$). Instead, xylose concentration decreased to 6.3 $g.L^{-1}$, showing once more the preference of *P. variotii* for xylose in this assay. In this cycle the sugars uptake rate (2.1 $g.L^{-1}.day^{-1}$) was also higher than the acetic acid uptake rate (0.5 $g.L^{-1}.day^{-1}$). Substrate uptake rates were lower than in the previous cycle, except the glucose uptake rate that increased in the 3rd cycle to 0.3 $g.L^{-1}.day^{-1}$ but still lower than the xylose uptake rate (**Table 4.1**). Hence, a 4th cycle was not attempted.

Table 4.1. Uptake rates and biomasses concentrations rates and yields from the HSSL fermentation supplemented with salts and without salts supplementation.

	HSSL with salts				HSSL without salts			
	1st Cycle	2nd Cycle	3rd Cycle	Total	1st Cycle	2nd Cycle	3rd Cycle	Total
r_{xylose} ($g.L^{-1}.day^{-1}$)	0.6	2.5	1.8	4.9	0.6	1.7	1.0	3.3
$r_{glucose}$ ($g.L^{-1}.day^{-1}$)	0.1	0.1	0.3	0.4	0.2	0.3	0.1	0.6
r_{sugars} ($g.L^{-1}.day^{-1}$)	0.6	2.6	2.1	5.3	0.8	2.0	1.1	3.9
$r_{acetic\ acid}$ ($g.L^{-1}.day^{-1}$)	0.7	1.0	0.5	2.2	1.3	2.2	1.7	5.3
Total Biomass ($g.L^{-1}$)				15.2				10.3
Biomass production rate ($g.L^{-1}.day^{-1}$)				0.4				0.6
Overall biomass yield ($g.g^{-1}$)				2.0				1.1

In this assay, *P. variotii* consumed preferentially sugars leaving in the medium most of the acetic acid. This metabolic behaviour is also observed in the uptakes rates (**Table 4.1**) where the sugars uptake rate (5.3 $g.L^{-1}.day^{-1}$) was higher than the acetic acid uptake rate (2.2 $g.L^{-1}.day^{-1}$). The HSSL with the salts supplementation led to 15.2 $g.L^{-1}$ of fungal biomass (**Table 4.1**). Consequently, the biomass yield was 2.0 $g.g^{-1}$ and the biomass production rate was 0.4 $g.L^{-1}.day^{-1}$.

4.3.2 HSSL without salts supplementation

Since the HSSL fermentation with the salts supplementation resulted in a high biomass production, the fermentation of HSSL without salts supplementation was attempted in order to observe how the *P. variotii* performance was affected by the presence of salts. Both approaches were compared in order to evaluate the best one for a

future industrial application. The evolution of sugars and acetic acid concentration along the three SBR cycles is displayed in **Figure 4.2**.

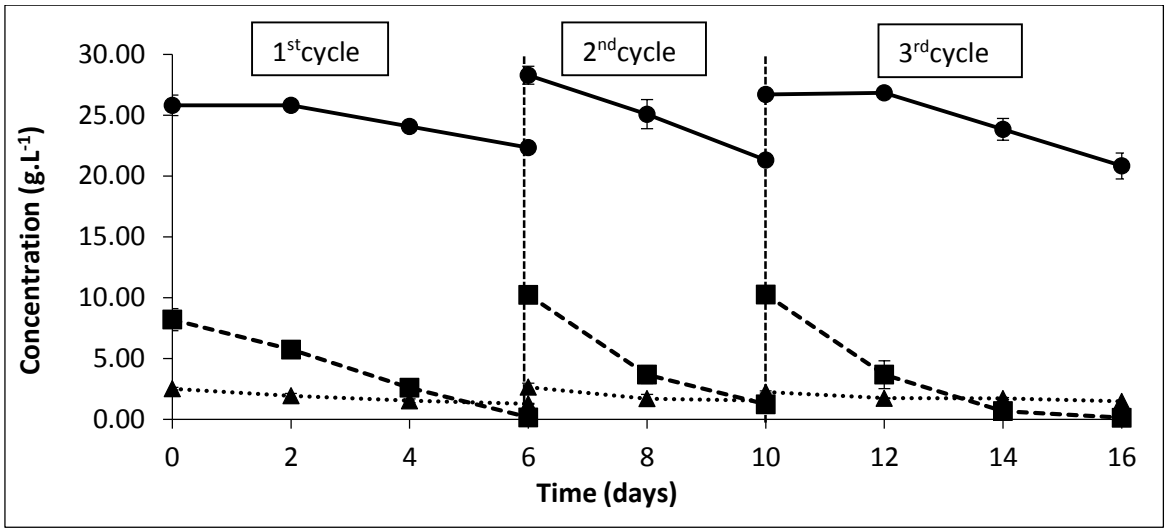

Figure 4.2. Fermentation profile of *P. variotii* in a SBR fed with HSSL without salts supplementation: (○)Xylose , (■) Acetic acid, (▲) Glucose.

In the 1st cycle, acetic acid consumption started immediately at the beginning of the cycle. *P. variotii* consumed preferentially acetic acid despite the uptake of some xylose and glucose observed. Hence, acetic acid uptake rate was higher (1.3 $.L^{-1}.day^{-1}$) than the sugars uptake rate (0.8 $g.L^{-1}.day^{-1}$) as it is shown in **Table 4.1**. This cycle ended after six days due to the depletion of acetic acid. In this case, *P. variotii* preferred acetic acid over xylose, which was the opposite from what was observed in the SBR with the salts supplementation (section 4.3.1).

The 2nd cycle ended after 4 days since the decrease in acetic acid concentration was faster than in the previous cycle (2.2 $g.L^{-1}.day^{-1}$). As expected, acetic acid uptake rate, as well as the sugars uptake rate (2.0 $g.L^{-1}.day^{-1}$), were higher than the values obtained previously (**Table 4.1**). These facts indicate that after the 1st cycle *P. variotii* was adapted to HSSL (**Table 4.1**), thus consuming substrates faster.

In the 3rd cycle, *P. variotii* was able to deplete acetic acid after 6 days, as in the 1st cycle. Although lower uptake rates were obtained 1.7 and 1.1 $g.L^{-1}.day^{-1}$ for acetic acid and sugars, respectively. These results showed that *P. variotii* metabolism was probably affected by the inhibitors present in HSSL. Hence, the fungus was unable to keep the performance observed during the 2nd cycle. The results showed that two SBR cycles were

the best option or, at least, no more than three SBR cycles should be performed. The acetic acid and sugars uptake rates of the 3rd cycle were similar to those of 1st cycle. This inhibition in sugars and acetic acid uptake probably was due to the presence of several microbial inhibitor compounds in the HSSL. Recently it was shown that *P. variotii* is able to consume and degrades several organic constituents of HSSL [7], known as phenolic microbial inhibitors. However after three SBR cycles, probably *P. variotii* cannot withstand the metabolic stress caused by inhibitory compounds present.

Considering *P. variotii* general behaviour, the glucose uptake rate was always lower than the xylose one, showing its preference for xylose. However, comparing the overall uptake rates, the acetic acid uptake rate was the highest (5.3 $g.L^{-1}.day^{-1}$), which indicates that *P. variotii* prefers acetic acid as substrate than sugars. This was the major difference relatively to the fermentation with the salts supplementation. When salts were added, the sugars uptake rate (5.3 $g.L^{-1}.day^{-1}$) was significantly higher than the acetic acid uptake rate (2.2 $g.L^{-1}.day^{-1}$). Without the salts supplementation, after *P. variotii* growth by the consumption of acetic acid (from the first day to the 6th day) and only a small fraction of sugars, the remaining sugars in HSSL can be used for further bioprocessing. This fact can contribute for integrating different bioprocesses such as SCP and bioethanol production with the industrial pulping process according to the biorefinary concept, in order to improve the sustainability of the pulping industry [7].

Comparing the biomass produced in both fermentation strategies (**Table 4.1**), the absence of salts led to lower fungal biomass, 4.9 $g.L^{-1}$, and consequently, this approach resulted in a lower biomass yield (1.1 $g.g^{-1}$). Nevertheless, the biomass production rate was higher (0.6 $g.L^{-1}.day^{-1}$) due to the lower time needed to finish the 3 cycles of fermentation, that was only 16 days.

4.3.3 Protein and DNA content of biomass obtained in both SBR strategies

As mentioned before, the biomass that resulted from *P. variotii* growth could be used for animal, or even, human feeding as single cell protein (SCP), if certain constrains are confirmed. The final product should present a good nutritional value and fulfil all toxicity requirements for its commercialization as a food product. The SCP safety tests

varies according to the final destination of the product (animal or human feeding). Commercialization of SCP for human feeding must undergo a series of expensive scientific studies. These include short-term acute toxicity testing with several animal species, followed by extensive long term studies of the final product. Also, a thorough analysis of composition, impurities, digestibility and human studies are usually done for safety assessment. The nutritional value is based on its composition namely, vitamins, nitrogen, carbohydrates, fats, proteins and amino acids.

The protein and DNA content of biomasses produced in the two fermentation approaches are presented in **Table 4.2**. The protein content of biomass obtained in HSSL supplemented with salts was slightly higher (91.4 %) than the one obtained in HSSL without salts supplementation (82.8 %). These protein values are higher than those obtained in the industrial production of *P. variotii* biomass (Pekilo process) between 55 and 60 % [23]. The difference can be explained by the different substrate composition. In this study eucalypt SSL rich in pentoses was used while in Pekilo process, the substrate was softwood SSL rich in hexoses [3, 24]. The SCP composition could also change with the microorganism used and the growth conditions. In the Pekilo process *P. variotii* D-75018 is usually used at 37 ^{o}C [25] in a continuous fermenter during several weeks [23]. In this study, the strain *P. variotii* D-83214T (corresponding to NRRL-1115) at 28 ^{o}C was used in a SBR configuration. Apunama and Ravindra (2000) refers that in general the fungal protein content can vary between 30 and 70 %, depending on the substrate and fungal species.

Table 4.2. Protein and DNA contents of biomasses from the fermentations studied: HSSL supplemented with salts and without salts supplementation.

	HSSL with salts	HSSL without salts
% Protein (w/w)	91.4 ± 1.0	82.8± 7.0
Protein production rate ($g.L^{-1}.d^{-1}$)	0.4	0.5
Overall protein yield ($g.g^{-1}$)	1.8	0.9
% DNA (w/w)	1.6 ± 0.1	1.1 ± 0.1
Full SBR duration (days)	35	16
Time to attain 41 % of protein in biomass (d)	16	8

Comparing the values of protein production rate, the observed in HSSL supplemented with salts was slightly lower than in HSSL without salts supplementation (0.4 and 0.5 $g.L^{-1}.day^{-1}$, respectively). This small difference can be explained by the time duration of both fermentations, as mentioned previously. The overall protein yield was higher in the HSSL with salts (1.8 $g.g^{-1}$) due to the higher biomass and protein concentration.

Considering the DNA composition, the biomass from the HSSL without salts supplementation had the lowest DNA content (1.1 %) while the salts supplementation resulted in a DNA content of 1.6 % (**Table 4.2**). According to Apunama and Ravindra (2000) these are lower than the values of DNA content usually obtained for fungal species, 9.7 %. This is an important result because SCP for human feeding requires a low amount of nucleic acids, since it was proved that a consumption higher than 2 g per day can lead to the formation of kidney stones and gout [11]. For this reason, the nucleic acid content should be as low as possible, and consequently, a low DNA content corresponds to a high quality SCP. Moreover, the SBR approach with 3 cycles, using as substrate an industrial by-product as HSSL probably resulted in metabolic stress for *P. variotii*, decreasing the DNA content of the biomass at the end of the 3rd cycle.

The only SCP from fungus commercialized exclusively for human food is the mycoprotein, from *Fusarium venenatum* (Quorn™). The minimum protein value acceptable for commercialization of a mycoprotein is 41 % [26]. With this information together with the fact that SCP from *P. variotii* could be implemented industrially, eventually for human feeding, it was determined the time needed to achieve 41 % of protein in the biomass for both assays (**Table 4.2**). In the case of the fermentation with the salts addition, it would take around 16 days to achieve a biomass with 41% of protein. Without the salts only 8 days would be needed to attain the same amount of protein. In this way and for a possible industrial application, the fermentation approach without the salts was the best one for SCP production from the HSSL.

4.3.4 Amino acids analysis of biomass obtained without salts supplementation

The amino acids (AA) profile is an important information for SCP acceptance in the market. Since the biomass obtained using HSSL without salts supplementation was the best approach for SCP production taking in consideration an industrial application, the AA analyses were carried out only for this biomass. The experimental results were compared with data obtained from literature: SCP from *Aspergillus niger*, the Food and Agriculture Organization (FAO) standard and the mycoprotein Quorn™, shown in **Table 4.3**.

The SCP produced with HSSL without salts had 17 % of AA, a value lower that those determined for *A. niger*, and indicated by FAO Standard (28.46 and 28.0 %, respectively). These differences could be due to the different method used for AA analysis. Although the hydrolysis method was similar, the majority of the AA profiles are determined using an AA analyzer with cation-exchange column and post-column with the ninhydrin reaction [27]. In this work the AA were derivatized into N-heptafluorobutyryl isobutyl esters and were separated by GC.

Table 4.3. Comparison of amino acid composition of HSSL without salt supplementation. *A. niger* SCP, the essential AA from mycoprotein (Quorn™) and the FAO standard.

	AA	% (w/w)			
		HSSL without salts	*A. niger* SCP	Mycoprotein	FAO standard
Non-essential AA	Alanine	0.53	--**	--	--
	Glycine	0.32	--	--	--
	Serine	0.46	--	--	--
	Proline	0.72	--	--	--
	Cysteine	--	Trace	--	2.8
	Asparagine/Aspartic acid	3.25	--	--	--
	Tyrosine	0.54	3.00	--	2.8
	Glycine/Glutamic acid	5.59	--	--	--
Essential AA	Histidine	--	--	0.39	--
	Isoleucine	0.87	3.75	0.57	4.2
	Leucine	1.87	6.80	0.95	4.8
	Lysine	1.09	4.50	0.91	4.2
	Methionine	--	0.35	0.23	2.2
	Phenylalanine	0.65	5.70	0.54	2.8
	Tryptophan	--	--	0.18	--
	Threonine	0.46	--	0.61	--
	Valine	0.80	4.36	0.60	4.2
	Total	17.16	28.46	--	28.0

Regarding the essential AA, the SCP from HSSL without salts supplementation presented almost all the AA except histidine, tryptophan and methionine (**Table 4.3**). Leucine and lysine were the essential AA present in higher amounts (1.87 and 1.09 % respectively). The Pekilo SCP contains all the essential AAs except histidine [12]. As mentioned before, the strain of *P. variotii* studied in this work was not the same as in Pekilo, as well the growth conditions and substrates were different. Moreover, SCP from *A. niger*, that is considered to meet FAO requirements, does not have all the essential AAs. In SCP of *A. niger*, threonine, tryptophan and histidine were missing (**Table 4.3**). In comparison with mycoprotein, the SCP produced without the salts supplementation showed a higher content for almost all the detected essential AA. Only threonine percentage is lower in the SCP from HSSL without salts supplementation (0.46 %), than for mycoprotein (0.61 %). These results show the potential of the SCP produced from *P. variotii* in HSSL for commercial animal feeding.

4.4 Conclusions

The best approach studied was the use of HSSL without the salts supplementation, considering a minimum of 41 % of protein in the biomass based on the mycoprotein for human feeding. The biomass produced in this approach showed a high protein content (82.8 %), a low DNA content (1.1 %) and a good variety of AA with six of the essential AA. Therefore the SCP produced by *P. variotii* from HSSL is a good candidate for animal feeding. For industrial implementation this is an important advantage since HSSL can be used just as it is produced as raw material for a biorefinery integration in the industrial plant.

4.5 References

1. Kamm, B., P.R. Gruber, and M. Kamm, Editors. *Biorefineries - Industrial Process and Products: Status Quo and Futures Directions.* Vol. 1., 2005, Wiley-VCH.
2. Fernandes, D.L.A., S.R. Pereira, L.S. Serafim, D. Evtuguin, and A. Xavier, *Second Generation Bioethanol from Lignocellulosics: Processing of Hardwood Sulphite Spent Liquor*, in *Bioethanol*, M.A.P. Lima and A.P.P. Natalense, Editors. 2011, Intech Rijeka. p. 123-152.
3. Pereira, S.R., D.J. Portugal-Nunes, D.V. Evtuguin, L.S. Serafim, and A.M.R.B. Xavier, *Advances in ethanol production from hardwood spent sulphite liquors.* Process Biochemistry, 2013. **48**(2): p. 272-282.

4. Sjöström, E., *Wood Chemistry. Fundamentals and Applications*. 2nd Ed., 1993, New York, USA: Academic Press. 293.
5. Lawford, H.G. and J.D. Rousseau, *Production of ethanol from pulp-mill hardwood and softwood spent sulfite liquors by genetically-engineered Escherichia coli.* Applied Biochemistry and Biotechnology, 1993. **39**(1): p. 667-685.
6. Xavier, A.M.R.B., M.F. Correia, S.R. Pereira, and D.V. Evtuguin, *Second-generation bioethanol from eucalypt sulphite spent liquor.* Bioresource Technology, 2010. **101**(8): p. 2755-2761.
7. Pereira, S.R., Š. Ivanuša, D.V. Evtuguin, L.S. Serafim, and A.M.R.B. Xavier, *Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol.* Bioresource Technology, 2012. **103**(1): p. 131-135.
8. Almeida e Silva, J.B., U.A. Lima, M.E.S. Taqueda, and F.G. Guaragna, *Use of response surface methodology for selection of nutrient levels for culturing Paecilomyces variotii in eucalyptus hemicellulosic hydrolyzate.* Bioresource Technology, 2003. **87**(1): p. 45-50.
9. FAO. *Protein sources for the animal feed industry*. in *Expert Consultation and Workshop*. 2002. Bangkok: FAO.
10. Nigam, P., *Single-Cell Protein Mycelial fungi*, in *Encyclopedia of food microbiology*, R.K. Robinson, C.A. Batt, and P.D. Patel, Editors. 1999, Academic Press: Great Britan p. 2034-2044.
11. Anupama and P. Ravindra, *Value-added food: Single cell protein.* Biotechnology Advances, 2000. **18**(6): p. 459-479.
12. Ugalde, U.O. and J.I. Castrillo, *Single Cell Proteins from Yeasts and Fungi* in *Applied Mycology and Biotechnology*. G.G. Khachatourians and D.K. Arora, Editors. 2002, Elsevier Amsterdam. p. 123-149.
13. Mølck, A.-M., M. Poulsen, H.R. Christensen, S.T. Lauridsen, and C. Madsen, *Immunotoxicity of nucleic acid reduced BioProtein—a bacterial derived single cell protein—in Wistar rats.* Toxicology, 2002. **174**(3): p. 183-200.
14. Adedayo, M.R., E.A. Ajiboye, J.K. Akintunde, and A. Odaibo, *Single Cell Proteins: As Nutritional Enhancer.* Advances in Applied Science Research 2011. **2**(5): p. 396-409.
15. Barber, R.S., R. Braude, and K.G. Mitchell, *The value of 'Pekilo protein' for growing pigs.* Animal Feed Science and Technology, 1977. **2**(2): p. 161-169.
16. Parker, B.J., R.G. Veness, and C.S. Evans, *A biochemical mechanism whereby Paecilomyces variotii can overcome the toxicity of the wood protectant borate.* Enzyme and Microbial Technology, 1999. **24**(7): p. 402-406.
17. Tavares, A.P.A., M.S.M. Agapito, M.A.M. Coelho, J.A. Lopes da Silva, A. Barros-Timmons, J.A.J. Coutinho, and A.M.R.B. Xavier, *Selection and Optimization of Culture Medium for Exopolysaccharide Production by Coriolus (Trametes) Versicolor.* World Journal of Microbiology and Biotechnology, 2005. **21**(8-9): p. 1499-1507.
18. Nelson, D.L. and M.M. Cox, *Nucleotides and Nucleic acids*, in *Lehninger Principles of Biochemistry.* 4th Ed., 2004, W. H. Freeman: New York p. 273-303.
19. Walker, J., *Protein structure, purification and characterisation*, in *Principles and techniques of practical biochemistry*, K. Wilson and J. Walker, Editors., 5th Ed., 2000, Cambridge University Press: Cambridge. p. 312-356.

20. Zumwalt, R.W., J.S. Absheer, F.E. Kaiser, and C.W. Gehrke, *Acid hydrolysis of proteins for chromatographic analysis of amino acids.* Journal of the Association of Official Analytical Chemists, 1987. **70**(1): p. 147-151.
21. MacKenzie, S.L., D. Tenaschuk, and G. Fortier, *Analysis of amino acids by gas-liquid chromatography as tert.-butyldimethylsilyl derivatives. Preparation of derivatives in a single reaction.* Journal of Chromatography A, 1987. **387**: p. 241-253.
22. MacKenzie, S.L. and D. Tenaschuk, *Gas-liquid chromatography of N-heptafluorobutyryl isobutyl esters of amino acids.* Journal of Chromatography A, 1974. **97**(1): p. 19-24.
23. Romantschuk, H. *A time to invest in forestry - Feeding cattle at the pulp mill*. Unasylva **106**, 1974. FAO - Forestry Department.
24. Nasseri, A.T., S. Rasoul-Amini, M.H. Morowvat, and Y. Ghasemi, *Single cell protein: production and process.* American Journal of Food Technology, 2011. **6**(2): p. 103-116.
25. Storgårds, E., *VTT culture colection*. 2013 [accessed: 2013, 25th of February]; Available from:http://culturecollection.vtt.fi/ccdb/html?p=stl&type=D&g=Paecilomyces&s=variotii&n=P.
26. FDA, *Gras notification for mycoprotein.* 2001, Marlow Foods Ltd.: Food and Drug Administration: Center for Food and Safety and Applied Nutrition.
27. Yamada, E.A. and V.C. Sgarbieri, *Yeast (Saccharomyces cerevisiae) Protein Concentrate: Preparation, Chemical Composition, and Nutritional and Functional Properties.* Journal of Agricultural and Food Chemistry, 2005. **53**(10): p. 3931-3936.

CHAPTER V

Adaptation of *Scheffersomyces stipitis* to hardwood spent sulphite liquor

Hardwood Spent Sulphite Liquor (HSSL) is a by-product of acidic sulphite pulping process that is rich in monomeric sugars, mainly xylose, 40-45 $g.L^{-1}$, and that can be converted to ethanol by *Sheffersomyces stipitis*. However, HSSL also contains inhibitory amounts of acetic acid and lignosulphonates for yeast growth. Hence, the main objective of the study was to obtain a strain of *S. stipitis* with increased tolerance to the HSSL inhibitors and maintained ability to ferment xylose to ethanol. A continuous reactor with gradually increasing HSSL concentrations, from 20% to 60% (v/v) was operated. The final population, that was obtained after 382 generations in HSSL (POP), was characterized in a batch reactor with 60 % HSSL and compared with the parental strain (PAR). Fermentation with bio-detoxified HSSL was also performed with POP, in order to study the influence of the HSSL inhibitors in the fermentation profile.

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5.1 Introduction

The fossil resources are declining while the requirements of modern lifestyle for energy and materials are increasing [1]. Besides, environmental concerns about industrial by-products and their removal are increasing. Hence, the search for sustainable biotechnological processes, environmentally favourable and cost effective for obtaining energy and materials from residual raw materials is growing worldwide [1-3].

Among all biofuels, ethanol is currently being industrially produced from sugar-containing biomass such as sugarcane and corn. However these feedstocks are essential for human and animal feeding and their prices is rising all over the world [2, 4]. It is therefore crucial to develop processes for energy production from forestry wastes, residual agricultural biomass and industrial wastes, the so-called "second generation" biofuels [1, 2, 4].

Spent sulphite liquors (SSLs), that are by-products from the pulp and paper industry, are promising feedstocks for second generation bioethanol production [3-5]. SSL is the main effluent resulting from acidic sulphite pulping of wood. Of all chemical pulps produced, around 6 % correspond to acidic sulphite pulping [3]. SSL contains significant amounts of dissolved organic matter (COD > 100000 $mgO_2.L^{-1}$) [3, 4], hence it cannot be discharged into natural basins due to environmental concerns and must be further processed. SSL is normally concentrated by evaporation and then burned for the energy and base recovering [6, 7]. The composition of SSLs depends on the type of wood used by the pulp and paper industry (softwoods, hardwoods or mixture of both) [7].

Hardwood SSL (HSSL) is rich in pentoses, which is not fully exploited yet for bioprocessing [3, 5]. The sustainable fermentation of pentoses into bioethanol is a challenge to overcome since a majority of microorganisms are unable to use these sugars [3, 4, 8]. *Scheffersomyces stipitis* is one of the most efficient yeast to naturally ferment pentoses to ethanol [9]. However, besides sugars, HSSL contains microbial inhibitors that limit the possibility of its bioprocessing [3, 10]. The presence of high amounts of acetic acid (8-11 $g.L^{-1}$) and lignosulphonates (60-80 $g.L^{-1}$) is inhibitory for yeast growth [3, 5, 6]. However, two of the most common and studied inhibitors found in softwood SSLs, furfural and hydroxymethylfurfural, are not found in the HSSL used in this study [5]. Xavier *et al.*

(2010) previously showed that *S. stipitis* could not grow over 40 % (v/v) HSSL and that it was necessary to perform a previous detoxification process. A process for biodetoxification of HSSL was performed using the filamentous fungus *Paecilomyces variotii* providing a more suitable substrate for *S. stipitis* growth and fermentation [10]. However, even after the fungal biodetoxification step, other inhibitors were still present, since the obtained ethanol yield was only 0.24 $g.g^{-1}$ [10]. This was still a low value when compared with the attained (0.49 $g.g^{-1}$) in the fermentation of the HSSL sugars fraction, obtained after separating the liquor major components by ionic-exchange resins [5]. It is well known that yeasts can, to some extent, gradually adapt to tolerate inhibitors and grow in their presence [11, 12]. However this property was mostly used in *S. cerevisiae* [12, 13] and only few attempts were made to adapt *S. stipitis* to typical inhibitors present in SSLs [3].

Mohandas *et al.* (1995) adapted *S. stipitis* to high acetic acid concentrations using shake-flask cultures with increasing acid amounts. The mutant obtained showed faster fermentations and higher tolerance to acid in wood hydrolysates at lower pH [14]. Using also shake-flask cultures, Nigam (2001) was able to obtain a mutant of *S. stipitis* adapted to a hardwood hemicellulose acid prehydrolysate with improved growth and ethanol yield [11]. Later, the same author used this approach to adapt *S. stipitis* to a red oak HSSL, improving ethanol fermentation [15]. Random UV mutagenesis has been also used in recent years to induce mutations in yeasts. With this technique a mutant with the desired characteristics can be isolated after a screening of all the mutants produced during the mutagenesis. Bajwa *et al.* (2009) obtained mutants of *S. stipitis*, using this approach, with enhanced tolerance to HSSL inhibitors and capable of attaining higher ethanol yields than the parental strain [16]. Another mutant strain was obtained by Hughes *et al.* (2012) using UV mutagenesis. The mutants were capable of growing anaerobically on xylose/glucose substrate and showed a higher ethanol production than a *Saccharomyces cerevisiae* yeast strain used in industry for fuel ethanol production [17]. Therefore, natural selection of strains and random mutation can be a good alternative to the classical genomic approaches, to obtain more robust yeasts. These methods are particularly useful since they are non-invasive techniques that can provide yeasts resistant to multiple stresses [12].

The main purpose of this work was to improve the tolerance of *S. stipitis* by adapting the yeast to *E. globulus* HSSL by a long term fermentation in a continuous-stirred tank reactor (CSTR) with temperature, pH, agitation and oxygen feeding control. For this purpose, a CSTR with increasing HSSL concentrations, from 20 to 60 % was operated for 382 generations. The adapted population obtained (POP) was then compared with the original strain (PAR) in a batch reactor with 60 % (v/v) HSSL. POP was also tested with a HSSL where the major inhibitors were already consumed by *P. variotii* (bio-detoxified HSSL), in order to evaluate the influence of the low inhibitors concentration in the fermentation profile.

5.2 Materials and Methods

5.2.1 HSSL supply and pretreatment

Industrial HSSL from magnesium based acidic sulphite pulping of *E. globulus* was supplied by Caima-Indústria de Celulose SA (Constância, Portugal). Pre-evaporated HSSL was collected. The pretreatment of HSSL consisted in pH adjustment to 7.0 with KOH, followed by aeration with compressed air for 2 h per litre of HSSL processed. The precipitated colloids were centrifuged at 5000 rpm during 20 min at 4 ^{o}C and then the supernatant was filtered using a 1.0 μm glass microfiber filter (Fioroni).

The bio-detoxified HSSL was obtained through the procedure described by Pereira *et al.* (2012).

5.2.2 Microorganism and media

S. stipitis NRRL Y-7124 (PAR) was gently supplied by Agricultural Research Service Culture Collection at National Center for Agricultural Utilization Research, USDA. The yeast culture was grown at 28 ± 0.5 ^{o}C and maintained at 4 ^{o}C on YM agar slants (3.0 g yeast extract, 3.0 g malt extract, 5.0 g peptone, 10.0 g glucose and 20.0 g agar per liter of distilled water). All media and materials were autoclaved at 121 ^{o}C for 20 min.

Liquid cultures were performed in a chemically-defined media (CDM) with sugars matching HSSL concentrations [5] (25 g xylose and 2.3 g glucose per liter of distilled water). A buffer solution [18] at pH 5.5 (5.1 g.L^{-1} $C_8H_5KO_4$ and 1.1 g.L^{-1} KOH) and a salt solution [19]

(5.0 g.L^{-1} $(NH_4)_2SO_4$; 0.5 g.L^{-1} $MgSO_4.7H_2O$; 3.0 g.L^{-1} KH_2PO_4) were also part of the CDM. Vitamins and trace elements were also added according to Verduyn *et al.* (1992).

5.2.3 Precultivation

S. stipitis (PAR and POP) growth was performed by taking colonies from the YM plate to liquid CDM. Precultivations were prepared in CDM with a predetermined volume of grown cells in order to have an optical density at 620nm (OD_{620nm}) of 0.2. These were performed in Erlenmeyer flasks with a working volume that corresponded to 10 % (v/v) of the total flask volume, at 180 rpm and 28 ^oC.

5.2.4 HSSL screening trials

The ability of *S. stipitis* to grow in different HSSL percentages (v/v): 0, 10, 20, 30 and 40 was assessed in 5.0 mL of media in 50 mL conical tubes incubated at 28 ^oC and 180 rpm for 35 h. HSSL was supplemented with CDM and also compensated for vitamins and trace elements in order to avoid nutrients limitation. Each cultivation was inoculated with an initial OD_{620nm} of 0.5. The OD_{620nm} was measured over time.

5.2.5 *S. stipitis* adaptation

A CSTR with a working volume of 1 L (B. Braun Biotech International Controller micro DCU-300) was set up to perform the adaptation of *S. stipitis* to increasing concentrations of HSSL. Three different dilution rates (D) were tested: 0.10, 0.15 and 0.20 h^{-1}. The final D of 0.20 h^{-1} was selected to operate the CSTR with HSSL. Agitation was controlled at 300 rpm and temperature at 28 ^oC. The pH was maintained at 5.5 by supplementing the medium with 3 M KOH or H_2SO_4, and air was supplied at 0.3 L.min^{-1}. HSSL and CDM concentrations varied from 0% of HSSL (100% CDM) until 60% HSSL (40 % CDM). The HSSL percentage was increased after *S. stitipis* attained a stable growth state. The reactor was operated for 382 generations in HSSL and samples were taken every day for OD_{620nm} and HPLC analysis. The final population obtained from the CSTR was used in this work (POP).

5.2.6 Batch experiments

Three different batch experiments were performed. A first cultivation ("(-)PAR") was inoculated with the PAR strain. The second cultivation ("(-)POP") was performed with POP obtained in the CSTR. Both experiments were performed with 60 % (v/v) HSSL and 40 % (v/v) CDM, without buffer solution. The medium of the third cultivation ("(*detox*(-)POP)") contained 60 % (v/v) of bio-detoxified HSSL and 40 % CDM, without buffer solution and was inoculated with POP.

The experiments were run for 60 h in a B. Braun BioLab reactor with temperature, agitation, aeration and pH control and 800 mL of working volume. In all experiments the temperature was controlled at 28 ^{o}C, stirring at 240 rpm, the aeration at 0.24 L.min^{-1} and pH to 5.5 by addition of 3 M KOH or H_2SO_4. Samples were taken for OD_{620nm} and HPLC analysis.

5.2.7 Analytical methods

Dry weight was determined in the end of the bioreactor fermentations. Three 5 mL samples were filtered with a 0.45 µm pore diameter filter (Whatman ME 25/21 ST) and washed with 15 mL of distilled water. Each filter was then dried at 100^{o}C until constant weight.

Colony forming units (CFUs) was measured at the end of each fermentation. The serial dilutions were made with samples of 0.5 mL into 4.5 mL of sterile 0.9% NaCl. 100 µL of the selected dilutions were then plated in YM solid media and left to grow for 48 h at 28 ^{o}C.

Glucose, xylose, acetic acid and ethanol were analysed by HPLC (Hitachi) using a 10 µm EurokatH (Knauer) ion-exchange column, 300 × 7.5 mm with an oven (Gecko 2000) set at 40 ºC, and refraction index detector (Hitachi RI Detector L-2490). The eluent was sulphuric acid 0.01 N, with a flow rate of 0.4 mL min^{-1}. The injected volume was 20 µl. All samples were centrifuged and filtered off with 0.20 µm filters (CoStar Spin-X) before the analysis. An absolute calibration was applied for all analysed compounds.

For phenolic compounds analysis, 20 ml samples of HSSL collected at the beginning and end of each cultivation were acidified until pH 2 with concentrated HCl. Three liquid-

liquid extractions (1:1, v/v) were made to each sample using diethyl ether. The organic phase was collected and passed through anhydrous sodium sulphate to remove traces of aqueous phase. Then, the collected organic phase was concentrated on a vacuum rotor evaporator at 40 ºC until complete dryness. The remaining solids were dissolved in 0.5 ml solution of pyridine with the internal standard, tetracosane (2 $mg.ml^{-1}$). Then, 200 µl BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) and 50 µl TMSC (trimethylchlorosilane) were added and the mixture was left to react at room temperature during 24 h. Samples were analysed by GC-MS, as trimethylsilane (TMS) derivatives according to Marques *et al.*(2009) and results obtained are related to the amount of internal standard added to the samples.

5.3 Results and discussion

5.3.1 HSSL screening trialss

In order to study the ability of *S. stipitis* to grow in HSSL and select the initial conditions for the following adaptation studies in a CSTR, five tests with increasing percentages of HSSL (0, 10, 20, 30 and 40) were performed in batch. The values of lag phase duration and maximum specific growth rate obtained are shown in **Table 5.1**.

Table 5.1. Maximum specific growth rate (μ_{max}) and lag phase duration for different HSSL concentrations.

	HSSL (% v/v)				
	0%	10%	20%	30%	40%
μ_{max} (h^{-1})	0.36	0.28	0.28	0.25	0.13
Lag (h)	1.5	1.5	1.5	3.0	24.0

Table 5.1 confirms the negative influence of HSSL on *S. stipitis* growth. With the raise of the HSSL concentration, the μ_{max} decreased and the lag phase increased. These results were confirmed by the previous studies done by Xavier *et al.*(2010) that showed the inhibition of *S. stipitis* growth with HSSL concentrations above 40 %. Furthermore, these authors observed a drop in the maximum specific growth rate when HSSL percentage rose [5].

In light of these results, the studies in the CSTR with the objective to adapt *S. stipitis* were initiated at 20 % (v/v) HSSL, since this was the maximum concentration with a high

μ_{max} (0.28 h^{-1}) and low lag phase (1.5 h). In this way a possible washout of the reactor in the adaptation studies was prevented.

5.3.2 *S. stipitis* adaptation

The adaptation of *S. stipitis* was performed by a direct adaptation in a CSTR operated during 382 generations. The results of substrate consumption and ethanol production along the CSTR operation are shown in **Figure 5.1**. After a batch operation of 42 h in CDM aiming at increasing biomass, the continuous operation was started. First several dilution rates, 0.10, 0.15 and 0.20 h^{-1}, were tested in CDM in order to maximize the number of generations in the reactor before HSSL started to be supplied. During the dilution rate increase from 0.10 to 0.15 h^{-1}, *S. stipitis* xylose consumption did not vary. For example, xylose consumption was 8.06 $g.L^{-1}$ with the dilution rate of 0.10 h^{-1} and 7.76 $g.L^{-1}$ with 0.15 h^{-1}. With 0.20 h^{-1}, the xylose consumption decreased to 4.99 $g.L^{-1}$ so, the dilution rate was not increased to higher values. Hence, when the culture was stable at 0.20 h^{-1}, HSSL started to be pumped in order to feed a concentration of 20 % (v/v) to the reactor (**Figure 5.1**). With CDM at a dilution rate of 0.20 h^{-1}, *S. stipitis* was consuming 4.99 $g.L^{-1}$ of xylose and 1.1 $g.L^{-1}$ of glucose. When HSSL started to be pumped into the reactor, the xylose consumption decreased to 3.69 $g.L^{-1}$ and the glucose consumption increased to 2.43 $g.L^{-1}$.

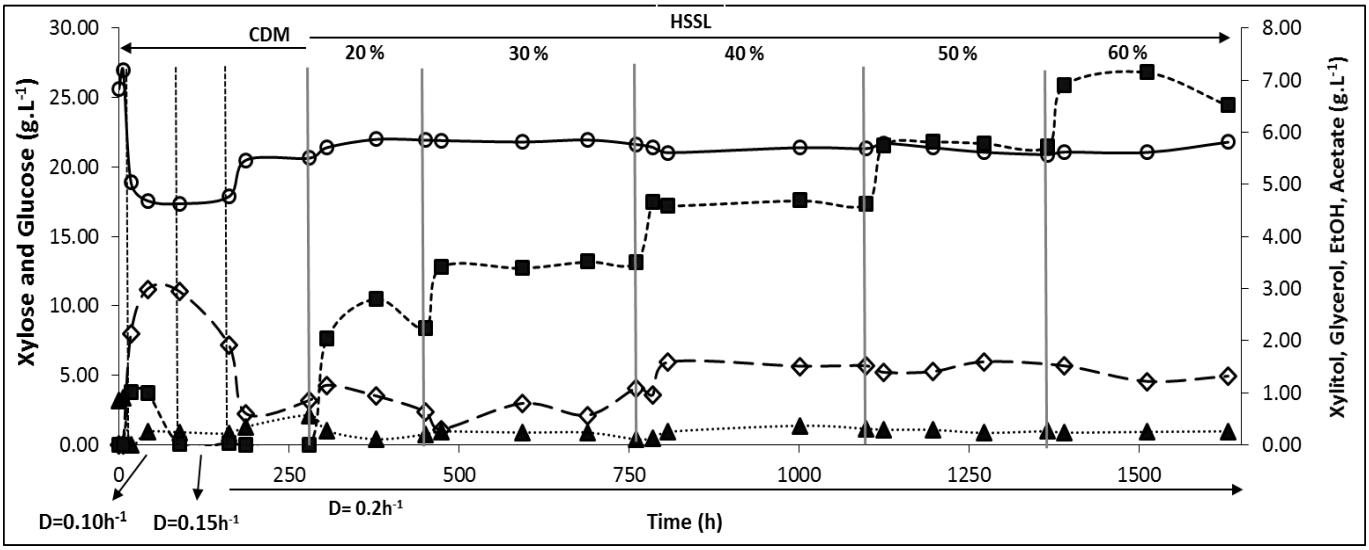

Figure 5.1. Adaptation of *S. stipitis* to HSSL in a CSTR: xylose (○), glucose (▲), acetic acid (■) and ethanol (◇).

Along the several increments in HSSL concentration no substantial variation in the substrate consumption was shown (**Figure 5.1**). The amount of xylose consumed averaged

4.11 ± 0.41 $g.L^{-1}$ and glucose, 2.08 ± 0.68 $g.L^{-1}$. The maintenance of the substrate consumption amounts after increasing HSSL concentrations showed that *S. stipitis* was able to tolerate the inhibitors without damaging xylose or glucose metabolism. No consumption of acetic acid was shown during the CSTR operation.

Ethanol production was affected by the several alterations made to the reactor (dilution rate and HSSL percentage). Ethanol concentration decreased as the dilution rate increased, from 2.99 $g.L^{-1}$ attained with dilution rate of 0.10 h^{-1} to 0.85 $g.L^{-1}$ at 0.20 h^{-1}. When the HSSL started to be pumped, ethanol concentration decreased again to 0.63 $g.L^{-1}$. On the opposite, when the HSSL increased to 30 %, ethanol concentration also increased to a final concentration of 1.08 $g.L^{-1}$ and with 40 % HSSL to 1.51 $g.L^{-1}$. For the 50 and 60 % HSSL the ethanol concentration was stabilized around 1.45 $g.L^{-1}$.

The CSTR ended when the culture reached a steady state with 60 % (v/v) of HSSL, which corresponds to the HSSL percentage that was normally used in fermentations with the bio-detoxified HSSL by *S. stipitis* [5, 11]. This CSTR adaptation resulted in a *S. stipitis* POP capable of withstanding 60 % of HSSL without a preliminary HSSL biodetoxification step.

5.3.3 *S. stipitis* PAR Vs. POP

5.3.3.1 (-)PAR experiment

Results for batch test with PAR are presented in **Figure 5.2**. *S. stipitis* did not have any lag phase when growing in 60 % (v/v) HSSL. Glucose was the first sugar to be consumed, being depleted from the media after 33 h of fermentation. Xylose consumption only started after glucose depletion. However, after 60 h, only 6.06 $g.L^{-1}$ of xylose were consumed by *S. stipitis.* Ethanol production started at 11h and a maximum ethanol concentration of 1.76 $g.L^{-1}$ was achieved after 60 h.

S. stipitis growth in HSSL was already studied by Xavier *et al.*(2010). In that study *S. stipitis* growth and fermentation metabolisms were severely affected at 40 % (v/v) HSSL. With 60 % (v/v) HSSL no growth was observed. Also, in the screening test performed (section 3.1) at 40 % (v/v) of HSSL a 24 h lag phase was shown showing the difficulties of *S. stipitis* when growing in this amount of HSSL.

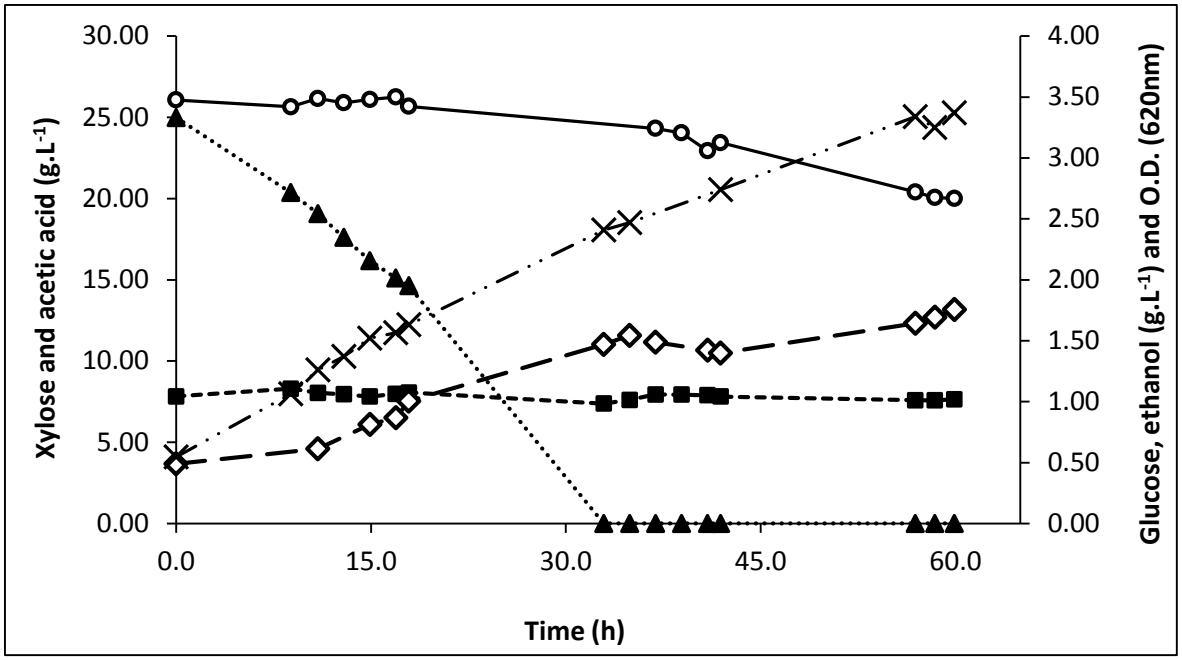

Figure 5.2. *S. stipitis* PAR grown in 60 % (v/v) HSSL: xylose (O), glucose (▲), acetic acid (■) and ethanol (◇) and Optical Density (×).

However, the previous tests were performed in Erlenmeyers flasks without pH and aeration control. In the (-)PAR experiment (**Figure 5.2**) pH and aeration were controlled, which is expected to positively affect the biomass growth. Recently the importance of pH control in *S. stipitis* fermentation ability was stated [20]. Portugal-Nunes *et al.*(2013) showed that the control of process parameters, namely pH, in the fermentation lead to improvements in the microbial performance, maximizing the fermentation metabolism.

5.3.3.2 (-)POP experiment

POP also did not show any lag phase (**Figure 5.3**), similar to the observed in the PAR cultivation (**Figure 5.2**). However glucose started to be consumed earlier, *i.e.* after 10 h, and was depleted from the media after 18 h. Xylose started to be consumed after 10 hours, although at a low rate (0.12 $g.L^{-1}.h^{-1}$).

Only after the glucose depletion, xylose consumption rate increased almost four times to 0.42 $g.L^{-1}.h^{-1}$. In total the POP consumed 20.71 $g.L^{-1}$ of xylose, a higher consumption than the attained in (-)PAR experiment. Acetic acid started to be consumed after 24 h. At the end of the fermentation, 62 h, POP was able to consume 3.04 $g.L^{-1}$ of acetic acid. Ethanol started to be produced at 10 h achieving a maximum concentration of

6.93 g.L^{-1} at the 63 h. Overall, POP consumed higher concentrations of xylose and also, produced more ethanol than in the (-)PAR experiment, which demonstrated that *S. stipitis* adaptation was a successful approach to improve the yeast HSSL inhibitor tolerance.

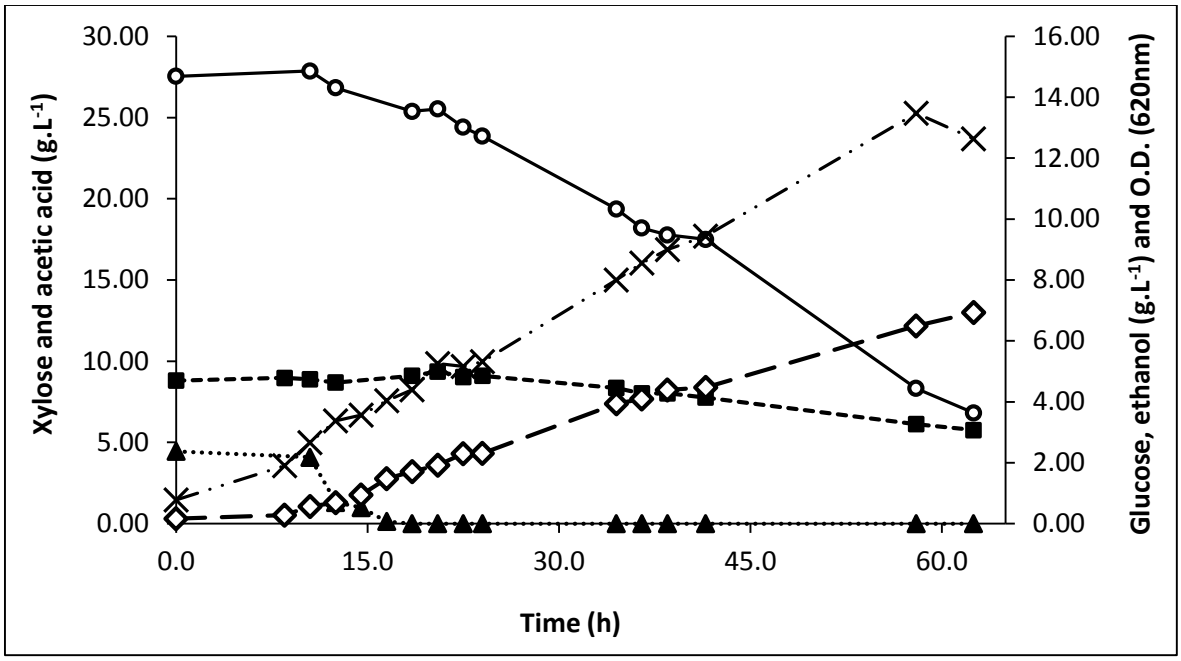

Figure 5.3. *S. stipitis* POP grown in 60 % (v/v) HSSL: xylose (O), glucose (▲), acetic acid (■) and ethanol (◇) and Optical Density (×).

5.3.3.3 *detox*(-)POP experiment

The bio-detoxified HSSL results from the growth of the fungus *P. variotii,* so bears lower amounts of inhibitors than the HSSL used in the previous experiment [10]. The fungus consumes most of the inhibitory compounds present, such as gallic acid, pyrogallol, syringaldehyde and vanillic acid. Hence, in order to study the inhibitors influence in the fermentation profile of POP, an experiment with the bio-detoxified HSSL was performed and the results shown in **Figure 5.4**.

Glucose was the first sugar to be consumed, starting after 5 h of lag phase. At 23 h, glucose was depleted from the media and as observed before, only then, xylose started to be consumed. During the 60 h of fermentation of POP consumed 17.59 g.L^{-1} of xylose. Besides, at the 53 h xylose consumption seemed to cease, and simultaneously cells entered in stationary phase (**Figure 5.4**). Throughout the fermentation, a low concentration of acetic acid was consumed, 0.61 g.L^{-1}. Ethanol production started at the 9th h and stopped

around 53rd h with a maximum concentration of 2.94 g.L^{-1}. Though the similarity in the substrate consumption with (-)POP, the ethanol concentration was lower in this experiment and closer to the one detected in (-)PAR.

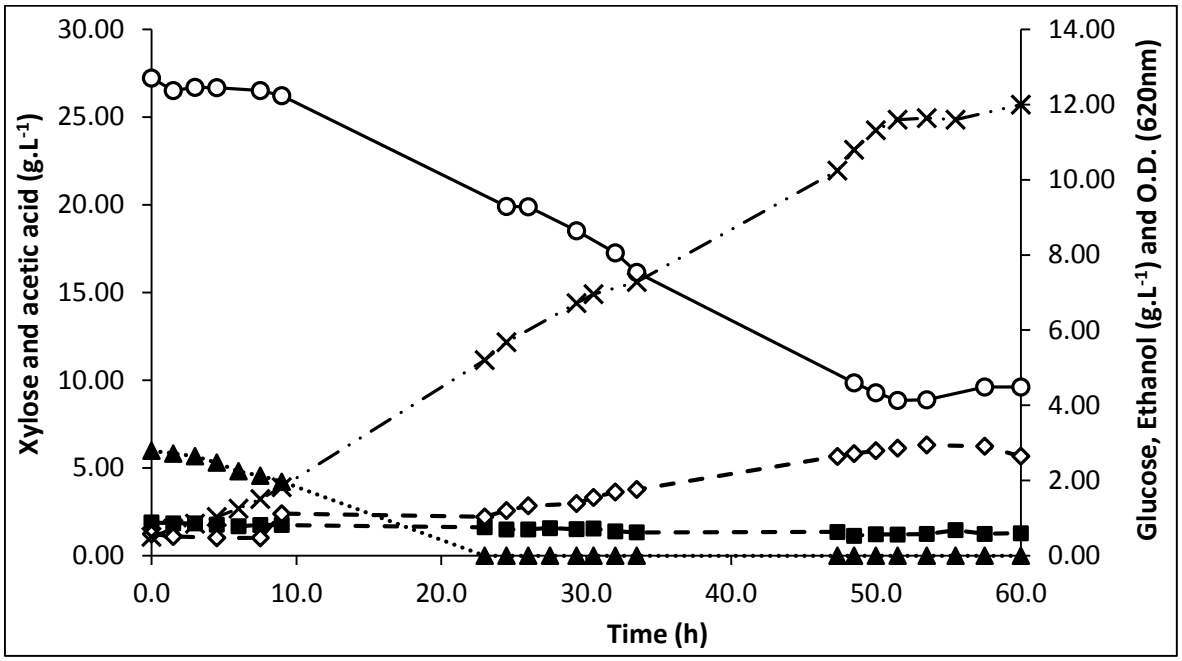

Figure 5.4. *S. stipitis* POP grown in 60 % (v/v) bio-detoxified HSSL: xylose (O), glucose (▲), acetic acid (■) and ethanol (◇) and Optical Density (×).

5.3.3.4 Fermentation experiments comparison

Several differences in the fermentation parameters were observed, between PAR strain and POP (**Table 5.2**). Despite similar maximum specific growth rate (μ_{max}) (0.037 h^{-1}), the final biomass achieved was significantly different (**Table 5.2**). The biomass achieved by (-)POP (3.75 g.L^{-1}) was 2.5 higher than the one formed in (-)PAR (1.45 g.L^{-1}). Colony-forming units (CFU) shows as well, that the viable colonies from (-)POP (3.5×10^8 cells.mL^{-1}) were 25 times higher than the ones from (-)PAR (7.8×10^7 cells.mL^{-1}). These results show that POP possessed a higher tolerance to the HSSL inhibitors than PAR strain since it was able to grow more with a higher colony viability.

The substrates consumption rates were higher in (-)POP fermentation (**Table 5.2**). Also, the maximum ethanol concentration was 3.8 times higher in (-)POP, achieving 6.93 g.L^{-1}. The ethanol conversion efficiency was 51 % for POP, which corresponds to almost the double as the one obtained for PAR (26 %). Again, these data showed that POP could better

withstand the HSSL inhibitors. POP, was able to consume more substrates and consequently was able to produce a higher ethanol concentration (**Table 5.2**). Overall, it was noticeable an improvement in the growth and fermentation profile of POP. It also showed a more robust growth and higher fermentations parameters when growing in the HSSL than PAR, showing the importance of the adaptation process in *S. stipitis.*

Table 5.2. Fermentation parameters of *S. stipitis* PAR and POP cultivations.

Fermentation parameters	(-)PAR	(-)POP	*detox*(-)POP
μ_{max} (h^{-1})	0.037	0.037	0.081
Final biomass ($g.L^{-1}$)	1.45 ± 0.08	3.75 ± 0.14	2.83 ± 0.40
Final CFU[a] ($cells.mL^{-1}$)	$7.8 \times 10^7 \pm 6.0 \times 10^6$	$3.5 \times 10^8 \pm 3.0 \times 10^7$	$1.9 \times 10^8 \pm 1.0 \times 10^7$
r_{xylose} ($g.L^{-1}.h^{-1}$)	0.10	0.33	0.34
$r_{glucose}$ ($g.L^{-1}.h^{-1}$)	0.10	0.13	0.12
$r_{acetic\ acid}$ ($g.L^{-1}.h^{-1}$)	0.003	0.05	0.01
$[EtOH]_{max}$ ($g.L^{-1}$)	1.76	6.93	2.94
$Y_{ethanol}$[b] ($g.g^{-1}$)	0.13	0.26	0.11
Conversion efficiency (%)	26	51	22

[a] Colony-forming unit

[b] Calculated at the maximum ethanol concentration

When comparing experiments with and without bio-detoxification, the use of bio-detoxified HSSL did not improve the overall fermentation profile (**Table 5.2**). Despite a higher μ_{max} (0.081 h^{-1}), a lower final biomass was achieved (2.83 $g.L^{-1}$) in the *detox*(-)POP. The final CFU was in the same order of magnitude as (-)POP. Likewise, xylose and glucose uptake rates (0.34 and 0.12 $g.L^{-1}.h^{-1}$, respectively) were very similar to the ones determined for (-)POP. Only acetic acid uptake rate (0.01 $g.L^{-1}.h^{-1}$) was lower than the attained for (-)POP, probably due to the low (1.90 $g.L^{-1}$), non-toxic concentration present in the bio-detoxified HSSL. The xylose consumption rate was similar in both experiments of POP. However, the maximum ethanol concentration attained in the *detox*(-)POP trial was (2.94 $g.L^{-1}$) lower than the achieved in (-)POP (6.93 $g.L^{-1}$). Hence, the yield and the conversion efficiency achieved were closer to the obtained in the (-)PAR trial. Although the lower toxicity of the bio-detoxified HSSL in detox(-)POP experiment, a higher ethanol yield was achieved in the (-)POP experiment with the HSSL containing inhibitors.

PAR fermentation in 60 % (v/v) bio-detoxified HSSL was already studied by Pereira *et al.* (2012). In this study, a maximum ethanol concentration of 2.36 $g.L^{-1}$ with a 0.24 $g.g^{-1}$ yield was achieved. Despite the higher ethanol concentration achieved in *detox*(-)POP, since it was attained with higher substrate consumption, the ethanol yield was lower than the achieved by Pereira *et al.*(2012), 0.24 $g.g^{-1}$. POP was already adapted to the HSSL with all the inhibitors, probably the lower inhibitor concentration in the bio-detoxified HSSL did not favour the ethanol production as in (-)POP.

5.3.4 Phenolic profile

POP showed the best biomass growth and fermentation profile in HSSL bioprocessing. Aiming to relate these benefits of POP with the amount of low molecular weight compounds, extractable by diethyl ether, the analysis of phenolics was performed. Hence, liquor before and after fermentation experiments were analysed and compared so, the differences can be established and discussed. HSSL collected before (Control) and after the fermentation with POP in the CSTR and in the batch reactor ((-)POP) were carried out (**Table 5.3**).

In the HSSL control analysis (**Table 5.3**), the main compound present was gallic acid contributing to 15 % of liquor extractable compounds, followed by syringic acid (1.5 %), syringic aldehyde (1.4 %) and vanillic acid (0.70 %). These phenolics are normally formed due to the degradation of hydrolysable tannins and lignin during the cooking process [10, 21]. Some furanic compounds resulting from the degradation of hemicelluloses under acidic pulping conditions were also present (*e.g.* furoic acid). The fatty acids found in HSSL are typical extractives of *E. globulus* [7], which were released from wood during the cooking process.

Polyphenolic compounds are known for their inhibiting properties during ethanolic fermentation by *S. stipitis*, greatly affecting its metabolism [5, 10]. The presence of these compounds and lignin derivatives (lignosulphonates) are the major bottleneck in HSSL bioprocessing. Taking into account that during the CSTR adaptation *S. stipitis* was forced to grow in the presence of lignin derivatives and polyphenols, an eventual physiological response could be expected. The phenolics profile of the final HSSL from the CSTR

experiment was analysed in order to observe if the yeast metabolism was changing. The composition of the HSSL from the CSTR (**Table 5.3**) and the control HSSL shows marked differences in the amount of occurring extractable compounds of low molecular weight. The amount of phenolics derived from tannins (gallic acid and pyrogallol) in fermented HSSL increased almost twice, to 33 and 0.6 %, respectively. Hence, it may be proposed that the adapted *S. stipitis* developed metabolic pathways for the oxidation of proanthocyanidins dissolved in HSSL.

Similarly, the concentration of lignin derived aromatic compounds (syringic/vanillic acids and 3-vanillylpropanol) in HSSL from the CSTR was 2-3 times higher (4.5, 1.4 and 1.1 %, respectively) than in the control of HSSL (**Table 5.3**). This indicates an eventual assimilation of part of the soluble lignin oligomers by adapted *S. stipitis* with the release of oxidised lignin subunits like the ones identified in **Table 5.3**. Hence, the adaptation could result in a POP able to oxidise lignin oligomers and condensed tannins into lower molecular weight products, probably less harmful to the yeast. The contents of furanic compounds (mostly furfuryl alcohol and furoic acid) derived from dissolved pentoses in HSSL also increased noticeably. Concentrations of organic acids in processed HSSL increased as well. Since these are metabolic products of *S. stipitis*, from the tricarboxylic acid cycle (TCA), they were expected to be detected in HSSL after the fermentation.

The HSSL after the cultivation with POP showed a noticeable raise in aromatic oxidation products, when compared to the control HSSL, but less accentuated than in HSSL after the CSTR experiment (**Table 5.3**). At the same time, a depletion of several phenolic products (*e.g.* pyrogallol and syringic aldehyde) was also observed after the batch trial, meaning that POP could completely metabolise these compounds (**Table 5.3**). It may be expected that part of the released phenolics were intermediates in a series of lignin/tannins bio-oxidation reactions. The concentration of vanillic acid in HSSL after the batch cultivation was higher than in the CSTR HSSL (**Table 5.3**). The HSSL after the batch reactor demonstrated also relatively high concentrations of furanic compounds derived from degradation of hemicelluloses.

Table 5.3. Composition of HSSL without any fermentation (control), the final HSSL from the CSTR and HSSL after batch fermentation of POP ((-)POP) analysed by GC-MS.

	% (m/m)[a]		
Compound	**Control**	**CSTR**	**(-)POP**
Furan derivatives			
Furfuryl alcohol	0	31.0	7.9
Furoic acid	0.8	4.1	7.5
Polyphenols			
Pyrogallol	0.4	0.6	0
Syringic aldehyde	1.4	1.7	0
Vanillic acid	0.7	1.4	1.6
3-vanillylpropanol	0.4	1.1	0.7
Protocatechuic acid	0	0	0.8
Syringic acid	1.5	4.5	4.4
Gallic acid	15.0	33.0	14.0
4-methoxyphenyllactic acid	0	0.5	0.6
Phenyllactic acid	0	0	0.5
Extractives			
Palmitic acid	0.9	1.0	4.2
Stearic acid	0.5	0.40	2.3
Organic acids			
Propanoic acid	0	0.6	1.9
Pentanoic acid	0	0.6	0.8
α-hydroxyisoacetic acid	0	0	0.5
Succinic acid	0	1.6	2.9
Glutaric acid	0	0	0.5
Malic acid	0.8	1.0	1.9
Total identified [b]	27	83	53

[a] related to the internal standard used

[b] calculated relatively to the mass extracted from HSSL

New compounds were found in the HSSL from the batch, such as the phenyllactic acid (0.5 %) and 4-methoxyphenyllactic acid (0.6 %). The gallic acid concentration, the major phenolic component present (14.0 %), was similar in both analyses (control and batch).

The observed profiles of phenolic compounds in HSSL bioprocessed by adapted *S. stipitis* indicate an eventual partial assimilation of dissolved lignin oligomers and tannins (*e.g.* flavonoids) via oxidation pathways. Besides, some of these oxidation products, the pyrogallol and syringic aldehyde, known inhibitors of microorganisms, were completely consumed by POP. Targoński (1992) already showed the ability of *S. stipitis* CBS 5773 to convert some aromatic aldehydes, typically found in lignin moieties, to their corresponding

alcohols. However, these compounds were tested individually in a mineral medium only at the concentration of 0.5 $g.L^{-1}$ [22], not in a real substrate with several different type of lignin derivatives. Probably *S. stipitis* already had the metabolic pathways to perform this bio-transformation of low-molecular lignin derivatives since it seems that the adaptation performed activated this metabolic capacity. In this way, POP has a higher tolerance to HSSL inhibitors, consuming more xylose and producing more ethanol than PAR. This enhanced ability of POP can be a great advantage for the bioprocessing of HSSL and lignocellulosic biomass in general.

5.4 Conclusions

S. stipitis was successfully adapted to 60 % (v/v) eucalyptus HSSL. POP showed important improvements compared with PAR, when grown in 60 % (v/v) HSSL, with higher xylose consumption (20.71 $g.L^{-1}$) and ethanol concentration (6.93 $g.L^{-1}$).

The phenolic profile analysed showed that POP had the improved ability to assimilate dissolved lignin oligomers and tannins probably through oxidation pathways. Moreover, pyrogallol and syringic aldehyde, known microbial inhibitors were completely metabolised by POP. These improved abilities may represent a major advantage in the bioprocessing of HSSL and lignocellulosic biomass in general.

5.5 References

1. Sannigrahi, P., Y. Pu, and A. Ragauskas, *Cellulosic biorefineries-unleashing lignin opportunities.* Current Opinion in Environmental Sustainability, 2010. **2**(5): p. 383-393.
2. Bacovsky, D., W. Mabee, and M. Worgetter, *How close are second-generation biofuels?* Biofuels Bioproducts & Biorefining, 2010. **4**(3): p. 249-252.
3. Pereira, S.R., D.J. Portugal-Nunes, D.V. Evtuguin, L.S. Serafim, and A.M.R.B. Xavier, *Advances in ethanol production from hardwood spent sulphite liquors.* Process Biochemistry, 2013. **48**(2): p. 272-282.
4. Fernandes, D.L.A., S.R. Pereira, L.S. Serafim, D. Evtuguin, and A. Xavier, *Second Generation Bioethanol from Lignocellulosics: Processing of Hardwood Sulphite Spent Liquor*, in *Bioethanol*, M.A.P. Lima and A.P.P. Natalense, Editors. 2011, Intech Rijeka. p. 123-152.
5. Xavier, A.M.R.B., M.F. Correia, S.R. Pereira, and D.V. Evtuguin, *Second-generation bioethanol from eucalypt sulphite spent liquor.* Bioresource Technology, 2010. **101**(8): p. 2755-2761.

6. Marques, A.P., D.V. Evtuguin, S. Magina, F.M.L. Amado, and A. Prates, *Chemical Composition of Spent Liquors from Acidic Magnesium-Based Sulphite Pulping of Eucalyptus globulus.* Journal of Wood Chemistry and Technology, 2009. **29**(4): p. 322-336.
7. Sjöström, E., *Wood Chemistry. Fundamentals and Applications*. 2nd Ed., 1993, New York, USA: Academic Press. 293.
8. Hahn-Hagerdal, B., K. Karhumaa, C. Fonseca, I. Spencer-Martins, and M.F. Gorwa-Grauslund, *Towards industrial pentose-fermenting yeast strains.* Applied Microbiology and Biotechnology, 2007. **74**(5): p. 937-953.
9. Jeffries, T.W., I.V. Grigoriev, J. Grimwood, J.M. Laplaza, A. Aerts, A. Salamov, J. Schmutz, E. Lindquist, P. Dehal, H. Shapiro, Y. Jin, V. Passoth, and P.M. Richardson, *Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast Pichia stipitis.* Nature Biotechnology, 2007. **25**(3): p. 319-326.
10. Pereira, S.R., Š. Ivanuša, D.V. Evtuguin, L.S. Serafim, and A.M.R.B. Xavier, *Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol.* Bioresource Technology, 2012. **103**(1): p. 131-135.
11. Nigam, J.N., *Development of xylose-fermenting yeast Pichia stipitis for ethanol production through adaptation on hardwood hemicellulose acid prehydrolysate.* Journal of Applied Microbiology, 2001. **90**(2): p. 208-215.
12. Koffas, M. and S.d. Cardayre, *Evolutionary metabolic engineering.* Metabolic Engineering, 2005. **7**(1): p. 1-3.
13. Çakar, Z.P., U.O.S. Seker, C. Tamerler, M. Sonderegger, and U. Sauer, *Evolutionary engineering of multiple-stress resistant Saccharomyces cerevisiae.* FEMS Yeast Research, 2005. **5**(6-7): p. 569-578.
14. Mohandas, D., D. Whelan, and C. Panchal, *Development of xylose-fermenting yeasts for ethanol production at high acetic acid concentrations.* Applied Biochemistry and Biotechnology, 1995. **51-52**(1): p. 307-318.
15. Nigam, J.N., *Ethanol production from hardwood spent sulfite liquor using an adapted strain of Pichia stipitis.* Journal of Industrial Microbiology & Biotechnology, 2001. **26**(3): p. 145-150.
16. Bajwa, P.K., T. Shireen, F. D'Aoust, D. Pinel, V.J.J. Martin, J.T. Trevors, and H. Lee, *Mutants of the Pentose-Fermenting Yeast Pichia stipitis With Improved Tolerance to Inhibitors in Hardwood Spent Sulfite Liquor.* Biotechnology and Bioengineering, 2009. **104**(5): p. 892-900.
17. Hughes, S., W. Gibbons, S. Bang, R. Pinkelman, K. Bischoff, P. Slininger, N. Qureshi, C. Kurtzman, S. Liu, B. Saha, J. Jackson, M. Cotta, J. Rich, and J. Javers, *Random UV-C mutagenesis of Scheffersomyces (formerly Pichia) stipitis NRRL Y-7124 to improve anaerobic growth on lignocellulosic sugars.* Journal of Industrial Microbiology & Biotechnology, 2012. **39**(1): p. 163-173.
18. Hahn-Hägerdal, B., K. Karhumaa, C.U. Larsson, M. Gorwa-Grauslund, J. Görgens, and W.H. van Zyl, *Role of cultivation media in the development of yeast strains for large scale industrial use.* Microbial Cell Factories, 2005. **4**(31).
19. Verduyn, C., E. Postma, W.A. Scheffers, and J.P.V. Dijken, *Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation.* Yeast, 1992. **8**(7): p. 501-517.

20. Portugal-Nunes, D.J., V.S. Nogué, S.R. Pereira, S.C. Craveiro, A.J. Calado, M.F. Gorwa-Grauslund, and A.M.R.B. Xavier, *Effect of cell-immobilization and pH control on Scheffersomyces stipitis using Eucalypt Spent Sulphite Liquor* Letters Of Applied Microbiology 2013 (submitted).
21. Marques, A.P., D.V. Evtuguin, S. Magina, F.M.L. Amado, and A. Prates, *Structure of Lignosulphonates from Acidic Magnesium-Based Sulphite Pulping of Eucalyptus globulus.* Journal of Wood Chemistry and Technology, 2009. **29**(4): p. 337-357.
22. Targoński, Z., *Biotransformation of lignin-related aromatic compounds by Pichia stipitis PIGNAL.* Zentralblatt für Mikrobiologie, 1992. **147**(3–4): p. 244-249.

CHAPTER VI

Influence of the precultivation medium on *Scheffersomyces stipitis* growth and fermentation performances in Hardwood Spent Sulphite Liquor

Hardwood spent sulphite liquor (HSSL) is a by-product of pulp and paper industry, rich in fermentable sugars that can be used by the *Scheffersomyces stipitis,* to produce ethanol. However, HSSL also contains high amounts of compounds that can act as inhibitors for microbial growth. Evolutionary engineering approaches have been used to increase the natural tolerance of microorganisms to inhibitors. The main objective of this work was to evaluate if the presence of HSSL inhibitors during precultivation would affect the growth and fermentation profiles of a HSSL-adapted *S. stipitis* population (POP) and the corresponding parental strain (PAR). In this way, both POP and PAR cells were precultivated with the same media as subsequent batch fermentations studied and then compared with fermentations performed with cell precultivated in a chemically-defined media. HSSL with all the inhibitors and the bio-detoxified HSSL, where the majority of inhibitors were already consumed, were tested.

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6.1 Introduction

Nowadays, the increasing demand of fossil resources requirements for energy and materials raises several environmental questions besides social issues [1]. Also, the depletion of fossil resources, on which the world economy depends on, calls for a shift towards a more sustainable future with a bio-based economy [1, 2]. Bioethanol that can be either mixed with gasoline or used as a sole fuel, using dedicated engines [3], is currently produced in large-scale by using feedstocks like sugarcane and corn, the so-called first generation bioethanol [3-5]. However, the use of these raw materials can negatively affect the human and animal food supply chain [2, 4, 5]. Instead, the so-called "second generation" bioethanol involves the processing of cheap, abundant and renewable raw-materials from industrial by-products and wastes [2, 4-6].

In this context, spent sulphite liquor (SSL) that is a by-product from the pulp and paper industry, is a promising feedstock for bioethanol production. SSL that is generated from the sulphite pulping process is nowadays burned for energy and chemicals recovery [2, 5]. The use of liquors for bioethanol production represent major advantages when compared with the lignocellulosic biomass since they have already a high content in ready fermentable monosaccharides. Consequently, the hydrolytic step usually required to process lignocellulosic biomass for polysaccharides conversion into monosaccharides can be avoided [2, 5, 6]. SSL from hardwoods, namely *Eucalyptus globulus,* are composed by lignosulphonates (60-80 $g.L^{-1}$) and monosaccharides (40-45 $g.L^{-1}$), mainly xylose [5-7]. These sugars can be used by the *Scheffersomyces stipitis,* to produce ethanol [6, 7]. However the presence of lignosulphonates, together with acetic acid (9-11 $g.L^{-1}$) is a major drawback of this liquor since they inhibit the process by affecting not only cell growth but also ethanol production [6-8].

Inhibitors of ethanolic fermentation present in lignocellulosic are conventionally classified into four groups according to their origin: sugar hydrolysis products, lignin degradation products, compounds derived from extractives and heavy metal ions [5, 8]. The lignin degradation products resulting from the delignification occurring during the cooking process in pulping mills are considered to be the most toxic class of compounds to microorganisms, including *S. stipitis* [5, 6]. In HSSL, lignin oligomers and monomeric

phenolic compounds are partially sulphonated, with one or two sulphonic groups [9, 10]. Phenolic compounds can cause a loss of integrity of biological membranes, thus, affecting their ability as selective barriers and as enzyme matrices. Also, a decrease in cell growth and in sugar assimilation rates in the presence of these compounds is expected [4, 8]. Moreover, eucalypt hardwood contains high amount of hydrolysable tannins giving rise to gallic acid and pyrogallol during the pulping process [5, 10]. These phenolic compounds are known inhibitors of microbial growth, possessing strong anti-fungal properties [5, 7]. Xavier *et al.* (2010) and Pereira *et al.* (2012) highlighted the toxicity of HSSL from *E. globulus* as a biological substrate. With 60% (v/v) of HSSL, no growth of *S. stipitis* NRRL Y-7124 was observed [6]. Biological removal of acetic acid was carried out with several yeasts, being the best one *Saccharomyces cerevisiae* PYCC 4072, and still no ethanol was obtained [6]. *S. stipitis* was only able to produce ethanol after a previous step of bio-detoxification performed using a filamentous fungus, *Paecilomyces variotti* that converted the majority of the phenolics and acetic acid in HSSL [7].

Several technologies have been employed to improve yeasts tolerance to inhibitors. The selection of mutants under selective pressure of a given by-product, may improve their growth and fermentation performance [11, 12]. Evolutionary engineering strategies such as mimicking natural selection under pressure or random mutagenesis can be very useful to improve inhibitors tolerance of engineered/modified or natural strains [12, 13]. Since HSSL has a wide range of inhibitors [7, 10], the specific mechanisms of microorganisms tolerance are a crucial step in the HSSL bioprocessing. The full microbial inhibition mechanisms is still not yet fully understood [12] due to the huge diversity of possible inhibitors, from small organic acids, or simple phenolics, to sulphated dimers derived from lignin [10, 14]. Therefore, targeted engineering approaches to increase inhibitor tolerance remain a challenge [11, 15].

An adaptation of *S. stipitis* was previously performed by Pereira *et al.(*2013) using a continuous stirred tank reactor (CSTR) with increasing HSSL concentrations. The obtained population (POP) displayed an improvement in the fermentation performance, as compared with the parental strain (PAR) [16]. Pereira *et al.* (2013) also demonstrated the ability of POP to assimilate part of the soluble lignin oligomers present in HSSL, releasing

oxidised lignin subunits like syringic acid, 3-vanilpropanol or vanillic acid. The present work aims at studying how the presence of HSSL inhibitors, in the precultivation step affected further growth and fermentations profile for POP, without the HSSL pressure removed, and PAR. Hence, both POP and PAR were precultivated in the same media as the subsequent batch fermentations. Moreover, the effect of the amount of inhibitors in the precultivation was also accessed by using a bio-detoxified HSSL, where the majority of inhibitors were already consumed by *P. variotii*. Three batch bio-reactors were performed using 60 % (v/v) HSSL with the PAR or POP and 60 % (v/v) bio-detoxified HSSL with POP and 40% of chemically-defined media (CDM). These data were compared to the previous fermentations performed with POP and PAR, where the precultivation was performed only with CDM [16].

6.2 Materials and Methods

6.2.1 HSSL supply and pretreatment

Industrial HSSL from magnesium based acidic sulphite pulping of *E. globulus* was supplied by Caima-Indústria de Celulose SA (Altri Group, Portugal). Pre-evaporated HSSL was collected. The pretreatment of HSSL consisted in pH adjustment to 7.0 with KOH, followed by aeration with compressed air for 2 h per litre of HSSL processed. The precipitated colloids were centrifuged at 5000 rpm during 20 min at 4 ^{o}C and then the supernatant was filtered using a 1.0 μm glass microfiber filter (Fioroni).

The bio-detoxified HSSL was obtained through the procedure described by Pereira *et al.*(2012).

6.2.2 Microorganism and media

S. stipitis NRRL Y-7124 (PAR) was gently supplied by Agricultural Research Service Culture Collection at National Center for Agricultural Utilization Research, USDA. The yeast culture was grown at 28 ± 0.5 ^{o}C and maintained at 4 ^{o}C on YM agar slants (3.0 g yeast extract, 3.0 g malt extract, 5.0 g peptone, 10.0 g glucose and 20.0 g agar per litre of distilled water). All media and material were autoclaved at 121 ^{o}C for 20 min.

S. stipitis NRRL Y-7124 was adapted by operating a CSTR between 20% and 60% (v/v) of HSSL, for 382 generations at a dilution rate of 0.20 h^{-1} as described by Pereira *et al.*(2013). POP corresponds to the final population obtained in the CSTR.

Liquid cultures were performed with 100 % chemically-defined media (CDM) or 60% HSSL or bio-detoxified HSSL and 40 % (v/v) CDM depending on the cultivation. The CDM was composed by sugars matching HSSL concentrations [6] (25.0 $g.L^{-1}$ xylose and 2.3 $g.L^{-1}$ glucose). A buffer solution [17] at pH 5.5 (5.1 $g.L^{-1}$ $C_8H_5KO_4$ and 1.1 $g.L^{-1}$ KOH) and a salt solution [18] (5.0 $g.L^{-1}$ $(NH_4)_2SO_4$; 0.5 $g.L^{-1}$ $MgSO_4.7H_2O$; 3.0 $g.L^{-1}$ KH_2PO_4) were also part of the CDM. The media was also supplemented with a vitamins and trace elements solution according to Verduyn *et al.*(1992).

6.2.3 Precultivation

S. stipitis both POP and PAR, were grown by taking colonies from the YM plates to the liquid CDM, into conical tubes. Precultivations were prepared with 60 % (v/v) of HSSL (for (+)PAR and (+)POP cultivations) or bio-detoxified HSSL (for *detox*(+)POP cultivation) and 40 % (v/v) of CDM. A predetermined volume of grown cells was added to the precultivation media, in order to obtain an initial optical density of 0.5 at 620nm (OD_{620nm}). These were performed in Erlenmeyer flasks with a working volume that corresponded to 10 % (v/v) of the total flask volume, at 180 rpm and 28 ^oC.

6.2.4 Batch cultures

Three batch fermentations were performed. The first one prepared with 60 % (v/v) HSSL and 40 % (v/v) CDM, without buffer solution and performed with POP ((+)POP). The second, prepared as the first one but performed with PAR ((+)PAR). The third cultivation was done with 60 % (v/v) bio-detoxified HSSL and 40 % (v/v) CDM without buffer solution. This cultivation was performed with POP (*detox*(+)POP).

A 1L B. Braun BioLab reactor was used in all the batch fermentations with 800 mL of working volume. The reactors were operated at 28 ^oC, 240 rpm and with an aeration of 0.24 $L.min^{-1}$, in order to maintain the final conditions in the CSTR. The culture pH was maintained at 5.5 by supplementing the medium with 3 M KOH or H_2SO_4. Samples were

taken over 60 hours for OD_{620nm} and HPLC analysis. Growth and fermentations profiles of *S. stipitis* PAR and POP were analysed and compared with the cultivations previously studied by Pereira *et al.*(2013).

6.2.5 Analytical methods

Dry weight was determined in the end of the bioreactor fermentations. A 5 mL sample was filtered with a 0.45 µm pore diameter filter (Whatman ME 25/21 ST) and then washed with 15 mL of distilled water. The filter was then dried at 100 ^{o}C until constant weight.

Glucose, xylose, acetic acid and ethanol were analysed by HPLC (Hitachi) using a 10 µm Eurokat H (Knauer) ion-exchange column, 300 × 7.5 mm with an oven (Gecko 2000) set at 40 ^{o}C, and refraction index detector (Hitachi RI Detector L-2490). The eluent was sulphuric acid 0.01 N, with a flow rate of 0.4 mL min^{-1}. An autosampler (Hitachi L-2200) was used to inject each sample with a volume of 20 µl. All samples were centrifuged and filtered off with 0.20 µm filters (CoStar Spin-X) before the analysis. An absolute calibration was applied for all analysed compounds.

For the phenolic compounds analysis, 20 ml of HSSL before and after fermentations were acidified until pH 2 with concentrated HCl. Three liquid-liquid extractions (1:1, v/v) were made to each sample using diethyl ether. The organic phase was collected and passed through anhydrous sodium sulphate to remove traces of aqueous phase. Then, the collected organic phase was concentrated on a vacuum rotor evaporator at 40 ^{o}C until complete dryness. The remaining solids were dissolved in 0.5 ml solution of pyridine with the internal standard, tetracosane (2 $mg.ml^{-1}$). Then, 200 µl BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) and 50 µl TMSC (trimethylchlorosilane) were added and the mixture was left to react at room temperature during 24 h. The samples were analysed by GC-MS, as trimethylsilane (TMS) derivatives according to Marques *et al.*(2009) and results obtained are related to the amount of internal standard added to the samples.

6.3 Results and discussion

6.3.1 Effect of the presence of HSSL inhibitors in the precultivation step

6.3.1.1 Using PAR: (+)PAR

The growth and fermentation profile of the parental strain in 60 % (v/v) HSSL is presented in **Figure 6.1**. No lag phase was observed and glucose was completely depleted after 21 hours (**Figure 6.1**). Xylose only started to be consumed after 19 h (**Figure 6.1**), when glucose concentration was lower than 0.50 $g.L^{-1}$. During the 62 h of fermentation 8.86 $g.L^{-1}$ of xylose were consumed by *S. stipitis.* Regarding the acetic acid almost no consumption was observed during the fermentation, only 0.58 $g.L^{-1}$ of acetic acid. The ethanol production started after 15 hours and the maximum ethanol concentration of 2.93 $g.L^{-1}$ was achieved at the end of fermentation (60 hours).

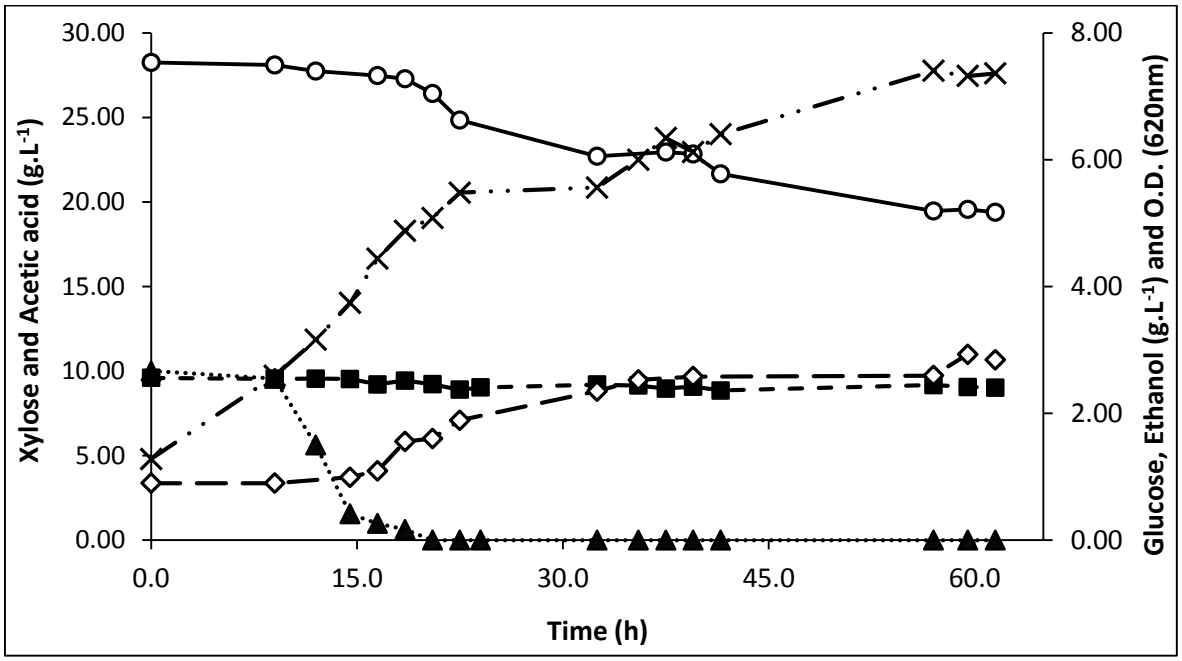

Figure 6.1. *S. stipitis* PAR strain grown in 60 % (v/v) HSSL: xylose (O), glucose (▲), acetic acid (■), ethanol (◇) and Optical Density (×).

The xylose and acetic acid consumption increased as compared with the previously performed experiment using PAR strain, pre-grown in CDM (and referred to as "(-)PAR" in [16]), where only 6.06 and 0.18 $g.L^{-1}$ of xylose and acetic acid, respectively, were consumed. Regarding the ethanol production, a plateau was attained after 40 hours. Moreover, the ethanol concentration in (-)PAR was also lower (1.76 $g.L^{-1}$) [16] than the

achieved in this cultivation, (+)PAR (2.93 g.L^{-1}). Therefore, the HSSL precultivation improved the overall fermentation profile PAR strain. This approach seemed to act as a short-term adaptation previous to the HSSL fermentation performed and, consequently *S. stipitis* metabolism was better prepared for withstanding the inhibitors present in HSSL. Hence, *S. stipitis* was able to consume more sugars (11.53 g.L^{-1}) and produce a higher amount of ethanol (2.93 g.L^{-1}). This was an expected result since pre-exposing the cells to the same kind of pressure as the subsequent fermentation has already been proven to provide positive effects in the fermentation profiles for the yeast *S. cerevisiae* [19, 20].

6.3.1.2 Using POP: (+)POP

The growth and fermentation profile obtained with POP is shown in **Figure 6.2**. In this case, POP presented around 4 h of lag phase, probably due to the lower initial biomass (O.D. of 0.53) compared to the (+)PAR fermentation (O.D. of 1.28). Glucose started to be consumed from time zero, being depleted after 24 h of fermentation (**Figure 6.2**). Xylose started to be consumed after 10 hours, when glucose concentration was around 1.0 g.L^{-1} (**Figure 6.2**). During the fermentation, 15.13 g.L^{-1} of xylose were consumed.

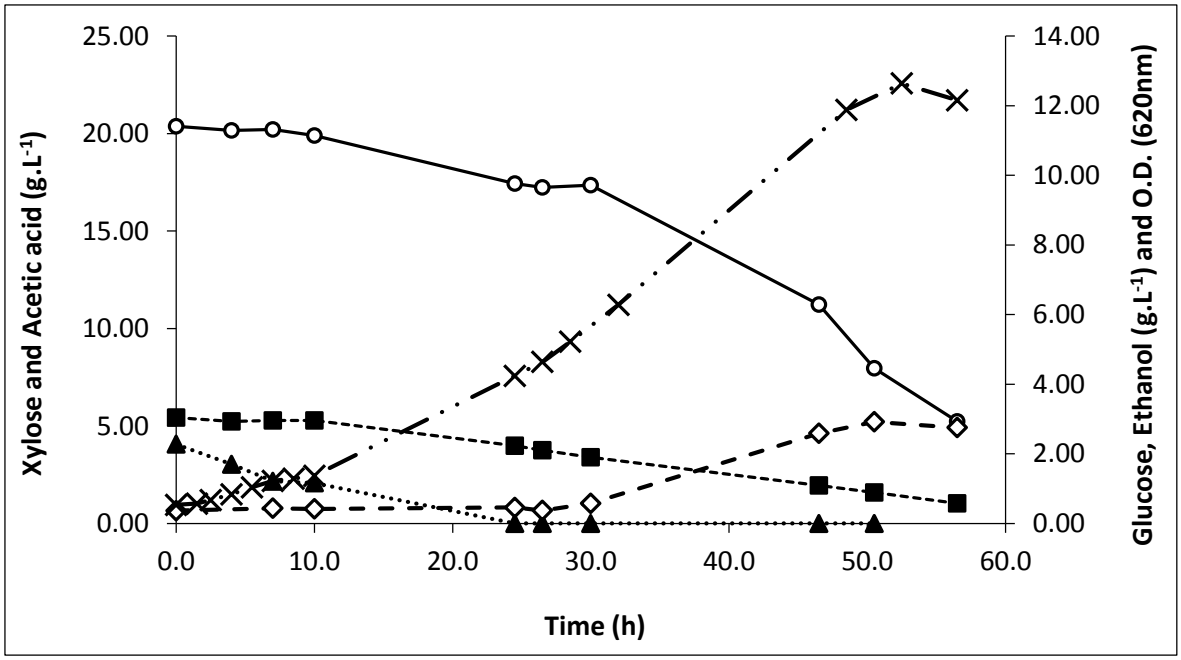

Figure 6.2. *S. stipitis* POP grown in 60 % (v/v) HSSL: xylose (O), glucose (▲), acetic acid (■), ethanol (◇) and Optical Density (×).

The acetic acid consumption started at the same time as xylose consumption, leading to a total of 4.40 $g.L^{-1}$ acetic acid consumption. The ethanol production started after 24 h of fermentation (**Figure 6.2**). The ethanol attained a plateau after 47 hours and the maximum concentration reached 2.92 $g.L^{-1}$ after 51 hours.

POP consumed in general more xylose (15.13 $g.L^{-1}$) and acetic acid (4.40 $g.L^{-1}$) than the parental strain in (+)PAR. However, in terms of ethanol concentration, similar concentrations (2.92 $g.L^{-1}$) were achieved in both cultivations, (+)POP and (+)PAR. When compared with the previous study from Pereira *et al.* (2013), the (-)POP showed a better fermentation profile than (+)POP. The (-)POP attained higher xylose consumption rate (0.33 $g.L^{-1}.h^{-1}$) and a higher ethanol yield (0.26 $g.g^{-1}$) [16].

6.3.1.3 Using bio-detoxified HSSL: *detox*(+)POP

Bio-detoxified HSSL fermentation by POP is shown in **Figure 6.3**. The growth profile was very similar to the observed in the (+)POP fermentation. During all the fermentation, a total of 13.65 $g.L^{-1}$ of xylose was consumed. Since this HSSL already passed through the bio-detoxification step, the initial acetic acid concentration was lower (3.64 $g.L^{-1}$) than the observed in the previous cultivations.

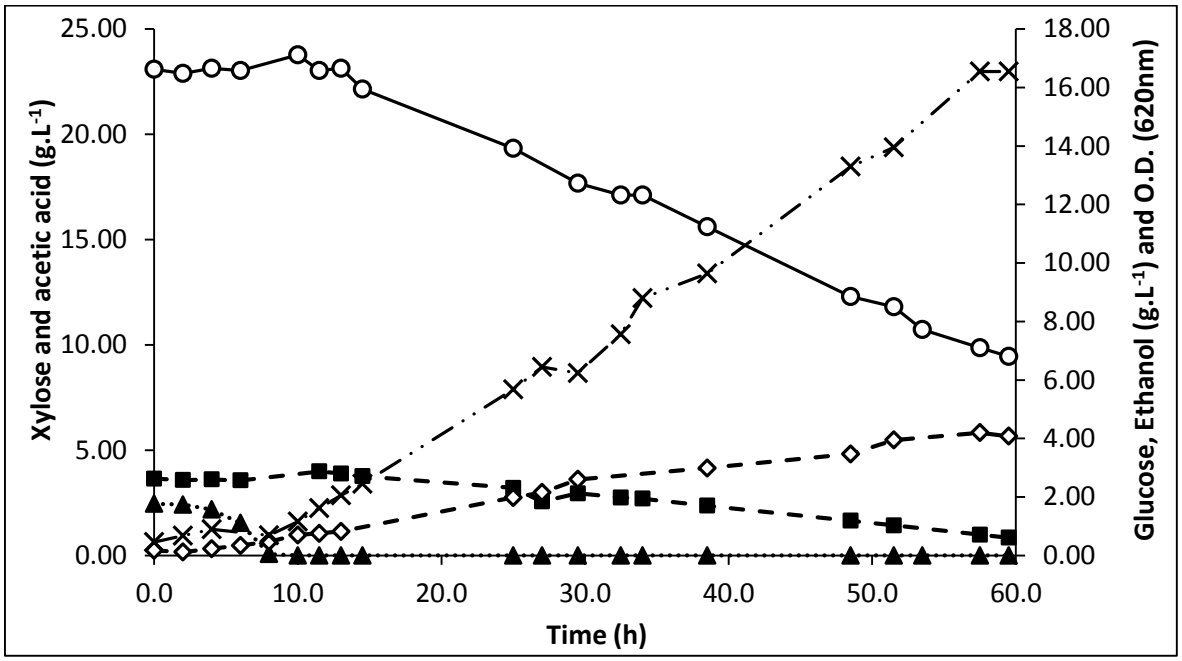

Figure 6.3. *S. stipitis* POP grown in 60 % (v/v) bio-detoxified HSSL: xylose (O), glucose (▲), acetic acid (■), ethanol (◇) and Optical Density (×).

Still, POP began acetic consumption around the 24th h (**Figure 6.3**). In total, POP consumed 2.80 g.L^{-1} of acetic acid. Regarding ethanol, the yeast started to produce it at the 13th h. At the 58th h ethanol reached 4.20 g.L^{-1}, the maximum concentration detected.

Moreover, a higher ethanol yield (0.23 g.g^{-1}) was achieved in this cultivation comparing with (+)POP (0.14 g.g^{-1}). Also, when comparing with the cultivation performed by Pereira *et al.*(2013), *detox*(-)POP, higher ethanol yield was achieved also in this cultivation. Hence, the low concentration of inhibitors present in the precultivation step allowed POP to produce more ethanol, in comparison with the (+)POP. Also, the fact that it was used bio-detoxified HSSL in the precultivation step revealed to improve the fermentation ability of POP, compared with *detox*(-)POP cultivation [16].

6.3.1.4 Fermentations trials comparison

The fermentation parameters of all the cultivations performed in this work and also the ones from the fermentations performed by Pereira *et al.* (2013), are presented in **Table 6.1**. Regarding (-)PAR, it was shown that the fermentation parameters of (+)PAR were in general higher. Both the final biomass (3.15 g.L^{-1}) and the maximum specific growth rate increased (0.055 h^{-1}) comparing with (-)PAR (1.45 g.L^{-1} and 0.037 h^{-1}, respectively).

Table 6.1. Comparison between the fermentation parameters of the experiments performed in this work and the ones performed in Pereira *et al.*(2013) study.

Fermentation parameters	(+)PAR	(-)PAR	(+)POP	(-)POP	*detox*(+) POP	*detox*(-) POP
μ_{max} (h^{-1})	0.055	0.037	0.070	0.037	0.063	0.081
Final biomass (g.L^{-1})	3.15 ± 0.17	1.45 ± 0.08	3.60 ± 0.03	3.75 ± 0.14	5.05 ± 0.18	2.83 ± 0.40
r_{xyl} (g.L^{-1}.h^{-1})	0.15	0.10	0.25	0.33	0.23	0.34
r_{glc} (g.L^{-1}.h^{-1})	0.13	0.10	0.093	0.13	0.18	0.12
$r_{acetic\ acid}$ (g.L^{-1}.h^{-1})	0.01	0.003	0.08	0.05	0.05	0.01
$[EtOH]_{max}$ (g.L^{-1})	2.93	1.76	2.92	6.93	4.20	2.94
$Y_{ethanol}$[a] (g.g^{-1})	0.16	0.13	0.14	0.26	0.23	0.11
Conversion efficiency (%)	31	26	27	51	45	21
Reference	This work	[16]	This work	[16]	This work	[16]

*Calculated at the maximum ethanol concentration

A general increase in the xylose and acetic acid uptake rates were observed in (+)PAR, comparing with (-)PAR. Also, ethanol maximum concentration (2.93 $g.L^{-1}$) as well as ethanol yield (0.16 $g.g^{-1}$) were higher. Therefore, the conversion efficiency increased from 26 % in (-)PAR to 31 % in (+)PAR. These facts showed that preculturing *S. stipitis* in HSSL gave the yeast an advantage in the fermentation process over the one precultured in defined medium. This preculturing step worked as a short-term adaptation step for the yeast, so when the fermentation started, *S. stipitis* could better withstand better the inhibitors in HSSL. Despite the observed improvements, the HSSL fermentation parameters were still lower for PAR than for POP. Also, xylose uptake rate and ethanol yield obtained with (+)PAR were still lower than the ones obtained in (-)POP [16]. Hence, even with the HSSL precultivation step, PAR was not as efficient to fermented HSSL as POP.

In the case of POP fermentation, the (+)POP showed a higher maximum specific growth rate (0.070h^{-1}), almost the doubled as observed for (-)POP (0.037 h^{-1}) [16]. However, the final biomass was very similar for both cultivations, 3.85 $g.L^{-1}$ for (+)POP and 3.60 $g.L^{-1}$ for (-)POP. In general, the (+)POP revealed lower fermentation parameters compared with (-)POP but, still higher when compared with both PAR cultivations. However, the acetic acid consumption rate was the highest (0.076 $g.L^{-1}.h^{-1}$) in the (+)POP cultivation when compared with all the other cultivations. The maximum ethanol concentration attained in (+)POP (2.92 $g.L^{-1}$) was equal to the obtained in (+)PAR and lower than the achieved in (-)POP (6.93 $g.L^{-1}$). Consequently, the ethanol yield was lower in (+)POP (0.14 $g.g^{-1}$) than in (-)POP (0.26 $g.g^{-1}$) and similar to the ones obtained in the PAR cultivations. From all these results, it seems that preculturing the cells with HSSL did not further improve the fermentation profile of POP. Instead, preculturing POP with HSSL negatively affected the fermentation capabilities. Contradicting the observed for PAR, it seemed that the short-term adaptation to POP (resulting from a long-term adaptation to HSSL) did not favour its fermentation metabolism. This fact could indicate that POP cells were already growing at the maximum capability allowed by the long-term adaptation performed. Hence, preculturing these cells in HSSL did not improved further this yeast performance in the liquor.

Considering the *detox*(+)POP cultivation, the parameters determined were very similar to the ones observed in the (+)POP. The μ_{max} was very close to the determined for (+)PAR and slightly lower than the attained in (+)POP. However, the final biomass achieved (5.05 $g.L^{-1}$) was almost the double than the achieved in (+)POP and (+)PAR. This biomass was also the double than the achieved in the previous cultivation *detox*(-)POP [16]. Regarding the substrates consumption rates, the similarity with the (+)POP was evident. Nevertheless, the maximum ethanol concentration achieved (4.20 $g.L^{-1}$) and the ethanol yield (0.23 $g.g^{-1}$) in *detox*(+)POP were higher than the achieved in (+)PAR, (+)POP and also *detox*(-)POP. Hence, a 45 % of ethanol conversion was attained, a closer percentage to the achieved in the cultivation (-)POP [16]. Improved fermentation parameters were obtained when the cells were precultivated with bio-detoxified HSSL, in the *detox*(+)POP. Hence, the low amount of inhibitors present during precultivation, improved the fermentation ability of POP. Due to the *S. stipitis* HSSL long-term adaptation with all the inhibitors, preculturing POP with a HSSL in which the majority of the inhibitor compounds were already removed turned to be the best option for the fermentation of this bio-detoxified HSSL. In this way, the short-term adaptation to the bio-detoxified HSSL improved the fermentation profile of POP in this bio-detoxified HSSL.

Moreover, in order to fully understand the differences observed between the long-term and short-term adaptation effect in *S. stipitis*, the POP should be further analysed in a single-clone level without the HSSL pressure. In this way, it should be perceptible if the improvements observed in POP were at the genetic level and if so, the yeast performance should be stable through generations. However if the removal of HSSL pressure, leads to the reversion of the previously observed features, POP will revert to PAR state.

6.3.2 Phenolic profile

6.3.2.1 The effect of the inhibitors presence in the inocula

In our previous study (Pereira *et al.* (2013)), POP showed the ability to assimilate part of the soluble lignin oligomers and proanthocyanidins, releasing to the media derived phenolic subunits. Similar analysis was performed here for both strains that were precultivated in HSSL. The composition of inhibitors of HSSL, after the different

fermentation, namely low molecular weight compounds, extractable by diethyl ether was analysed and presented in **Table 6.2**.

Table 6.2. Composition of ethyl acetate extractable compounds from HSSL without any fermentation (control), the final HSSL from the (+)Original and (+)Population fermentations.

	%(m/m)[a]		
Compound	**Control**	**(+)PAR**	**(+)POP**
Furan derivatives			
Furfuryl alcohol	0	3.0	0
Furoic acid	0.8	6.8	32.3
Polyphenols			
Pyrogallol	0.40	0	3.8
phenyllactic acid	0	0	0
4-hydroxyphenylethanol	0	0	1.2
Hydroxycinnamic acid	0	0	1.2
Syringic aldehyde	1.4	0	0
2-(4-hydroxy-3-methoxyphenyl)ethanol	0	0.9	3.3
Vanillic acid	0.7	2.5	0.9
3-vanillylpropanol	0.4	1.0	0
Protocatechuic acid	0	1.0	0
4-methoxyphenyllactic acid	0	0.7	0
Syringic acid	1.5	4.8	3.8
Homosyringic acid	0	0	2.0
Gallic acid	15.0	23.1	24.2
Fatty acids			
Palmitic acid	0.9	2.5	1.7
Stearic acid	0.5	1.1	2.0
Organic acids			
Propanoic acid	0	2.0	2.6
Levulinic acid	0	1.1	0
Pentanoic acid	0	0	0.5
Succinic acid	0	1.9	0
Malic acid	0.8	1.6	1.2
Total identified[b]	27	54	83

[a] calculated relatively to an internal standard

[b] calculated relatively to the mass extracted from HSSL

The HSSL from (+)PAR cultivation showed increased concentrations of several extractable compounds, when compared with those in the control HSSL sample (**Table 6.2**). Thus, furanic compounds derived from the degradation of hemicelluloses, namely furfuryl alcohol and furoic acid increased noticeably in (+)PAR HSSL. In particular, furoic acid increased to 6.8 %, while furfuryl alcohol was detected with a concentration of 3.0 %.

Phenolic compounds derived from tannins such as pyrogallol and gallic acid could also be found in HSSL. Interestingly, pyrogallol was not detected in HSSL after (+)PAR cultivation, whereas the amount of gallic acid increased (23.1 %). It is clear that *S. stipitis* was able to metabolise pyrogallol completely. At the same time, *S. stipitis* may have metabolic pathways for the oxidation of proanthocyanidins present in HSSL with the release of corresponding phenolic subunits, thus explaining the increased amount of gallic acid in the liquor. These abilities were already proposed to POP in the previous study by Pereira *et al.*(2013). This fact indicates that the presence of some HSSL inhibitors in the precultivation step, activated *S. stipitis* metabolism towards HSSL inhibitors. Some organic acids were detected in HSSL after (+)PAR cultivation at higher concentrations when compared to the control analysis of HSSL. These acids come from the yeast metabolism since the majority of the acids detected belong to the tricarboxylic acid cycle (TCA).

Likewise, the concentration of some lignin derived aromatic compounds increased in the liquor after fermentation with POP, in (+)POP. Thus the concentrations of vanillic acid, 3-vanillylpropanol and syringic acid have increased 3 to 4 fold in the HSSL after (+)PAR cultivation, when compared to their content in control HSSL (**Table 6.2**). Moreover, several new aromatic compounds were detected in liquor after that fermentation, such as protocatechuic acid, 2-(4-hydroxy-3-methoxyphenyl)ethanol and 4-methoxyphenyllactic acid. All these features indicate the possible metabolization of the soluble lignin oligomers and proanthocyanidins by POP with the release of oxidised phenolic subunits as depicted in **Table 6.2**. This capability was previously suggested by Pereira *et al.*(2013) in the study with the POP denoted as the (-)POP cultivation. In fact, the phenolic profile obtained in the presently studied trial, (+)PAR, was very similar to that one obtained in (-)POP [16]. This fact indicates the significant influence of pre-culturing of *S. stipitis* with HSSL, on the yeast metabolism. After pre-culturing *S. stipitis* in HSSL, the yeast seemed to withstand better HSSL inhibitors and, therefore, growth and fermentation of *S. stipitis* improved as compared to previously reported (-)PAR fermentation [16]. Nevertheless, the (-)POP cultivation [16] attained better fermentation parameters than (+)PAR thus indicating that the CSTR long-term adaptation process performed to *S. stipitis* was more effective than the precultivation approach.

The HSSL from (+)POP cultivation showed several differences relatively to the HSSL from control and the (+)PAR cultivations (**Table 6.2**). For example, (+)POP HSSL analyses revealed increased concentration of furanic compounds derived from degradation of hemicelluloses. Thus, the concentration of furoic acid in HSSL after (+)POP cultivation showed of 40-fold increase in relation to control HSSL and almost 5-fold increase in relation to HSSL from (+)PAR fermentation. However, on the contrary to the (+)PAR cultivation, no furfuryl alcohol was detected in HSSL after the (+)POP fermentation (**Table 6.2**). In the same way, a noticeable raise in the total amounts of oxidised aromatic products was detected in the (+)POP cultivation. In fact, four new lignin derived compounds were detected in (+)POP HSSL, such as hydrocinnamic acid and 4-hydroxyphenylethanol, contributing to extractable compounds of 1.2 % each. In addition, 2-(4-hydroxy-3-methoxyphenyl)ethanol and homosyringic acid were detected (3.3 and 2.0 %, respectively). In the face of those new compounds, syringic aldehyde and 3-vanillylpropanol were not detected in (+)POP HSSL (**Table 6.2**), which could be due to the yeast metabolism. Vanillic and syringic acids (2.50 and 4.8 %, respectively) were detected in (+)PAR HSSL with higher amounts than in (+)POP HSSL. At the same time, the content of gallic acid (23-24 %) was similar in both HSSL after the fermentation, (+)PAR and (+)POP. For the first time, in the batch fermentation cultivation, the amount of pyrogallol increased significantly as in the case of (+)POP trial (**Table 6.2**). In previously performed cultivations, as in (+)PAR and in (-)POP [16] the pyrogallol was not detected at all. As expected, POP was able to assimilate dissolved lignin oligomers and tannins (*e.g.* flavonoids) probably via oxidative pathways.

Comparing both cultivations, (+)PAR and (+)POP, the amount of identified compounds in (+)POP HSSL was much higher (83 %) than in the (+)PAR HSSL where only 54 % of the diethyl ether extracted products were identified. These facts may indicate that HSSL resulting from (+)PAR cultivation still possessed low molecular weight lignin oligomers and condensed tannins that could not be identified through the used GC-MS technique. These differences in the two cultivations could be due to the different metabolisms performed by *S. stipitis* PAR and POP. It seems that the PAR can metabolise complex lignin oligomers and condensed tannins into low molecular weight phenolics in lower extent than

POP. This confirms that the long-term adaptation of *S. stipitis* increased the yeast capacity to metabolise HSSL compounds.

Considering the cultivations performed with POP, (+)POP and (-)POP [16], preculturing POP in HSSL showed to be an important fact to interfere in the yeast metabolism. The total amount of extractable with ethyl acetate compounds either resulting from carbohydrates or lignosulphonates/tannins, were lower in the HSSL from (-)POP cultivation [16] than from (+)POP fermentation. These data show that POP metabolism was more active towards the HSSL ingredients in (+)POP. Apparently, the preculture of POP in HSSL activated the metabolization of dissolved HSSL lignin oligomers and tannins, the potential inhibitors of ethanolic fermentation. In this way, (+)POP metabolism was redirected towards the HSSL inhibitors while in (-)POP the ethanol fermentation metabolism was more active. Accordingly, a higher concentration of low molecular weight phenolics was detected in liquor from (+)POP than from (-)POP, whereas a higher ethanol conversion efficiency was attained in (-)POP cultivation.

6.3.2.2 The effect of the inhibitors concentration in precultivation

POP showed improved fermentation profile in the *detox*(+)POP cultivation, comparing with (+)POP. To verify if this fact is related to the profile of polyphenolics these were analysed in the bio-detoxified HSSL before and after POP fermentation (**Table 6.3**).

The bio-detoxified HSSL possesses very low concentrations of the typical inhibitors usually found in the non-detoxified HSSL [7] (**Table 6.3**). Thus, pyrogallol, gallic acid, syringic aldehyde and vanillic acid were consumed by *P. variotii* almost entirely during the bio-detoxification process and only syringic acid (1.7 %), 3-vanilpropanol (0.8 %) and 2,6-dimethoxy-4-(1-propenyl)-phenol (2.4 %) could still be detected in the bio-detoxified HSSL. Also, several organic acids coming from the fungal metabolism were present.

After the bio-detoxified HSSL fermentation, a general increase in the diethyl ether extractable compounds was detected (**Table 6.3**) [7]. Newly formed lignin derived molecules were found, such as 2-(4-hydroxy-3-methoxyphenyl)ethanol (0.8 %) and vanillic acid (2.2 %). Syringic acid was detected in *detox*(+)POP with a higher amount (4.5 %) than

in the analysis of control bio-detoxified HSSL. Furthermore, gallic acid resulting from tannin oxidation was also detected in *detox*(+)POP HSSL at the increased level of 1.6 %.

Table 6.3. Composition of ethyl acetate extractable compounds from bio-detoxified HSSL prior to POP fermentation, citied from Pereira *et al.*(2012) (bio-detoxified control) and after the fermentation of POP, *detox*(+)POP.

	% (m/m)*	
Compound	**Bio-detoxified control [7]**	***detox*(+)POP**
Furan derivatives		
Furoic acid	0.8	15.0
Polyphenols		
phenyllactic acid	0	3.4
2,6-dimethoxy-4-(1-propenyl)-phenol	2.4	0
2-(4-hydroxy-3-methoxyphenyl)ethanol	0	0.8
Vanillic acid	0	2.2
3-vanillylpropanol	0.8	0
Syringic acid	1.7	4.5
Gallic acid	0	1.6
Fatty acids		
Palmitic acid	0	1.1
Stearic acid	0	0.6
Organic acids		
Lactic acid	1.0	0
Propanoic acid	0	3.3
Oxalic acid	1.0	1.8
Levulinic acid	1.0	0
Succinic acid	18.6	31.0
Fumaric acid	0	3.7
Glutaric acid	0	1.4
3,6-dihydroxyhexanoic acid	0	1.9
2-Methyl-4-ketogluconate	0	8.5
Malic acid	4.5	17.0

*calculated relatively to an internal standard

Therefore, all these results show that POP was able to metabolise dissolved lignin oligomers and tannins still remaining in the bio-detoxified HSSL. Moreover, the high amount of organic acids detected could be the result of the bio-oxidation of aforementioned compounds by the yeast. Since POP suffered a long-term adaptation to the HSSL with all the inhibitors, preculturing these cells with the bio-detoxified HSSL, resulted in a short-term adaptation step for POP. This approach resulted in a POP, which was able to metabolise

the remaining lignin and tannin compounds in HSSL without affecting critically the yeast fermentation metabolism.

6.4 Conclusions

Preculturing *S. stipitis* with HSSL increased its tolerance to the inhibitors present in the liquor. With this approach, PAR improved the HSSL fermentation profile. In the case of POP, the preculturing in HSSL enhanced the lignin and tannin metabolism despite the decreased observed in the fermentation metabolism. Moreover, preculturing POP with a HSSL in which the majority of the inhibitor compounds were already removed turned to be the best option for the fermentation of this bio-detoxified HSSL, most probably due to the long-term adaptation suffered by POP. Hence, preculturing these cells with the bio-detoxified HSSL, resulted in a short-term adaptation step for POP, improving its fermentation performance in the bio-detoxified HSSL.

6.5 References

1. European economic and social committee and the committee of the regions, *Innovating for Sustainable Growth: A Bioeconomy for Europe*, 2012, European Commission: Brussels. p. 9.
2. Lima, M.A.P. and A.P.P. Natalense, eds. *Bioethanol*. 2011, Intech Rijeka. 123-152.
3. Hahn-Hägerdal, B., M. Galbe, M.F. Gorwa-Grauslund, G. Lidén, and G. Zacchi, *Bio-ethanol – the fuel of tomorrow from the residues of today.* Trends in Biotechnology, 2006. **24**(12): p. 549-556.
4. Haghighi Mood, S., A. Hossein Golfeshan, M. Tabatabaei, G. Salehi Jouzani, G.H. Najafi, M. Gholami, and M. Ardjmand, *Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment.* Renewable and Sustainable Energy Reviews, 2013. **27**(0): p. 77-93.
5. Pereira, S.R., D.J. Portugal-Nunes, D.V. Evtuguin, L.S. Serafim, and A.M.R.B. Xavier, *Advances in ethanol production from hardwood spent sulphite liquors.* Process Biochemistry, 2013. **48**(2): p. 272-282.
6. Xavier, A.M.R.B., M.F. Correia, S.R. Pereira, and D.V. Evtuguin, *Second-generation bioethanol from eucalypt sulphite spent liquor.* Bioresource Technology, 2010. **101**(8): p. 2755-2761.
7. Pereira, S.R., Š. Ivanuša, D.V. Evtuguin, L.S. Serafim, and A.M.R.B. Xavier, *Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol.* Bioresource Technology, 2012. **103**(1): p. 131-135.
8. Palmqvist, E. and B. Hahn-Hagerdal, *Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition.* Bioresource Technology, 2000. **74**(1): p. 25-33.

9. Sjöström, E., *Wood Chemistry. Fundamentals and Applications*. 2nd Ed., 1993, New York, USA: Academic Press.
10. Marques, A.P., D.V. Evtuguin, S. Magina, F.M.L. Amado, and A. Prates, *Chemical Composition of Spent Liquors from Acidic Magnesium-Based Sulphite Pulping of Eucalyptus globulus.* Journal of Wood Chemistry and Technology, 2009. **29**(4): p. 322-336.
11. Sonderegger, M. and U. Sauer, *Evolutionary engineering of Saccharomyces cerevisiae for anaerobic growth on xylose.* Applied and Environmental Microbiology, 2003. **69**(4): p. 1990-1998.
12. Hasunuma, T. and A. Kondo, *Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering.* Biotechnology Advances, 2012. **30**(6): p. 1207-1218.
13. Koffas, M. and S.d. Cardayre, *Evolutionary metabolic engineering.* Metabolic Engineering, 2005. **7**(1): p. 1-3.
14. Marques, A.P., D.V. Evtuguin, S. Magina, F.M.L. Amado, and A. Prates, *Structure of Lignosulphonates from Acidic Magnesium-Based Sulphite Pulping of Eucalyptus globulus.* Journal of Wood Chemistry and Technology, 2009. **29**(4): p. 337-357.
15. Hahn-Hagerdal, B., K. Karhumaa, C. Fonseca, I. Spencer-Martins, and M.F. Gorwa-Grauslund, *Towards industrial pentose-fermenting yeast strains.* Applied Microbiology and Biotechnology, 2007. **74**(5): p. 937-953.
16. Pereira, S.R., V.S. Nogué, C. Frazão, D.V. Evtuguin, M.F. Gorwa-Grauslund, L.S. Serafim, and A.M.R.B. Xavier, *Adaptation of Scheffersomyces stipitis to hardwood spent sulphite liquor*, 2013 (*in preparation*).
17. Hahn-Hägerdal, B., K. Karhumaa, C.U. Larsson, M. Gorwa-Grauslund, J. Görgens, and W.H. van Zyl, *Role of cultivation media in the development of yeast strains for large scale industrial use.* Microbial Cell Factories, 2005. **4**(31).
18. Verduyn, C., E. Postma, W.A. Scheffers, and J.P.V. Dijken, *Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation.* Yeast, 1992. **8**(7): p. 501-517.
19. Sànchez i Nogué, V., V. Narayanan, and M. Gorwa-Grauslund, *Short-term adaptation improves the fermentation performance of Saccharomyces cerevisiae in the presence of acetic acid at low pH.* Applied Microbiology and Biotechnology, 2013. **97**(16): p. 7517-7525.
20. Tenreiro, S., P.C. Rosa, C.A. Viegas, and I. Sá-Correia, *Expression of the AZR1 gene (ORF YGR224w), encoding a plasma membrane transporter of the major facilitator superfamily, is required for adaptation to acetic acid and resistance to azoles in Saccharomyces cerevisiae.* Yeast, 2000. **16**(16): p. 1469-1481.

Chapter VII

Final Remarks

Future Perspectives

7.1 Final Remarks

Throughout this work, several biotechnological developments were achieved, regarding HSSL bioprocessing.

The major HSSL inhibitors such as, gallic acid, pyrogallol, syringaldehyde and also acetic acid were successfully identified and removed by the growth of the filamentous fungus *P. variotii* in a SBR approach. With this technology a higher amount of detoxified liquor could be obtained when compared to the processing in a traditional batch. This bio-detoxification process showed to be a promising strategy to ennoble HSSL. After this bioprocess, the HSSL was bio-transformed from a toxic by-product into a substrate which could be used by *S. stipitis* for growth and ethanol production. The bio-detoxification by *P. variotti* allowed a successful ethanolic fermentation of HSSL by *S. sitipitis* providing a maximum ethanol concentration of 2.36 $g.L^{-1}$ with a yield of 0.24 $g.g^{-1}$.

The growth of *P. variotii* in a SBR was also the best approach for the production of SCP. The HSSL was supplemented with a salts mixture to improve *P. variotii* growth and maximize the SBR cycles. However, when salts were added to the HSSL a shift in the fungus metabolism occurred, the acetic acid uptake rate decreased and the sugars uptake rate increased. Therefore, each SBR cycle was longer making the SBR with 3 cycles last for more than a month. Hence, the best operation strategy to produce SCP with the fungus *P. variotii* was the use of HSSL without salts supplementation. The fungal biomass produced in this way, possessed a high content of protein (82.8 %) and a low amount of DNA (1.1 %). The protein present in this biomass possessed also a good variety of AAs, six of which were essential AAs. These characteristics made the SCP from *P. variotii* growth on HSSL, a good candidate for animal feeding. In this way, the pulp and paper industry can integrate the bioethanol production with *S. stipitis* after the SCP production with *P. variotii* and thus improving the plant sustainability and contributing to the pulp mill profits.

In order to improve further the fermentative performance of *S. stiptis* in the HSSL, an adaptation experiment of the yeast to the liquor was performed using a CSTR. The final *S. stipitis* population from the CSTR with 60 % HSSL (POP) was recovered. POP could withstand the HSSL inhibitors better than the parental strain (PAR) so, substrates consumption rates and ethanol concentration were improved. With POP, 6.93 $g.L^{-1}$ of

ethanol was achieved with a yield of 0.26 $g.g^{-1}$. Moreover, POP showed the enhanced ability to assimilate dissolved lignin oligomers and tannins probably through activation of oxidative pathways. However, when POP fermented the bio-detoxified HSSL, the ethanol concentration and yield were lower than the obtained in the HSSL with all the inhibitors. As POP was adapted to the HSSL with all the inhibitors, the lower inhibitor concentration in the bio-detoxified HSSL did not favour the ethanol production.

A precultivation step was introduced both in PAR and POP, to access if the fermentation profile could be improved. It was established that this approach improved PAR tolerance towards the HSSL inhibitors and also the yeast fermentation ability in HSSL. However, precultivating POP in HSSL redirected its metabolism to the inhibitors assimilation, reducing the ethanol production. Nevertheless, preculturing POP in a bio-detoxified HSSL, turned out to be the best approach, most probably due to the long-term adaptation suffered by POP. Hence, preculturing these cells with the bio-detoxified HSSL, resulted in a short-term adaptation step for POP, improving its fermentation performance in the bio-detoxified HSSL. Furthermore, these capacity of POP to activate the metabolic pathways necessary to bio-transform the tannin and lignin inhibitors, represents a major advantage in the bioprocessing of HSSL and lignocellulosic biomass in general.

7.2 Future perspectives

Although the developments achieved during this work, three main research lines still require further investigation and optimization:

1) Optimization of SCP production and HSSL detoxification by *Paecilomyces variotii*:

P. variotii growth in a SBR should be optimized in a bioreactor with some operational parameters controlled (aeration, pH, temperature and agitation). The SBR cycles should be maximized and optimized. The fungal biomass obtained should be further analysed for lipids, minerals and potential contaminants coming from HSSL.

2) Accessing the stability of the adapted population of *Scheffersomyces stipitis*:

The final population obtained from the CSTR (POP) should be accessed for its stability by removing the HSSL pressure during some generations. Clones should be selected in the beginning of this procedure. After passing through several generations without being submitted to the HSSL pressure, the clones should be tested again in HSSL. In this way, it should be perceptible if the improvements observed in POP were at the genetic level and if so, the yeast performance should be stable through generations. In this case, the clone with the best performance in HSSL should then be compared, genetically, with the parental strain (PAR) and POP.

3) Optimization of bioethanol production:

S. stipitis is a yeast that induces its fermentation mechanism with micro-aerophilic conditions in the media. Hence, the ethanol production should be optimized within this conditions. Bioethanol fermentation should be studied in a bioreactor with oxygen control so, a micro-aerophilic environment could be created and studied. This study should be performed with the clone from the previous topic and also with PAR.