THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Concepts for improving ethanol productivity from lignocellulosic materials: Encapsulated yeast and membrane bioreactors

PÄIVI YLITERVO

Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden

UNIVERSITY OF BORÅS
SCHOOL OF ENGINEERING

Borås, Sweden

Concepts for improving ethanol productivity from lignocellulosic materials: Encapsulated yeast and membrane bioreactors

PÄIVI YLITERVO

ISBN 978-91-7385-988-2

Doktorsavhandlingar vid Chalmers tekniska högskola

Ny serie nr 3669

ISSN 0346-718X

Department of Chemical and Biological Engineering

Chalmers University of Technology

SE-412 96 Gothenburg

Sweden

Telephone + 46 31 772 1000

Skrifter från Högskolan i Borås, nr. 49

ISSN 0280-381X

School of Engineering

University of Borås

SE-501 90 Borås

Sweden

Telephone: +46 33 435 4000

Cover illustration: Picture of alginate-chitosan capsules (left) containing low amounts of yeast, and a submerged membrane bioreactor (right). Photographs by Päivi Ylitervo.

Printed by Chalmers Reproservice

Gothenburg, Sweden 2014

Concepts for improving ethanol productivity from lignocellulosic materials: Encapsulated yeast and membrane bioreactors

PÄIVI YLITERVO

School of Engineering, University of Borås
Department of Chemical and Biological Engineering, Division of Life Sciences — Industrial Biotechnology, Chalmers University of Technology

ABSTRACT
Lignocellulosic biomass is a potential feedstock for production of sugars, which can be fermented into ethanol. The work presented in this thesis proposes some solutions to overcome problems with suboptimal process performance due to elevated cultivation temperatures and inhibitors present during ethanol production from lignocellulosic materials. In particular, continuous processes operated at high dilution rates with high sugar utilisation are attractive for ethanol fermentation, as this can result in higher ethanol productivity. Both encapsulation and membrane bioreactors were studied and developed to achieve rapid fermentation at high yeast cell density.

My studies showed that encapsulated yeast is more thermotolerant than suspended yeast. The encapsulated yeast could successfully ferment all glucose during five consecutive batches, 12 h each at 42 °C. In contrast, freely suspended yeast was inactivated already in the second or third batch. One problem with encapsulation is, however, the mechanical robustness of the capsule membrane. If the capsules are exposed to e.g. high shear forces, the capsule membrane may break. Therefore, a method was developed to produce more robust capsules by treating alginate-chitosan-alginate (ACA) capsules with 3-aminopropyltriethoxysilane (APTES) to get polysiloxane-ACA capsules. Of the ACA-capsules treated with 1.5% APTES, only 0–2% of the capsules broke, while 25% of the untreated capsules ruptured within 6 h in a shear test.

In this thesis membrane bioreactors (MBR), using either a cross-flow or a submerged membrane, could successfully be applied to retain the yeast inside the reactor. The cross-flow membrane was operated at a dilution rate of 0.5 h^{-1} whereas the submerged membrane was tested at several dilution rates, from 0.2 up to 0.8 h^{-1}. Cultivations at high cell densities demonstrated an efficient *in situ* detoxification of very high furfural levels of up to 17 g L^{-1} in the feed medium when using a MBR. The maximum yeast density achieved in the MBR was more than 200 g L^{-1}. Additionally, ethanol fermentation of nondetoxified spruce hydrolysate was possible at a high feeding rate of 0.8 h^{-1} by applying a submerged membrane bioreactor, resulting in ethanol productivities of up to 8 g L^{-1} h^{-1}.

In conclusion, this study suggests methods for rapid continuous ethanol production even at stressful elevated cultivation temperatures or inhibitory conditions by using encapsulation or membrane bioreactors and high cell density cultivations.

Keywords: Encapsulated yeast, Biofuel, *S. Cerevisiae*, Membrane bioreactors, Thermotolerance, Furfural, Acetic acid

LIST OF PUBLICATIONS

This thesis is based on the following research papers, referred to as **Papers I–V** in the text:

Paper I **Päivi Ylitervo**, Carl Johan Franzén, Mohammad J. Taherzadeh, (2011), Ethanol production at elevated temperatures using encapsulation of yeast, Journal of Biotechnology, 156, 22 – 29

Paper II **Päivi Ylitervo,** Carl Johan Franzén, Mohammad J. Taherzadeh, (2013), Mechanically robust polysiloxane-ACA capsules for prolonged ethanol production, Journal of Chemical Technology & Biotechnology, 88, 1080–10

Paper III **Päivi Ylitervo**, Carl Johan Franzén, Mohammad J. Taherzadeh, (2013), Impact of furfural on rapid ethanol production using a membrane bioreactor, Energies, *6*:3, 1604-1617

Paper IV **Päivi Ylitervo**, Carl Johan Franzén, Mohammad J. Taherzadeh, Continuous ethanol production in a membrane bioreactor at high acetic acid concentrations (Manuscript)

Paper V **Päivi Ylitervo**, Wim Doyen, Mohammad J. Taherzadeh, Fermentation of lignocellulosic hydrolysate using a submerged membrane bioreactor (sMBR) for continuous ethanol production (Submitted)

Additional publications during my doctoral research that are not included in this thesis:

Päivi Ylitervo, Julius Akinbomi, Mohammad J. Taherzadeh (2013), Membrane bioreactors' potential for ethanol and biogas production: A review, Environmental Technology, 34: 13–14, 1711–1723

Johan Westman, **Päivi Ylitervo**, Carl Johan Franzén, Mohammad J. Taherzadeh, (2012), Effects of encapsulation of microorganisms on product formation during microbial fermentations, Applied Microbiology and Biotechnology, 96, 1441–1454

Patrik R. Lennartsson, **Päivi Ylitervo**, Christer Larsson, Lars Edebo, Mohammad J. Taherzadeh, (2012), Growth tolerance of Zygomycetes *Mucor indicus* in orange peel hydrolysate without detoxification, Process Biochemistry, 47:5, 836-842

STATEMENT OF CONTRIBUTION

My contributions to each of the above publications are as follows:

Paper I: Conceived the idea together with the co-authors. Performed all experimental work, and was the main author for the manuscript.

Paper II: Responsible for the idea and planning. Performed all experimental work, and conducted all data analysis together with manuscript preparation and its revision.

Paper III: Responsible for most of the idea, all of the experimental work, and was the main author for the manuscript.

Paper IV: Responsible for the idea, performed all experimental work, and the writing of the manuscript.

Paper V: Responsible for the idea, performed all experimental work, and the writing of the manuscript.

‘I guess I should warn you, if I turn out to be particularly clear, you've probably misunderstood what I said’.

Chairman of the Federal Reserve Alan Greenspan
New York, 1988

TABLE OF CONTENT

CHAPTER 1

INTRODUCTION

1.1 Scope and outline

At present, we are consuming around 90 million barrels of oil each day [1]. Our transport sector is unfortunately heavily dependent on oil as raw material, and we know that we face a progressive depletion of oil and traditional fossil fuels. Environmental concerns due to greenhouse gas emissions from fossil fuel consumption has increased the interested in renewable alternatives. Usage of lignocellulosic materials has the potential to provide a large source of clean, low carbon and secure energy [2]. Nevertheless, there is still no large production of any substitute fuel. Today, most of the easily accessible oil has already been used, and the world's demand for more fuel has resulted in oil prospecting in deep waters and sensitive areas. Exploitation of oil sands for oil production has also been developed and started [3].

In the future, we probably can not only rely on one single substitute for replacing the fossil fuels used for transportation. There are several suggested substitutes for fossil fuels, such as dimethyl ether, biomethane, biodiesel, bioethanol or hydrogen [4]. Ethanol, which is the focus of my thesis work, can be produced by fermenting sugars derived from sugar-rich, starch-rich or lignocellulosic materials. Lignocellulosic materials are cheap and abundant materials, which make them attractive as feedstock for ethanol production. However, lignocellulosic materials are recalcitrant and its hydrolysis not only produces fermentable sugars but also toxic compounds, such as weak acids, furan aldehydes and phenolics [5]. Unfortunately, inhibitors or other stress factors such as elevated cultivation temperatures can lead to reduced capacity or complete failure of the yeast to ferment the sugars into ethanol. Ethanol is a low-value bulk chemical. It is, therefore, crucial that its production is relatively simple, robust and efficient. Suitable fermentation conditions are therefore needed to make the yeast be able to utilise the lignocellulose derived substrate, sometimes containing high concentrations of inhibitors.

The overall aim of my thesis research was to develop rapid ethanol fermentation, even at inhibiting conditions, with the aim of fermenting toxic media or lignocellulosic hydrolysates prepared from spruce. Two potential methods – yeast encapsulation and fermentation in membrane bioreactors (MBR) – were applied throughout the thesis research to gain high cell concentrations inside the bioreactor. The overall hypothesis was that a high local yeast cell density in a capsule or in the entire bioreactor would be beneficial for rapid fermentation at stressful conditions like elevated temperatures or in toxic media or wood hydrolysate. The physiological responses, such as ethanol and glycerol production, sugar consumption, viability and vitality, were used to evaluate the performance of the yeast. This thesis investigates the limitations of rapid yeast ethanol fermentation by using encapsulated yeast or MBR at increased stress conditions at elevated cultivation temperatures or high inhibitor levels, such as high furfural concentration, high acetic acid concentrations and high hydrolysate dilution rates. Additionally, the capsule robustness and MBR technology were developed further for ethanol production.

This thesis is divided into five main chapters:

- Chapter 1 introduces the thesis and explains the motivation and purpose of the present work, together with a description of the complex connection between our economical system and our immense usage of transportation fuels.

- In Chapter 2, both first- and second-generation ethanol production is reviewed in general and utilisation of lignocellulosic materials in more detail. A short discussion on different cultivation modes is also included.

- Chapter 3 presents a detailed description of, the two methods, encapsulation and membrane bioreactors, used throughout the entire thesis, together with descriptions of experimental methodologies (**Papers I–V**).

- Chapter 4 describes the complexity of lignocellulosic hydrolysate fermentation. Problems and solutions are discussed in relation to results from the research in **Papers I–V**.

- Chapter 5 discusses rapid ethanol production from toxic lignocellulosic media by using membrane bioreactors. Results from **Papers III–V** are included in this chapter.

1.2 Our oil dependence

The world demand for oil has steadily increased, accelerated by the fast economical growth especially in China and India [6]. It is uncertain for how long the oil production can satisfy the world's vast oil consumption. We are presently consuming approximately 90 million barrels of oil each day [1]; furthermore, many areas that contained easily accessible oil have already been depleted, and today, lower grade oil and less accessible oil are being exploited. There has been an intensive debate as to whether oil production has reached its maximum (peak oil) and will decline. Some people point out that vast oil resources are available in tar sands and oil shales. Oil production from these sources has started even though the process is expensive and environmentally hazardous [7].

Figure 1 Brent oil price (USD barrel^{-1}) between the years 2006 and 2014. Reprinted with permissions [8].

The stability of our economical and social system is heavily dependent on the constant supply of oil. Large increases in the oil price can destabilise the world's macro economy, similar to rapid increases in food and other raw material prices. A rapidly rising oil price was e.g. observed in 2007 and 2008, with a peak of 147 USD per barrel in July 2008, after which it fell sharply. Nevertheless, in 2009, it started to increase steadily again and during the past three years it has fluctuated between around 100–125 USD per barrel [7, 8] as illustrated in Figure 1.

Research focused on providing alternative fuels for the transportation sector is crucial. A great deal of research has already been conducted in the field but many issues remain. Conversion of renewable raw materials such as lignocellulosic agricultural- and forest- residues is especially attractive, as vast amounts are available for producing necessary chemicals or fuels.

1.3 Economical growth and fuel consumption

In many ways, society is based on a continual expansion of economical and material needs, which in reality is unsustainable. Prosperity is generally calculated in economical terms by the gross domestic product (GDP) per capita, and the general belief is that an increasing GDP per capita will result in an equivalent increase in prosperity. As a result, GDP growth has been encouraged. A common belief among economists is that economical growth can and will continue forever, and our modern economy is by its structure deeply dependent on growth for its stability [7]. Today, there is a general pursuit for economical growth, driving the need to sell more goods and stimulate an ever-increasing consumer demand. The question is, however, how can a continuously growing economical system be integrated within a finite ecological system?

After the immense financial turbulence in 2008, when the investment bank Lehman Brothers declared its bankruptcy, several bailouts were put together to stabilise failing banks, to motivate increased consumption and a continued economical growth. By the end of October 2008, governments around the world had allocated an incredible 7 trillion US dollars of public funds for the bailouts. Economical growth nevertheless has vast consequences on both resource utilisation and environment. A conventional belief is that the dilemma with growth can be solved by decoupling, that is, by making the economy less dependent on material utilisation and making things more efficient. However, increased production efficiency can not restrain growing resource utilisation [7]. There are, for example, limitations on how efficient a process can be as well as the availability of raw materials.

One example is the increased fuel usage for transportation. Fuel consumption in EU for road transportation has increased by 5% from 2001 to 2011 (Figure 2). However, the fastest growth in fuel consumption in the transportation sector has occurred in air transportations, which has increased by 16%. Biofuels accounted for 4% of the consumed fuels in 2011 [9]. The target for the EU transport sector is that 10% of the fuel in 2020 should have a renewable origin, according to the Renewable Energy Directive [10].

The need to reduce the transport sectors dependence on fossil fuels and the large emissions of greenhouse gases motivates profound studies concerning renewable biofuels. Bioethanol, which is the focus in this thesis, is one of the probable substitutes for fossil fuels in the land-based transport sector.

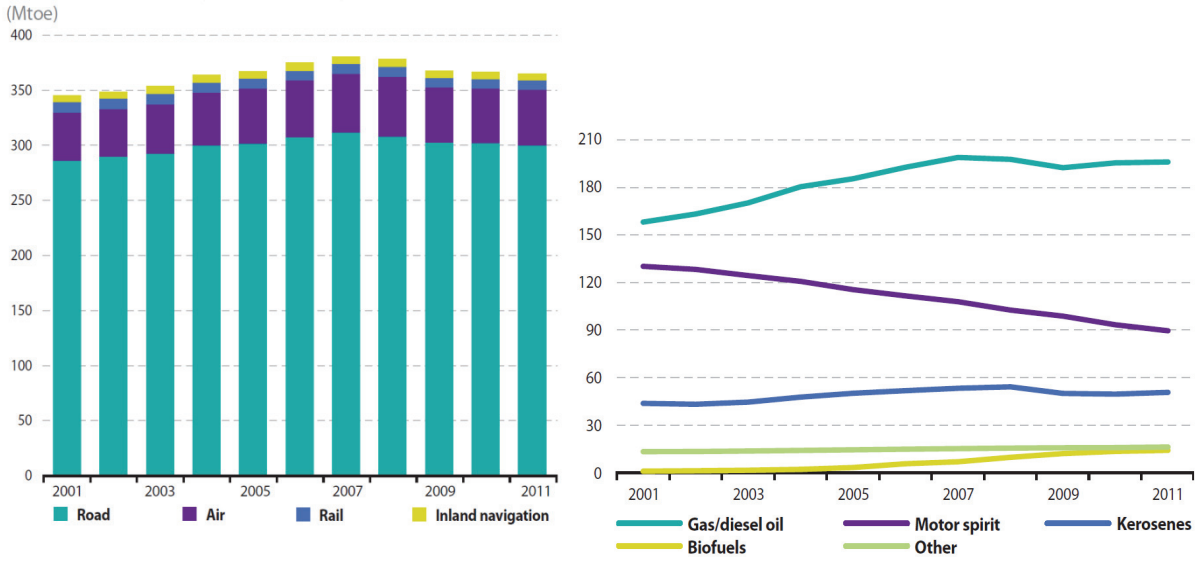


Figure 2 Energy consumption in Mtoe by the transportation sector in EU-28 and the utilised amounts of fuels. Reprinted with permissions [9].

CHAPTER 2

ETHANOL PRODUCTION

Today, first generation biofuels such as ethanol is produced in large scale at economical levels. However, it is obvious that the amounts of starch- and sugar-based material, in particular, required to substitute a major fraction of the fossil fuels in the transport sector are insufficient. As reviewed by Brennan and Owende [11], it is not possible since presently 1% or 14 million hectares of arable land is used to produce biofuels, which corresponds to 1% of the total consumption of transportation fuels. It is therefore not probable that the production of first generation biofuels can be increased anywhere close to 100%. This chapter will review the common ethanol fermenting yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) and first and second generation ethanol production together with different fermentation modes.

2.1 Saccharomyces cerevisiae

Several different microorganisms are being used for the fermentative production of ethanol. However, the yeast *Saccharomyces cerevisiae*, also known as baker´s yeast, is still the dominating organism for industrial ethanol production. It has received the most attention, for example, because of its GRAS status, long traditional use in both baking and alcoholic beverage production, high rate of fermentation of hexoses, high tolerance to ethanol, inhibitors, acidity and other process conditions; it has also been well researched. The long tradition of using *S. cerevisiae* has put selective pressure on certain useful properties such as ethanol tolerance, acid tolerance and osmotolerance [12, 13]. The wild-type *S. cerevisiae* is able to utilise sugars such as maltose, sucrose, glucose, mannose, fructose and galactose, but it is unable to utilise xylose and arabinose [14]. Glucose is the preferred carbon and energy source. Several other microorganisms have also been tested as possible ethanol producers. Among these are, for example, *Escherichia coli, Zymomonas mobilis, Scheffersomyces stipitis, Klyveromyces marxianus* and *Mucor indicus* [15].

S. cerevisiae belongs to the category of facultative anaerobes, which are able to grow both in the presence and absence of oxygen. However, at strictly anaerobic conditions it is unable to produce ergosterol and unsaturated fatty acids, which must be supplemented to enable anaerobic growth [16]. *S. cerevisiae* produces ethanol even in the presence of oxygen; it is

therefore a Crabtree-positive yeast. This mixed respiro-fermentative metabolism occurs when the glucose concentration is above a critical threshold concentration [17].

The main metabolic pathway involved in *S. cerevisiae* for ethanol fermentation is glycolysis, (synonymous to Embden-Meyerhof-Parnas or EMP pathway). During glycolysis, one glucose molecule is metabolised and generates two pyruvate molecules. In this reaction sequence, liberated free energy is used to form a net two ATP molecules, and two NAD^+ are converted into two NADH. Under anaerobic conditions, the two pyruvates are metabolised into two acetaldehyde molecules together with the release of two CO_2. In order to redox-balance the reaction, the two acetaldehydes are converted together with H^+ ions and NADH to produce ethanol, and thereby regenerate NAD^+ again. The theoretical yield on a mass basis on metabolised glucose is 0.511g g^{-1} for ethanol and 0.489 g g^{-1} for CO_2 [18]. Many yeast strains produce glycerol as a by-product during ethanol fermentation, and the glycerol concentration is generally between 2.5–3.6wt% of the produced ethanol [19]. Under anaerobic conditions, the formation of glycerol occurs because of the need to re-oxidise NADH, which is produced during biomass and acetic acid formation [20-22].

The main drawback of *S. cerevisiae* is its inability to ferment pentose sugars such as xylose and arabinose. Vast efforts have been made to construct *S. cerevisiae* strains by metabolic engineering to enable xylose fermentation as reviewed by [14, 23, 24]. In this work, pentose fermentation was not addressed, and the yeast strain *S. cerevisiae* CBS8066 was used in all studies.

2.2 Ethanol production from starch or sugar

At present, the prime raw materials for ethanol production by fermentation are starch- or sugar-rich crops. In USA, ethanol is mainly produced from corn, whereas in Europe sugar beets, cereals and wine alcohol are used [25]. In contrast, Brazil produces ethanol from sugarcane, where the sugar-rich extract from sugarcane can be utilised directly for ethanol fermentation. Starchy raw materials, on the other hand, need to be hydrolysed before they can be fermented, as they consist of two types of polymers: amylose and amylopectin. Both polymers consist of glucose units. In amylose, which is unbranched, the glucose units are connected by α-1,4 links, whereas amylopectin is a branched polymer with a main amylose-chain that is linked by α-1,6 bonds to short saccharide chains. The main procedure by which fermentable sugars are produced from starch is by first liquefying the starch at an elevated

temperature by adding α-amylase. To produce glucose a second enzyme named glucoamylase has to be supplemented [26].

However, the use of edible crops for ethanol production is controversial since it generates competition for the raw material, to either use it as food or for ethanol production. The cultivation of crops also takes agricultural land in possession [27]. Other limitations and uncertainties with using crops for generation of ethanol are its environmental impact because of the usage of fossil fuel for their production and their need for fertilizers and water for irrigation [28]. When ethanol is produced from sugar- or starch-rich feedstocks, about 40–70% of the production cost is attributed to the cost of the raw material [27].

2.3 Ethanol production from lignocelluloses

2.3.1 The raw material

Because lignocellulosic materials are abundant and cheap, they have attracted a great deal of interest as feed-stock for ethanol production. Unlike starch- and sugar-rich materials, which can be fermented into ethanol rather easily, lignocellulosic residues have a very complex and recalcitrant structure where the carbohydrates are tightly associated with lignin, as shown in Figure 3. Consequently, harsh treatment is needed to release fermentable sugars from the lignocellulose.

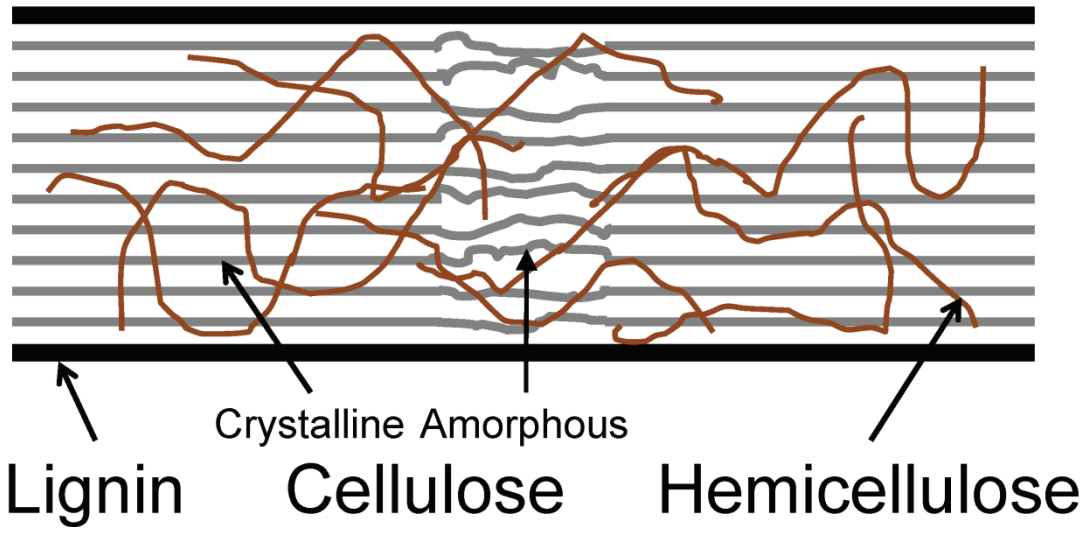


Figure 3 Illustration of the schematic structure of lignocellulose. Cellulose, hemicellulose and lignin are the three major components in lignocellulosic materials, forming a highly recalcitrant structure where the cellulose is tightly surrounded by both hemicellulose and lignin. Reprinted with permissions [29].

Lignocellulose is present in plant material and is constructed of three main components, namely, cellulose, hemicellulose and lignin. Up to 70% of the lignocellulosic plant residues consist of the carbohydrates cellulose and hemicellulose [30]. Generally, softwood and hardwood is composed of about 40–50% cellulose, 20–30% hemicelluloses and 15–30% lignin and other components. Herbaceous plants usually contain less lignin than wood [31, 32], see Table 1.

The cell wall of living plant cells is mainly composed of cellulose, having the main purpose to provide physical strength to the cell. Cellulose is a linear polymer of glucose molecules linked together by β-1,4 bonds, where the repeating unit is the disaccharide cellobiose [33]. The cellulose chain length is generally 2,000 to 20,000 glucose units, and the linear structure of the cellulose chains makes them able to connect strongly to each other by hydrogen bonding. The cellulose chains therefore form so-called microfibrils, which mainly have a crystalline structure [34]. The hemicellulose polymers, in contrast, have a more complex structure than cellulose and have a lower molecular weight. Hemicellulose consists of many different monosaccharides, such as arabinose, galactose, glucose, mannose and xylose. Softwood hemicellulose mainly consists of galactoglucomannan, whereas hemicellulose consists mainly of O-acetyl-4-O-methyl-glucuronoxylan in hardwood and of arabinoxylan in grass. In addition to a main hemicellulose, each lignocellulosic raw material also contains several different sorts of other hemicelluloses, for example, arabinoglucuronoxylan, arabinogalactan and glucomannan [32, 33].

Table 1 Composition of lignocellulosic materials (% of dry material).

Lignocellulosic material		Components					References
		Cellulose	Hemicellulose	Lignin	Extracts	Ash	
Straw	**Wheat straw**	33.0	33.0	20.0	na	na	[35]
	Corn stover	34.4	29.0	17.2	na	na	[36]
	Sugarcane leafs	40.8	30.8	25.8	na	2.6	[37]
Softwood	**Scots pine (Stem wood)**	40.7	26.9	27.0	5.0	na	[38]
	Norway spruce (Stem wood)	42.0	27.3	27.4	2.0	na	[38]
Hardwood	**Silver/Downy birch (Stem wood)**	43.9	28.9	20.2	3.8	na	[38]

na = not analysed

The hydrolysis of hemicellulose will, therefore, result in very different sugar composition depending on the origin of the raw material used. In softwood, the main sugar building blocks are glucose and mannose, whereas the amount of xylose is rather low, compared to the amounts in hardwood and straw [33]. The composition of the plant material can also vary significantly depending on growth conditions. Hemicellulose is also acetylated to a different degree. Usually, hardwood hemicellulose is more acetylated than softwood hemicellulose [39].

Different wood materials generally have similar cellulose content [30], but softwood commonly contains a larger portion of lignin and less hemicellulose than hardwood. In softwood, around 25–30% of the wood's dry weight is lignin, while in hardwood the lignin content is around 20–25% [40]. Lignin has a very amorphous structure where the lignin attaches to both cellulose and hemicellulose polymers by covalent or hydrogen bonding. The lignin part in the biomass consists of a three-dimensional polyphenolic polymer consisting of *p*-hydroxyphenyl-propanoid units bound together. The lignin monomers are derivatives of *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. How these momomers build up the plant and at what ratios they occur depends on the plant [30]. The complex structure of lignin makes it very resistant to both enzymatic and chemical degradation [41-43].

Plant material also contains a portion of extractives or secondary metabolites such as resins, terpenes, phenols, quinines and tannins together with non-extractives, which are mainly inorganic ash components e.g. silica and alkali, but also pectin, proteins and starch [30]. Several different treatment methods have been tested in order to pre-treat or hydrolyse lignocelluloses prior to ethanol fermentation. To write a complete overview of all these methods is outside of the scope of this thesis. Instead, only two hydrolysis methods are reviewed briefly. These are acid hydrolysis and enzymatic hydrolysis.

2.3.2 Hydrolysis

In order to utilise the carbohydrates in lignocellulosic material, they need to be degraded to fermentable sugars. There are several principal methods for degrading cellulose and hemicellulose to monosaccharides and fermenting them into ethanol as illustrated in Figure 4.

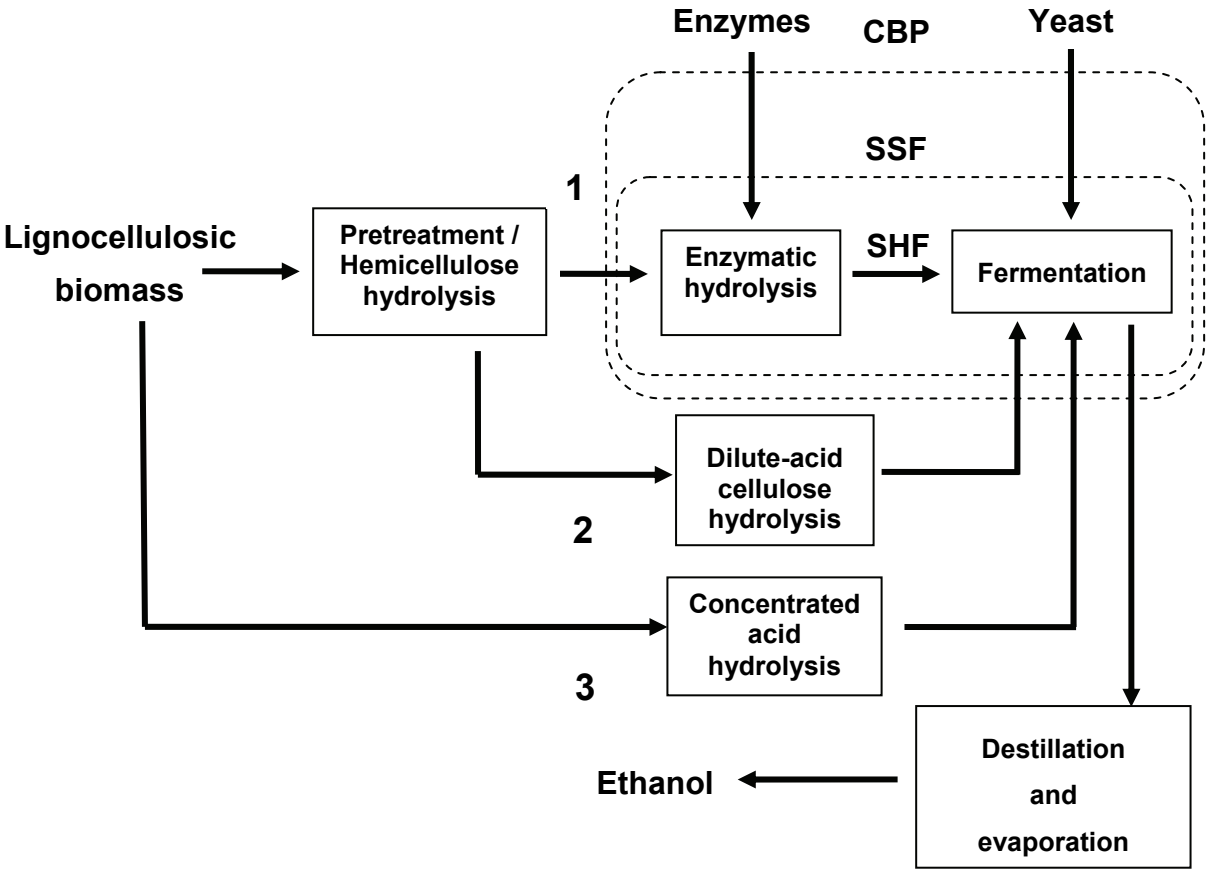


Figure 4 Illustration of the different concepts and steps involved in lignocellulosic hydrolysis and conversion into ethanol: enzymatic hydrolysis (1), dilute-acid hydrolysis (2) and concentrated acid hydrolysis (3). SHF: separate hydrolysis and fermentation, SSF: simultaneous saccharification and fermentation, CBP: consolidated bioprocessing.

2.3.2.1 Enzymatic hydrolysis

In enzymatic hydrolysis, cellulolytic and hemicellulolytic enzymes are added to a pretreated lignocellulosic material in order to hydrolyse the carbohydrates. The pretreatment is necessary to break up the recalcitrant structure of the lignocellulosic material and make it more accessible for the hydrolysing enzymes [44]. There are three principally different enzymatic hydrolysis concepts by which lignocellulosic materials are hydrolysed and fermented: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP), as illustrated in Figure 4. In the SHF process, enzymatic hydrolysis and fermentation of the released sugars are preformed in separate vessels, while the SSF is conducted in the same reactor. The sugars produced in the SSF process can thereby be consumed directly by the present microorganism, which hinders product inhibition of the enzymes. However, since both fermentation and hydrolysis occur in the same reactor, operating conditions need to be suitable for both enzymes and cells. Both

the SHF and SSF process require addition of enzymes whereas no enzyme addition is needed in the CBP since in this case, the fermenting microorganism can produce hydrolysing enzymes [45, 46].

The advantage of enzymatic hydrolysis is that it can be conducted at mild conditions, e.g. at pH 4.8 and a temperature of 45–50 °C. The utility cost and corrosion problems are therefore low compared to treatments with acid or alkaline [47]. The current interest in enzymatic hydrolysis has been high since it can give high saccharide recovery and a low inhibitor formation compared to acid hydrolysis. Additionally, the hydrolysis can be performed at moderate temperatures. However, to get a successful enzymatic hydrolysis, it is necessary that the material be first pretreated properly in order for the enzymes to get access to the carbohydrates. Without any pretreatment, cellulose hydrolysis will be extremely slow, since the enzymes will have difficulty gaining access to the cellulose polymer chains due to the crystalline structure of the lignocellulose [45].

In nature there are several organisms which produce enzymes capable of degrading cellulose and hemicellulose [48]. Many of these are filamentous fungi, such as *Aspergillus, Penicillium, Sporotrichum* and *Trichoderma* [49]. Filamentous fungi are able to produce and secrete large amounts of proteins. Industrially, as high titres as 100 g L^{-1} can be reached. The mould *Trichoderma reesei* (syn. *Hypocrea jecorina*) is the species mostly used for commercial production of cellulases, since it produces relatively large amounts of potent cellulose degrading enzymes, which are released into the substrate. The produced enzyme cocktail consists mainly of cellobiohydrolases, which cleave off cellobiose molecules from the ends of cellulose fibres, and of endoglucanases, which act preferably on amorphous cellulose by cleaving β-1,4-glycosidic bonds randomly in cellulose chains. Additionally, the enzyme cocktail contains β-glucosidase, which hydrolyses oligosaccharides and cellobiose into glucose [47, 48].

Unfortunately, when pretreated lignocellulosic material is hydrolysed enzymatically the hydrolysis is often inhibited. Inhibition occurs via product inhibition by e.g. glucose or cellobiose [49]. Product inhibition leads to reduced degradation of the carbohydrates, unless the formed products are further hydrolysed or consumed by a microorganism during, for example, SSF. However, new enzymes less sensitive to product inhibition have been developed. The major disadvantages of enzymatic hydrolysis are the moderate reaction rates

of the enzymes and the high cost of the enzymes. Enzymes costs have been reduced 30 fold [50], but further reductions in enzyme cost are necessary to make the process economically viable. Another difficulty is that enzymes may adsorb to lignin present in the substrate, thereby rendering the enzyme unproductive [51].

SSF is a commonly used way to degrade lignocellulosic material with enzymes, since product inhibition can be avoided when both fermentation and the hydrolysis is occurring in the same vessel [52]. A dilemma is that the optimal temperature is 45–50 °C for enzymatic hydrolysis and around 30 °C for fermentation, with conventional strains of *S. cerevisiae*. Consequently, SSF is operated at around 35–37 °C as a compromise [27]. It would be very attractive for the fuel ethanol industry if yeast strains, which are more thermally tolerant could be used. The thermal tolerance of yeast will be discussed in section 4.4.

Another problem with the SSF process is that fresh cells are required when starting a new SSF, because separation of the cells and particulate material is difficult [53]. It is, however, possible to reuse cells in a SSF, but then the process design has to be altered. Ishola et al. [54] demonstrated a SSFF (simultaneous saccharification, filtration and fermentation) process where sugar-rich liquid is removed from the slurry containing pretreated lignocellulosic material and hydrolysing enzymes. The liquid part containing dissolved sugars is added to a bioreactor containing high levels of cells where fermentation occurs. After fermentation, the liquid is transported back to the hydrolysis container. This configuration makes it possible to reuse the yeast in several SSF batches, and it is possible to operate the two reactors at different conditions e.g. different temperature and pH [54].

2.3.2.2 Acid hydrolysis

The original method to hydrolyse cellulose was by acid, either with concentrated or dilute acids. In the beginning, hydrolysis with concentrated acid was mostly used. Prior to or during Second World War, a total of twenty alcohol plants using lignocellulosic materials as raw material were operated in Europe, Russia, China, Korea and US. The plants used either the Scholler dilute sulphuric acid process, where the acid concentration was between 0.2–1% or the Bergius process which used 40–45% hydrochloric acid to hydrolyse the wood [55]. Processes based on concentrated acid hydrolysis generally have a high hydrolysis yield

(≥90% of the theoretical glucose yield), but they result in problematic corrosion and require costly acid recovery. Since processes using concentrated acid require high investment and maintenance costs, the interest in the process has mostly been low [56].

Using dilute acid hydrolysis can reduce the consumption of acid considerably, and among the chemical hydrolysis methods, it is the most commonly applied. The method can be used either as a pretreatment method, followed by enzymatic hydrolysis of the carbohydrates, or as the actual hydrolysis method to produce fermentable sugars. The Scholler process was probably the first established dilute-acid hydrolysis process for wood. The process was run batch wise, where the wood was kept in 0.5% sulphuric acid at 11–12 bar for about 45 min. Today, when dilute acid hydrolysis is performed it is carried out in batch mode but with a hydrolysis time of only a few minutes [56].

The main drawbacks of dilute acid hydrolysis, especially those performed in batch processes, are sugar degradation during hydrolysis, low overall sugar yield and the formation of several by-products which inhibit the fermenting microorganism [57]. The high hydrolysis temperature also causes corrosion problems even at low acid concentration and accelerates the sugar degradation. One way to reduce the sugar degradation is to perform a two-stage dilute acid hydrolysis. Here, mainly hemicellulose is hydrolysed at relatively mild conditions during an initial step and afterwards, the cellulose is hydrolysed in a second stage at more harsh conditions at a higher temperature [58]. Between the two stages the liquid phase is removed so the formed sugars are not further degraded into inhibitors. A two-stage dilute acid hydrolysis is generally preferred over a one stage hydrolysis, since e.g. sugar degradation is reduced and less inhibitors are produced [56, 58]. Table 2 summarises the advantages and disadvantages of dilute acid and enzymatic hydrolysis of lignocellulosic materials.

Table 2 Summary of advantages and disadvantages of dilute-acid and enzymatic hydrolysis.

	Dilute-acid hydrolysis	**Enzymatic hydrolysis**
Hydrolysis time	Rapid	Slow
Hydrolysis condition	Harsh	Mild
Inhibitor production	High	Low
Product inhibition during hydrolysis	No	Yes
Sugar yield	Low	High
Catalyst cost	Low	High

2.4 Modes of fermentation – batch, continuous and fed-batch

Ethanol production with yeast is highly dependent on which substrate is utilised. However, it is also largely affected by the cultivation mode. This section tries to visualise the advantages and disadvantages of the traditional cultivation methods when inhibitory media are used.

2.4.1 Batch cultivations

Batch technology has been preferred in the past since batch cultivation plants are fairly simple and inexpensive to construct and get running. Alcoholic beverages are therefore often produced in batch mode. One major disadvantage of batch systems is that it is time consuming and labour intensive since time is spent on cleaning, sterilisation, filling, lag phase, cell growth and harvesting in each batch. The overall productivity of the process is therefore low. One way to improve the process productivity is to reuse the produced cells by separating the cells from the media by centrifugation or sedimentation and then recycle the cells to the next batch [59]. In **Papers I** and **II,** the yeast could easily be separated from the medium and reused several times in consecutive batch cultivations since the cells were encapsulated.

Batch cultivation in dilute acid hydrolysate is usually not appropriate since the cells are exposed directly to a high concentration of inhibitors [60]. Depending on the inhibitor concentration in the used hydrolyzate, the fermentation can either be prolonged while some inhibitors are detoxified by the yeast cells, or fail completely as the cells are completely inactivated in the toxic environment [61, 62]. To improve the fermentability, the hydrolysate can be detoxified, a more inhibitor tolerant yeast can be used, or the yeast inoculum size can be increased to improve the microorganism's own *in situ* detoxification. From an economical point of view, *in situ* detoxification is more advantageous than a more expensive detoxification step.

2.4.2 Continuous cultivations

During continuous cultivations, fresh media is continuously added into the bioreactor at the same rate as cultivation broth is removed. This results in a constant liquid volume inside the reactor. In traditional continuous cultivations, yeast cells are constantly drained from the cultivation as fermentation broth is removed. The yeast therefore needs to grow at the same

rate as the dilution rate in order to avoid washout of the cells. Consequently, inhibiting medium that affects the specific growth rate will reduce the maximum possible dilution rate, which directly affects productivity. A major problem if the hydrolysate is very inhibiting and significantly reduces the specific growth rate, is that cell washout may take place unless a very low dilution rate is used, resulting in reduced productivity and economic utilisation of the system [63]. Another well-known weakness of continuous cultivations is the risk of contamination. One solution is to use flocculating yeast, which can be kept inside the bioreactor whereas the contaminating bacteria are washed out with the fermentation broth [64].

During continuous cultivation, the concentration of convertible inhibitors can be kept low throughout the cultivation, if the dilution rate is appropriate. Yeast possesses the capability to detoxify some inhibitors present in the added hydrolysate as long as the feed-rate is not increased beyond the yeast's maximum detoxification rate. The detoxification rate and productivity can be improved by having a higher yeast biomass concentration inside the reactor. Approaches to retain the yeast cells in the system are by immobilisation [65-67] or different yeast recirculation methods [68]. By retaining the yeast cells, the reactor can be operated at much higher dilution rates since the dilution rate is no longer restricted by the specific growth rate of the yeast. The maximum specific growth rate of *S. cerevisiae* CBS 8066 is 0.46 h^{-1} when batch cultivations are performed with non-inhibiting medium at anaerobic conditions [69]. However, in the presence of inhibitors the specific growth rate can be much lower. For example, when 4 g L^{-1} of furfural was pulse injected into batch cultivations containing *S. cerevisiae* CBS 8066, the specific growth rate fell from 0.4 to 0.03 h^{-1} until the furfural was completely consumed [70].

Most of the cultivations performed in this thesis (**Papers I** and **III–V**) were conducted in continuous mode, commonly at rapid dilution rates of up to 0.8 h^{-1} (**Paper V)**. In **Paper I,** where the thermal tolerance of encapsulated yeast was investigated, the dilution rate was rather low at only 0.2 h^{-1}. Continuous cultivations were chosen because of the goal of achieving a rapid ethanol production with high productivity, and because convertible inhibitor concentrations can be kept low by *in situ* detoxification.

In **Paper I,** when encapsulated yeast was grown inside the bioreactor, the liquid level inside the bioreactor was kept constant by placing a sieve at the appropriate liquid level and

withdrawing consumed fermentation broth with a peristaltic pump. Thereafter, feed medium was added into the bioreactor using a peristaltic pump to gain the appropriate dilution rate. In **Papers III–V** in which membrane bioreactors were applied, a level probe was used instead to regulate the liquid level inside the bioreactor. By connecting the level probe to a pump coupled to the permeate side of the membrane module, fermentation broth could be removed when needed.

2.4.3 Fed-batch cultivations

In fed-batch cultivations, the system is operated in a semi-open mode with a variable volume. During fed-batch cultivations the yeast inoculum is first added to a small amount of media. After a batch process, fresh medium is added continuously or periodically to the cultivation, without removing any culture fluid [71].

The fed-batch process was originally developed in the 1910s to produce bakers´ yeast [72]. It can result in a considerably higher biomass yield compared to batch processes because glucose repression and overflow metabolism can be avoided [73]. Fed-batch cultivations combined with cell recycling is the mostly used mode for bioethanol production due to the high volumetric productivity of the process [71]. For example Souza et al. [74] reported a volumetric ethanol productivity of up to 25.4 g L^{-1} h^{-1} in a fed-batch process using medium containing 150 g L^{-1} of sucrose.

One reason for operating in fed-batch mode is to minimise inhibition effects, caused by the carbon source or inhibiting compounds present in the medium [75]. Fed-batch cultivations are therefore very appropriate for fermentation of dilute-acid hydrolysate, because high concentrations of convertible inhibitors can be avoided [76]. In fed-batch cultivations some advantages of batch and continuous cultivations are combined, e.g. no washout of cells can occur, and inhibitor levels can be kept low by adjusting the fed rate.

CHAPTER 3

CELL RETENTION BY ENCAPSULATION AND MEMBRANE BIOREACTORS

Cell immobilization has attracted much interest. One reason is that it can provide a high cell concentration in the bioreactor. For example, Webb et al. [77] have shown that the cell biomass level in immobilised systems can be considerably higher than in free cell systems. High cell concentrations can likewise confer higher ethanol production rates. Immobilisation can additionally provide an easy separation of the microorganism from the fermentation broth and thereby reduce downstream processing [78].

The disadvantages of immobilised cell systems include: the additional cost for immobilisation, the mass transfer resistance in the immobilised matrix, and that the robustness of the immobilisation matrix may be insufficient for extended use. The utilisation of immobilised cells has usually been motivated by the higher ethanol productivity [79] and higher ethanol yield which can be achieved [80]. The higher ethanol yield is attributed to reduced biomass and acetic acid formation in the immobilised system, which also results in a reduced glycerol yield. A larger part of the available sugars can therefore be used for ethanol production, which improves the ethanol yield. Several different immobilisation methods exist, namely adsorption, covalent bonding, cross-linking, entrapment and encapsulation. These methods are illustrated in Figure 5.

3.1 Encapsulation – capsule formation

One strategy to immobilise the cells is to use encapsulation. In the encapsulation process the cells are enclosed by a polymer membrane that is permeable to small substrate molecules and metabolites [81], see Figure 5. The capsule membrane will retain all cells inside the capsule as long as the membrane is intact. If the cells instead are immobilised in compact gel beads (entrapped in a matrix), cells can escape from the bead surface into the nearby medium, and cells will mainly grow close to the bead surface [82].

Encapsulation also has the advantage that a higher cell density can be reached inside the capsule, in the liquid core. Moreover, encapsulated yeast cells have been shown to perform better in toxic media with e.g. high levels of limonene [83] or furfural [84] or at elevated cultivation temperatures (**Paper I**) compared to freely suspended yeast.

3.1.1 Capsule materials

A multitude of different materials have been used to construct the capsule membrane. Natural polymers such as e.g. cellulose [85], chitosan [66], carrageenan [86], agar, alginate [84], collagen and albumin have been used. Additionally synthetic polymers such as polystyrene, nylon [87], polyacrylamide, polyamides [88], polyether-sulphone (PES) and polyvinylidene fluoride [89] have also been tested. Furthermore, some inorganic materials such as silica have been used [90], or hybrid materials prepared by combining organic and inorganic materials (**Paper II**).

Obviously, the selected material and the applied encapsulation technique will affect the capsules' characteristics, such as rigidity, permeability, hydrophilicity and chemical and mechanical stability. The performance and stability of the encapsulated system will be determined by e.g. the materials pore size, swelling behaviour, mechanical strength and compression properties. However, it is important that the encapsulation is performed at mild enough conditions in order not to impair the viability of the cells too much [90].

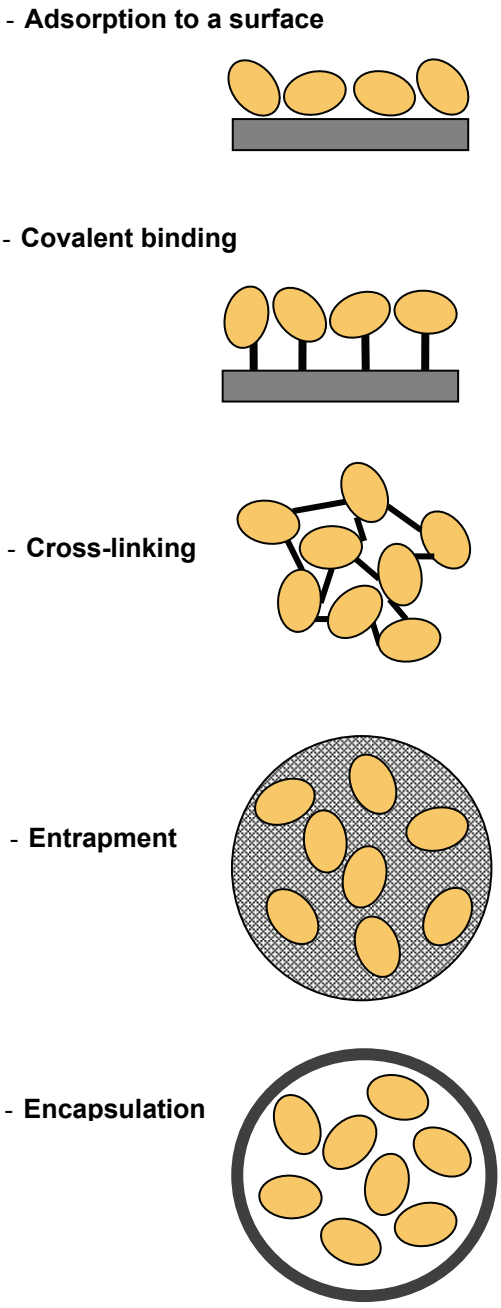


Figure 5 Common immobilisation methods used for microbial cells.

Different natural organic polyelectrolytes have been the most common materials used for encapsulating cells. Polyelectrolytes can easily form hydrogels when they are mixed with polyions of the opposite charge. The biopolymer alginate is a particularly popular material, because the encapsulation process is easy and can be conducted at mild conditions at neutral pH and room temperature. In both **Papers I** and **II,** alginate was the main building block for the capsule membrane. To ionically cross-link the alginate polymers, both Ca^{2+} ions and chitosan, which is a positively charged biopolymer, were used. It is the guluronic acid residues in the alginate polymer that can be ionically cross-linked by divalent cations such as Ca^{2+} or other polycations e.g. chitosan [91]. By using chitosan, the capsules can be made more robust.

Synthetic encapsulation materials usually have the advantage of being very mechanically stable. However, their production commonly demands high processing temperatures, reactive chemicals and organic solvents, which are all hazardous for living cells. Therefore, cells encapsulated in synthetic membranes are generally introduced after capsule formation [92]. It is furthermore important that the synthetic material used in the encapsulation is hydrophilic enough to allow dissolved nutrients to penetrate through the membrane.

Capsules prepared of e.g. silica or metal oxide can be made by different sol-gel methods where precursors of organometallic compounds are hydrolysed and then condensed to form a porous material. Unlike organic polymers, inorganic silicate materials are chemically inert, hard and have low swelling [93, 94]. The use of reactive precursors, acidic or basic catalysts and the formation of alcohols can, however, affect the viability of the cells and thereby restrict its use for cell encapsulation [93].

3.1.2 Encapsulation methods

There are several different methods by which encapsulated cells can be produced: liquid droplet formation (also called the one-step method), pregel dissolution (also called the two-step method), interfacial polymerisation, and coacervation.

In the *liquid droplet formation method,* capsules can be formed by just one step. Cells can be encapsulated with this method by dropwise addition of a solution of cell-containing hardening agent, such as Ca^{2+}, into a polymer solution of e.g. alginate. A capsule forms instantaneously around the droplet, placing the cells in the capsules inner liquid phase [95].

As the name indicates, the *pregel dissolving method* begins by first preparing a porous gel of e.g. calcium alginate by dripping a sodium alginate solution into a solution with calcium ions. Afterward, the prepared beads are, for example, treated with polycations such as chitosan or other polyions to create a membrane on the bead surface. A liquid core capsule can then be prepared by dissolving the inner core of e.g. calcium alginate with sodium citrate or other chelating agents [94, 95].

Encapsulation with the *interfacial polymerization method* is conducted by mixing a reactive water soluble monomer into the aqueous phase of an emulsion containing an organic phase. By stirring the emulsion steadily, the aqueous phase will form drops in the organic phase. When an appropriate monomer is dissolved in the organic phase, a polymeric membrane forms instantly at the interface between the organic and aqueous phase [88, 95].

The term *coacervation* is derived from the latin word *coacervare*, meaning "to assemble together or cluster" [96]. *Coacervation* is performed under continuous agitation by dispersing the core liquid in a solution of the coating polymer. The coating polymer is then induced to separate from the solution by e.g. changing the pH, temperature or addition of salt or nonsolvent. The core liquid is then coated by the deposition of the coating polymer on the droplets. Formed microcapsules are thereafter stabilised by, for example, cross-linking. [97].

3.2 Encapsulation procedures and robustness

Encapsulating the cells requires an extra investment, which increases the cost of the system. It is therefore important that the produced capsules are stable for long periods to make the method attractive at industrial scale. Capsule robustness is a major issue for the technique, and the membranes stability is a common problem especially in stirred reactors with high shear stress [98]. Several methods to improve the capsules have been investigated such as usage of cross-linking agents to introduce covalent bonds to bind together the polymers in the capsule membrane. Unfortunately, cross-linking agents are generally toxic for the cells. High molecular weight cross-linking agents, e.g. dextran dialdehyde, are usually less hazardous to the cells compared to low molecular weight agents, e.g. glutaraldehyde or carbodiimide [99].

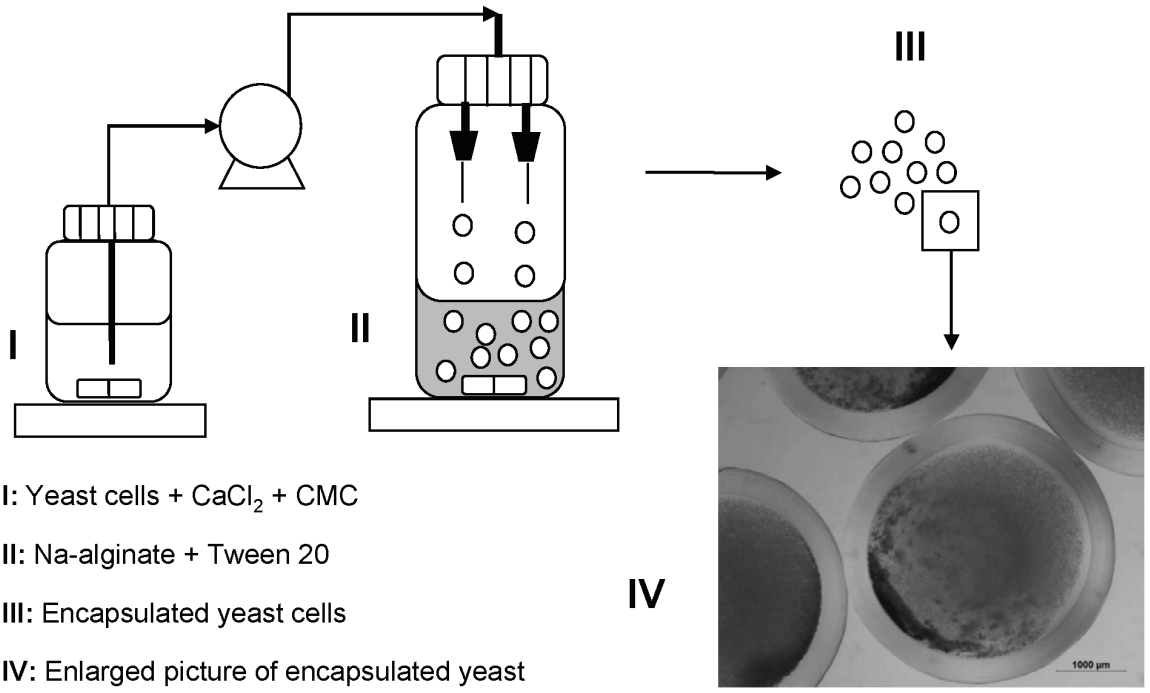
In Papers I and **II,** the liquid droplet method was employed to produce Ca-alginate capsules containing yeast cells. The methodology has previously been used for encapsulation of *Saccharomyces cerevisiae* by e.g. Talebnia et al. [81]. Calcium-alginate was used as the main capsule membrane material.

The stability of the formed capsules membrane is unfortunately strongly dependent on the presence of excess Ca^{2+} in the surrounding liquid. Otherwise, the weakly bound Ca^{2+} can slowly leak from the gel, which makes the membrane swell and finally rupture. Therefore, Ca^{2+} ions need to be added to the media, or the alginate capsules require some additional treatment to make the capsules more robust. In **Papers I** and **II**, the formed capsules were conjugated with low molecular weight chitosan to make the capsules more robust and avoid adding Ca^{2+} to the media, which is costly and may lead to solid salt formation. In **Paper II**, additional treatment was investigated.

Several different encapsulation methods were investigated during this thesis wor: cross-linking the alginate chitosan capsule matrix with glutaraldehyde and genipin or treating the capsules with sodium silicate solution or 3-aminopropyltrietoxysilane (APTES). However, only glutaraldehyde and APTES treated capsules were investigated in more detail. The encapsulation methodology and some results will be described in the following sections.

3.2.1 Chitosan alginate capsule production

In the first steps before encapsulation, the yeast cells were collected by centrifugation and thereafter re-suspended in a solution containing 1.3% (w/v) $CaCl_2$ and 1.3% (w/v) carboxymethylcellulose (CMC) with an average molecular weight of 250 kDa and degree of substitution 0.9. The addition of CMC increases the viscosity of the solution and makes it easier to form spherical capsules. The viscous yeast suspension containing $CaCl_2$ was then added dropwise to a 0.6% (w/v) sodium alginate solution containing 0.1% (v/v) Tween 20 to improve the permeability of the capsule membrane. The capsule membrane is formed when Ca^{2+} in the yeast suspension comes in contact with the alginate polymers, cross-linking and gelling them (see Figure 6). Capsules were produced for 5 min and were then left to gel for another 5 min, after which they were removed and washed with plenty of water. To harden the capsules, they were placed in a 1.3% (w/v) $CaCl_2$ solution for at least 20 min.



I: Yeast cells + $CaCl_2$ + CMC

II: Na-alginate + Tween 20

III: Encapsulated yeast cells

IV: Enlarged picture of encapsulated yeast

Figure 6 Schematic procedure for encapsulation of yeast cells in Ca-alginate capsules. The scale bar in the picture is 1000 µm.

One important factor to be considered when preparing spherical capsules is how the drops of yeast and $CaCl_2$ suspension fall into the alginate solution from the needles. If the drops fall in the vortex of the mixed sodium alginate solution, they become elongated creating capsules with a "tail". It is therefore important to make sure that the drops enter the alginate solution outside the vortex.

The ionic cross-link between alginate and Ca^{2+} is relatively weak. Thus, this will result in the loss of Ca^{2+} from the alginate matrix when the capsules are placed in media containing insufficient amounts of Ca^{2+}, or when the solution contains phosphate or citrate ions. When Ca^{2+} disappears, the membrane will lose its integrity and finally rupture. Adding Ca^{2+} to the medium can solve the problem, but this was not an alternative in this thesis work as it is both expensive and results in undesired salt precipitates.

To improve the robustness of the Ca-alginate capsule membrane, the formed capsules were treated in a 0.040 M acetate buffer solution of pH 4.5 containing 0.2% (w/v) low molecular weight chitosan and 300 mM $CaCl_2$. To incorporate the chitosan into the Ca-alginate matrix of the membranes, the capsules were treated at a 1:5 volume fraction of capsules to chitosan solution for 24 h at 30 °C at 140 rpm. After treatment the capsules were rinsed with NaCl and

water to remove any unbound chitosan before beginning any cultivation with the capsules. Since chitosan is a positively charged polyelectrolyte polymer, it can replace Ca^{2+} and crosslink the alginate matrix, thereby stabilising the membrane, as chitosan polymers are not removed as easily from the matrix as Ca^{2+}. The formed alginate-chitosan (AC) capsules were therefore much more robust than Ca-alginate capsules. The appearance of AC capsules both before and after cultivation is illustrated in Figure 7.

Figure 7 Pictures of AC capsules: Before cultivation (left), after cultivation with yeast pellet (middle) and after continuous cultivations in **Paper I** (right). The capsules size was 3-4 mm.

3.2.2 Glutaraldehyde treated chitosan alginate capsules

To improve the mechanical properties of gel capsules, different cross-linking agents can be used to covalently crosslink the gel polymers. Chemical cross-linking can be performed using the well known bifunctional reagent glutaraldehyde (GA) [100]. However, cross-linking the AC capsule with glutaraldehyde was found to be very detrimental to the yeast cells, even after first accumulating the yeast biomass in defined glucose medium with 50 g L^{-1} glucose for 30 h and then 14 h in new medium before treating the capsules in 1% GA. The thought was that by having a high level of yeast biomass inside the capsules not all the yeast cells would die because of the treatment. However, after treatment, none of the treated AC capsules managed to ferment any of the glucose in the medium, see Figure 8.

The failure to ferment the glucose did not depend on the permeability of the capsules. Diffusion tests of the treated and untreated capsules showed that the diffusion of glucose, glycerol and ethanol into GA treated capsules was only a little slower into the treated capsules compared with untreated (results not shown). However, equilibrium was reached for all compounds within 25 min.

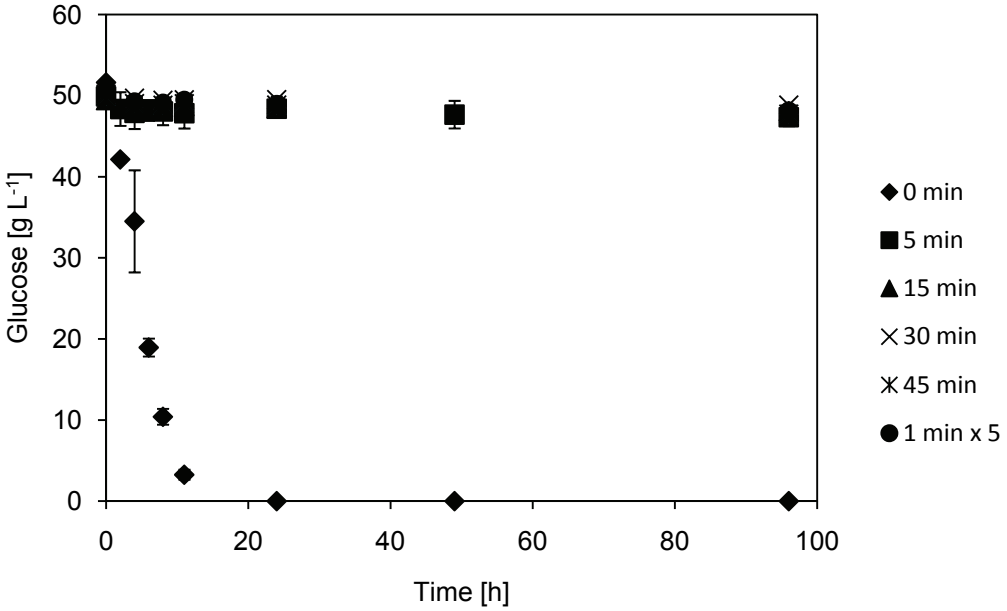


Figure 8 Glucose concentrations during cultivations with untreated and GA treated capsules. AC capsules were treated in 1% GA solution for: 5, 15, 30, 45 and 1 min five times. Data are averages of two cultivations with standard deviation bars.

The toxicity of GA was investigated by adding GA to cultivations in defined glucose medium to which 5 ml of yeast inoculum had been added. In none of the cultivations, to which GA was added, did the yeast survive not even when only 0.0025% GA was added, see Figure 9.

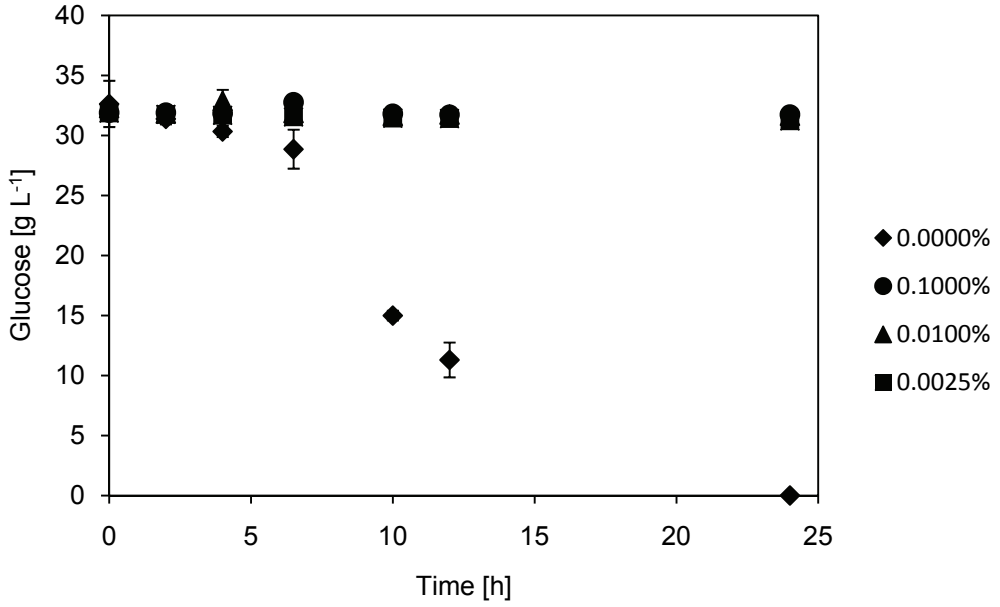


Figure 9 Glucose concentrations in suspended yeast cultivations containing different concentrations of GA. Data are averages of two cultivations with standard deviation bars.

In order to try to keep the cell viable, a shorter exposure time for GA was tested where the capsules were only dipped for 5 sec in the 1% GA solution after which they were immersed into a buffer solution of 0.05 M acetate buffer with pH 4.5. The treatment was repeated several times with some of the capsules, see Figure 10. As shown in Figure 10, the encapsulated yeast from all four tested treatments successfully fermented all the glucose within 48 h. However, the rate of glucose consumption was reduced the more times the capsules were treated. Because of the high toxicity of GA and no major improvement in capsule robustness after treatment, no further experiments were performed with the glutaraldehyde AC capsules.

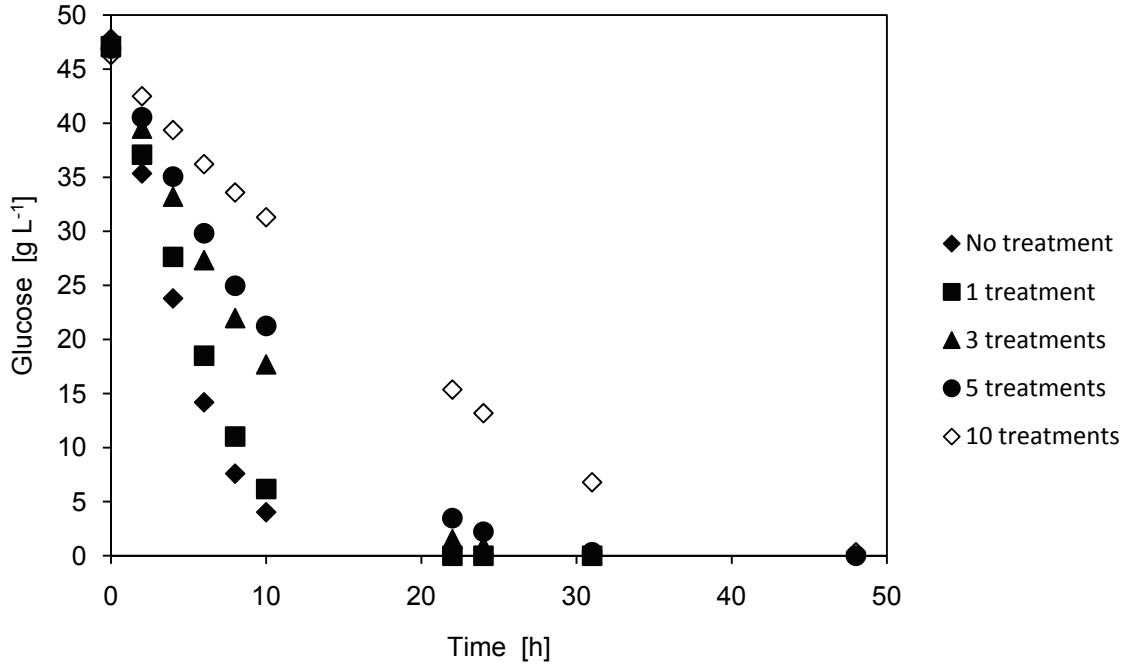


Figure 10 Glucose concentrations in yeast cultivations performed with 1% GA treated capsules. Several different treatments were tested where the ACA capsules were immersed in the GA solution for 5 sec and then placed in buffer solution. This procedure was repeated for some capsules up to 10 times.

3.2.3 Polysiloxane-ACA capsules

It is crucial that the produced capsules are robust enough to withstand chemical effects of the media and the shear forces occurring during stirring for prolonged periods. During the long continuous cultivations performed in **Paper I** at elevated temperatures, it was observed that the AC capsules were weakened. The encapsulation method was therefore further developed

to produce a new kind of polysiloxane (PS)-alginate-chitosan-alginate (ACA) capsules, which were more mechanically robust than the AC capsules.

Paper II describes how ACA capsules were further reinforced by treating the capsules with an organofunctional silane named 3-aminopropyltriethoxysilane (APTES). The treatment results in an adsorption of the hAPTES molecules on the alginate surface where a condensation reaction occurs between closely located hydroxyl groups on the hAPTES, which results in the formation of a polysiloxane coat on the capsule [101]. Although, alginate can react with APTES, AC capsules cannot. The AC capsules therefore needed to be covered with an additional layer of alginate. ACA capsules were prepared by producing the described AC capsules and thereafter removing any excess Ca^{2+} by immersing the capsules in 0.9% $CaCl_2$ solution for 15 min. The capsules were then placed in a 0.05% (w/v) alginate solution containing 0.008% (v/v) Tween 20 for 12 h at 130 rpm to get an alginate surface on the capsules. The APTES was first hydrolysed in an aqueous solution (hAPTES) before treating the alginate surfaced ACA capsules with the solution at room temperature. Four different hAPTES concentrations were tested 0, 0.5, 1.5 and 3.0% (w/w). Upon 90 min of treatment with hAPTES, the formed PS-ACA capsules became very glossy. After five consecutive cultivations the capsules became brown coloured, probably because of adsorption and/or absorption of coloured compounds present in the spruce hydrolysate. The differently treated capsules are illustrated in Figure 11.

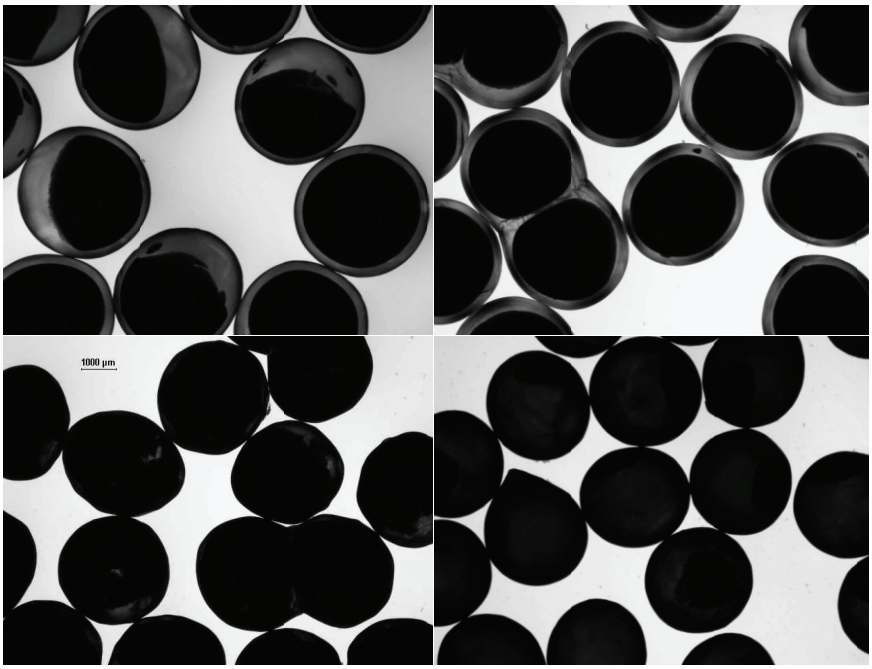


Figure 11 Picture of PS-ACA capsules treated with different concentrations of APTES after five consecutive batch cultivations in spruce hydrolysates; top left 0%, top right 0.75%, bottom left 1.5% and bottom right 3.0% APTES. The scale bar in the picture is 1000 µm.

3.2.4 Capsule robustness

The mechanical shear tests performed in **Paper II** to evaluate the PS-ACA capsules robustness revealed that the treated capsules were much more robust than untreated capsules, see Figure 12. Of the PS-ACA capsules produced by treatment with 1.5% or 3.0% hAPTES at the most 2% of the capsules were broken after a shear test of 6 h. Twenty-five per cent of the untreated capsules ruptured after the same treatment. However, fermentation tests were negative for PS-ACA capsules treated with 3.0% hAPTES since no ethanol was produced nor sugars consumed. The PS-ACA capsules treated with 1.5% hAPTES showed an almost equal fermentation profile as untreated capsules in the fifth consecutive batch fermentation in wood hydrolysate. The increased mechanical robustness of the PS-ACA capsules should make it possible to use the capsules for hydrolysate fermentation for much longer periods compared to untreated capsules. A longer capsule's lifetime would make encapsulation more economically attractive.

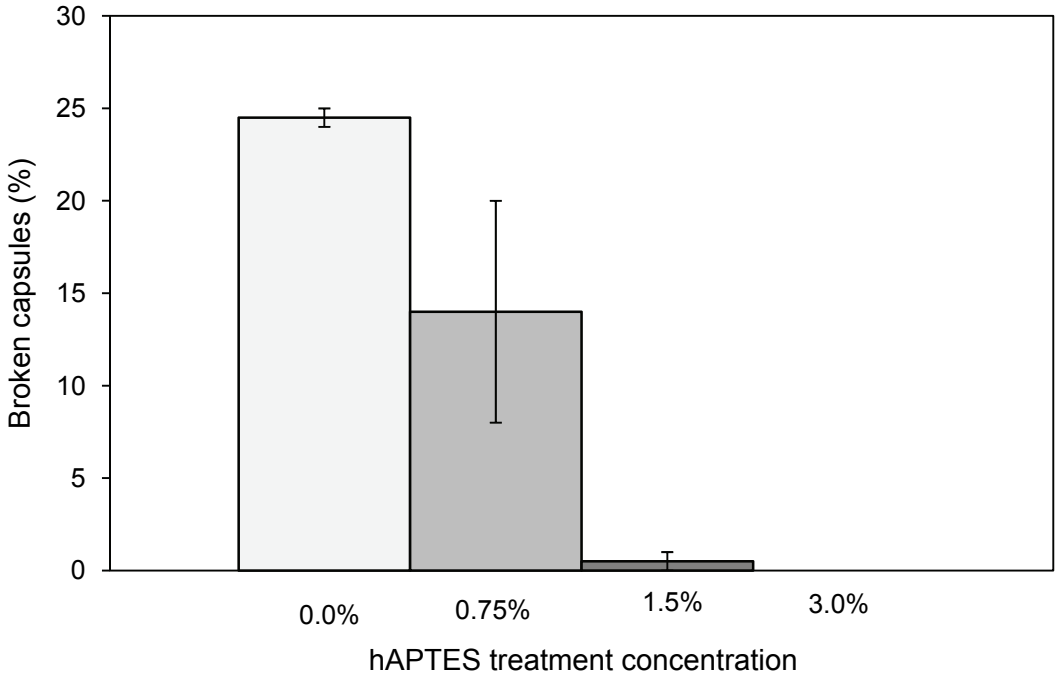


Figure 12 Results of the robustness of untreated and PS-ACA capsules, showing the percentage of broken capsules after 6 h at high shear stress. The concentrations in the figure denote the concentration of the hAPTES solution with which the capsules were treated. Data are averages of two cultivations with standard deviation bars.

3.3 Membrane bioreactors (MBRs)

Presently there is an emerging industrial usage of membrane technology for wastewater treatment. There are today numerous large companies such as GE Healthcare, Kubota, Merck-Millipore, Pall and Sartorius, which sell membranes for different applications. The purpose of the membrane is to separate compounds or cells, by allowing some components to pass the membrane but not others. Separation is driven by using pressure, vacuum or diffusion. The selectivity of the membrane is mainly determined by its pore size, although other membrane characteristics such as charge and hydrophilicity can also have an effect [102].

In MBRs, the purpose of the membrane can be to either retain cells inside the fermentor, or remove inhibitors or products from the broth. In **Papers III–V**, the bioreactor was combined with either of two different membrane modules to retain the cells in order to gain a very high yeast density inside the bioreactor. In **Papers III** and **IV**, a cross-flow module containing tubular ultrafiltration membranes was utilised whereas in **Paper V**, a MBR containing three flat membrane panels was operated, submerged in an external container outside the bioreactor.

Membranes can be manufactured using ceramic, metallic or polymeric materials; nowadays, many different configurations are available. All the membranes utilised in **Papers III–V** were polymer based since these are generally much cheaper.

3.3.1 Configuration

There are two different MBR designs, in which separation is induced by either pressure or vacuum [103]. When using direct pressure the membrane is placed externally to the bioreactor and the fermentation broth is circulated to the membrane under pressure to push liquid through the membrane. The external cross-flow MBRs (Figure 13A) are operated in a manner where the liquid to be filtered flows with a high velocity parallel to the membrane surface. This configuration prevents cake formation on the membrane surface and therefore reduces fouling. Fouling has been designated as one of the main hurdles when applying membranes; however, an external cross-flow MBR module applied in **Papers III** and **IV** showed only very minor problems with fouling and the membrane could be cleaned easily. No major fouling was observed even at very high yeast concentrations of approx. 180 g L^{-1} (**Paper III**) or 200 g L^{-1} (**Paper IV**).

The other MBR configuration is called submerged or immersed MBR. Here, the membrane, as implied by the name, is placed directly in the liquid (Figure 13B). The membrane is operated at vacuum or pressures below atmospheric to pull permeate through the membrane. Generally, submerged MBRs need less energy to operate compared to external cross-flow MBRs [102, 104]. Submerged MBRs, nevertheless, have the disadvantage of being problematic to operate at high particle or cell concentration because of fouling. Consequently, submerged MBRs generally require a larger surface area than cross-flow MBRs. Fouling can, however, be reduced by purging gas vigorously on the submerged membrane surface [102].

The submerged MBRs can be operated in two different configurations, either by immersing the membrane directly in the liquid in the bioreactor or in an external chamber as shown in Figure 13C. By having the submerged membranes in an external container, the membrane can be cleaned easier, but this design requires circulation of the liquid to and from the membrane container. In this thesis, two of the MBR configurations in Figure 13 (A and C) were used, in **Papers III–IV** the external cross-flow membrane bioreactor, and in **Paper V** the external submerged membrane bioreactor.

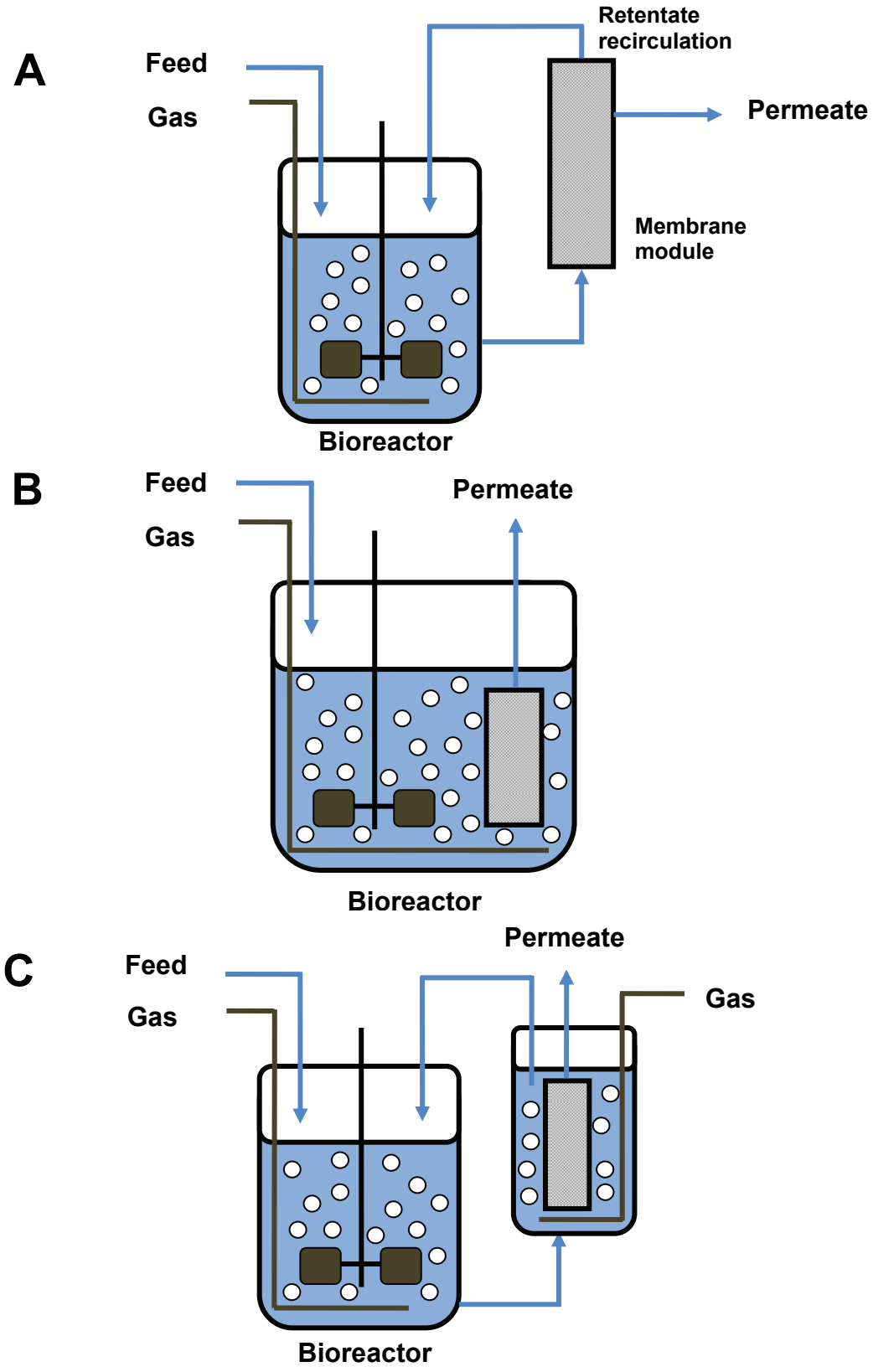


Figure 13 MBR configurations: A) external cross-flow membrane, B) internal submerged membrane and C) external submerged membrane.

3.3.2 Membrane fouling

Fouling is one of the main problems hampering the usage of membranes for separation purposes [105]. Fouling occurs during operation when compounds, particles or cells, which cannot pass through the membrane, accumulate on the membrane surface or inside of the membrane. This will affect the membrane performance. There are several methods which have been tested in order to reduce fouling and cake formation, e.g. purging gas on the membrane surface (**Paper V**), turbulence promoters [106], periodical inversion of flow [107], ultrasound [108] and gas injection [109]. The majority of these methods increase the turbulence close to the membrane surface. The turbulence reduces accumulation of e.g. particles and consequently improves the membrane performance.

No major fouling occurred in the cross-flow module used in both **Papers III** and **IV**. The operations of the submerged membrane panels were more problematic at high cell concentrations and high dilution rate. Consequently, the investigation in **Paper V** was conducted at a lower yeast cell concentration. Table 3 shows some studies made in MBRs together with the yeast density used.

Table 3 Summary of yeast cell density during some MBR cultivations.

Substrate	MBR	DW[a] L^{-1}	References
Glucose	Cross-flow	150	[109]
Glucose	Cross-flow	300	[110]
Glucose	Submerged	157	[111]
Tapioca hydrolysate	Submerged	42	[112]
Wood hydrolysate	Cross-flow	12	[68]
Wood hydrolysate	Submerged	60	**Paper V**
Sucrose	Cross-flow	200	**Paper IV**
Sucrose	Cross-flow	180	**Paper III**

[a] DW: Cell dry weight

3.4 Membrane bioreactors for ethanol production

3.4.1 Cross-flow membrane bioreactor

The cross-flow membrane module used in **Papers III** and **IV** was placed externally to the bioreactor, and the yeast suspension was circulated with a peristaltic pump through the module. Consumed fermentation broth could be removed by another peristaltic pump connected to the top part (permeate side) of the module, see Figure 14.

The used laboratory tubular cross-flow membrane module dizzer® LAB 1.5 MB 0.1 was purchased from inge Gmbh (Greifenberg, Germany). The module has been designed to treat drinking water, process water, sea water and waste water. According to the company, the module has a membrane surface area of 0.1 m^2, with a pore size of ~0.02 μm. The ultrafiltration membrane was manufactured of modified polyethersulfone. To get a satisfactory filtration through the module and to avoid fouling, the yeast suspension was continuously circulated with a flow of 0.5 L min^{-1} through the membrane module.

Since the membrane module could not be autoclaved, it was disinfected instead with sodium hypochlorite solution containing 100 mg L^{-1} of active chlorine for at least 30 min and rinsed with sterile water afterwards. Hydrogen peroxide (500 mg L^{-1}) was used between cultivations to clean the membrane unit in order to avoid fouling. The cross-flow MBR was operated in a continuous mode by adding medium at a certain dilution rate into the bioreactor and having a harvesting pump connected to the membrane modules' permeate outflow and a level probe inside the fermentor linked to the harvesting pump.

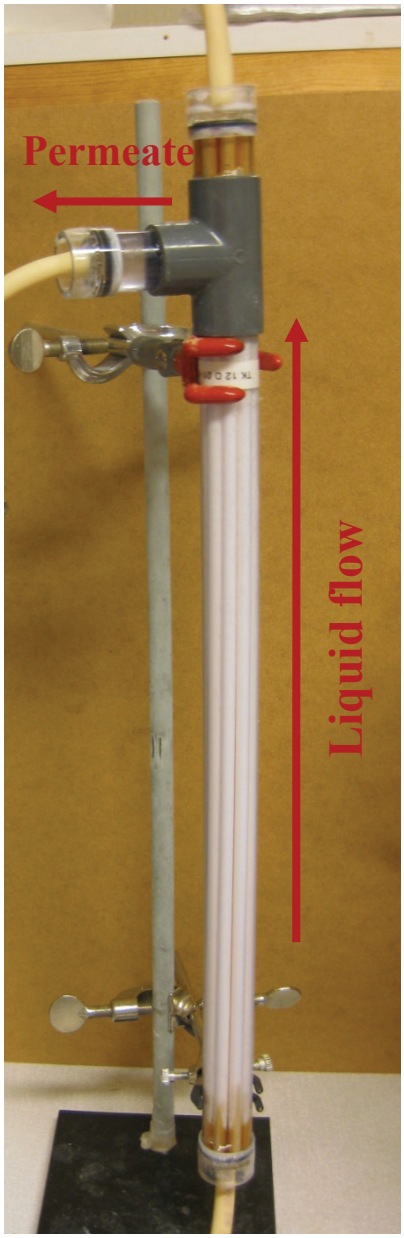
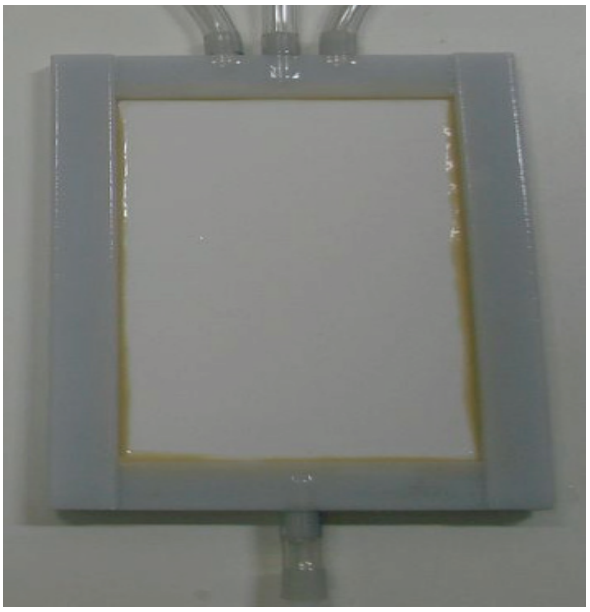


Figure 14 Cross-flow membrane module used in **Papers III** and **IV**.

3.4.2 Submerged membrane bioreactor

In **Paper V**, a submerged MBR was used, where three flat membrane panels were immersed in the yeast fermentation liquid in an external container. The external container was connected to a bioreactor in which the temperature, volume and pH was regulated. From the bioreactor, the fermentation liquid was continually circulated to the external container and back again. The membrane panels were manufactured and developed by the Flemish Institute for Technological Research (Vito NV, Belgium), see Figure 15. Every membrane panel had a total surface area of 0.0252 m^2.

To avoid fouling, the submerged MBR was operated at lower yeast concentrations below 60 g L^{-1} unlike the cross-flow membrane, which could be operated at a very high cell concentration of up to 200 g L^{-1}. A gas pump was also used to sparge large amounts of gas around the membrane panels to increase the liquid turbulence near the membrane surface. In order to get anaerobic conditions, the gas used to purge the membrane surface was collected and reused again. Membrane cleaning and disinfection was performed with 2% NaOH, 1% phosphoric acid and sodium hypochlorite solution with 100 mg L^{-1} active chloride.



The membrane panels consist of dual-layer membranes with a spacer fabric holding the membranes together [113]. Interposed to the two membranes is the internal permeate channel through which consumed fermentation broth can be removed. The membrane panel construction makes the membranes very robust; furthermore, it is able to withstand high pressure changes during filtration and backwashing.

Figure 15 Flat membrane panel used in the submerged membrane bioreactor in **Paper V**.

CHAPTER 4

INHIBITORS AND STRESS CONDITIONS

Lignocellulosic hydrolysates are difficult to ferment because they contain several compounds, formed during pretreatment or dilute acid hydrolysis, which inhibit both yeast growth and fermentation. In this thesis, dilute acid hydrolysates from spruce were used in **Papers II** and **V**. Defined media containing furfural or acetic acid were used in **Papers III** and **IV**. In the following sections, inhibitors and inhibiting mechanisms during ethanol fermentation are summarised together with some results from the mentioned papers.

4.1 Common inhibitors during ethanol production

Even the fermentation product ethanol is inhibitory for ethanol producing microorganisms. Ethanol's toxicity obviously depends on its concentration. The main reason for ethanol's inhibitory effect is believed to be caused by the reduced water activity. Ethanol can negatively affect hydrated cell components such as lipid bilayers, membrane-associated proteins and glycolytic enzymes [114]. Ethanol can easily diffuse past the cell membrane, where it reduces e.g. the glucose metabolism by lowering the activity [115]; in addition, ethanol has even been identified to denature glycolytic enzymes *in vitro* [116]. A high ethanol tolerance is crucial for the used ethanol producing strain. Generally, at concentrations of less than 2%, the ethanol's inhibitory effect is insignificant [117], but it can increase significantly at higher concentrations [118].

In processes using lignocelluloses as raw material, the ethanol concentration might be at levels of 4–5% [119]. The ethanol concentration did not reach over 60 g L^{-1} in any of the performed cultivations in **Papers I–V**. In most experiments the ethanol levels were well below this, except for **Paper III** where the ethanol concentration reached almost 60 g L^{-1}. The microbial inhibition due to ethanol probably exerts a minor effect on its own in **Papers I, II, IV** and **V**, since the ethanol level was generally below 15 g L^{-1}. Ethanol may nevertheless, act synergistically when combined together with other toxic compounds.

Additionally, ethanol productivity and yield can be negatively influenced by high sugar levels in the fermentation broth. High sugar levels will affect the fermentation by inactivating the

enzymes present in the fermentation pathway as reviewed by [120]. It is therefore important to keep the sugar levels low in the reactor, by e.g. maintaining a high biomass concentration like in **Paper III** where the sugar concentration was successfully kept low even at a high dilution rate of 0.5 h^{-1} and an inflow of 100 g L^{-1} sucrose.

Fermentation can also be inhibited by high salt concentration. Many substrates such as lignocellulosic hydrolysates and molasses contain high levels of alkali and heavy metal salts. High salt concentration results in osmotic stress, which can negatively influence the ethanol fermentation. In order to reduce the negative effect of the osmotic stress, *S. cerevisiae* can produce e.g. glycerol as a compatible solute [19, 121]. Upon increased osmolarity in the medium, *S. cerevisiae* enhances its glycerol production and accumulates glycerol intracellularly. Glycerol production has been identified as essential for yeast growth during reduced water availability. The key enzyme of glycerol synthesis during osmotic stress is the NADH dependent cytosolic glycerol-3-phospate dehydrogenase (GPD1) [21, 122].

In addition to ethanol, salt and substrate inhibition, extremely high yeast cell density cultivations have been reported as inhibitory. Toxic compounds, e.g. proteins or salts, can be produced by the growing biomass and may accumulate inside the reactor [123]. Very high biomass concentrations are possible by cell immobilisation or by membrane filtration [111, 124]. For example, Lee and Chang [125] used a membrane unit to retain the yeast cells in the reactor, in order to operate the system with 100-150 g L^{-1} yeast. They investigated the effect of cell concentration on specific growth rate and specific ethanol production rate and found that both the cell growth and ethanol production rates decreased when the cell concentration increased. By extrapolation, they found that cell growth and ethanol production would stop at cell concentrations of 255 and 640 g L^{-1}, respectively [125]. However, the maximum cell concentration that can be achieved probably lies around 300 g L^{-1} because the yeast forms an almost tissue like structure at this high yeast concentration [125, 126]. The main part of the liquid is then bound inside the cells making the cells form a solid structure.

Operating fermentations at extremely high cell concentrations is also problematic from the system management point of view because of the increased viscosity. This was especially observed during cultivations in **Papers III** and **IV** where the yeast concentration reached approx. 180 g L^{-1} and 200 g L^{-1}. However, the results illustrated in **Papers I–V** show that it is

possible to perform fermentation of either spruce hydrolysate (dilute acid) or sucrose containing medium for prolonged periods even at very high yeast concentrations.

4.2 Inhibitors in lignocellulosic hydrolysates

When lignocellulosic biomass is pretreated and hydrolysed, several compounds that can severely influence the following fermentation step are formed in addition to fermentable sugars, see Figure 16. Figure 17 illustrates the molecular structure of the most common organic acids and other inhibitory compounds present in the hydrolysate. The concentration of inhibitors in the final hydrolysate will depend on the selected pretreatment and hydrolysis strategy. Inhibitory compounds are formed when hemicellulose, wood extractives, phenolic derivatives and sugars are hydrolysed and degraded [42, 127]. The resulting inhibitors can be divided into three main groups: furan aldehydes, carboxylic acids and phenolic compounds.

Inhibitors present in lignocellulosic hydrolysate can further be divided into two additional types:

1. *Inhibitors not consumed or transformed by S. cerevisiae*

   The concentration of these inhibitors will be identical inside the reactor and in the feed medium, independent of the dilution rate and yeast concentration. Acetic acid is an example of this kind of inhibitor (**Paper IV**), however, only under anaerobic conditions.

2. *Inhibitors consumed or transformed by S. cerevisiae*

   These inhibitors can be biotransformed *in situ* by the cells, and the concentration of inhibitors can therefore be reduced, resulting in a less toxic medium. Inhibitors that can be detoxified *in situ* are e.g. furan aldehydes (**Paper III**) and some phenolic compounds. The concentration of these inhibitors can thereby be altered by changing the cell concentration and dilution rate.

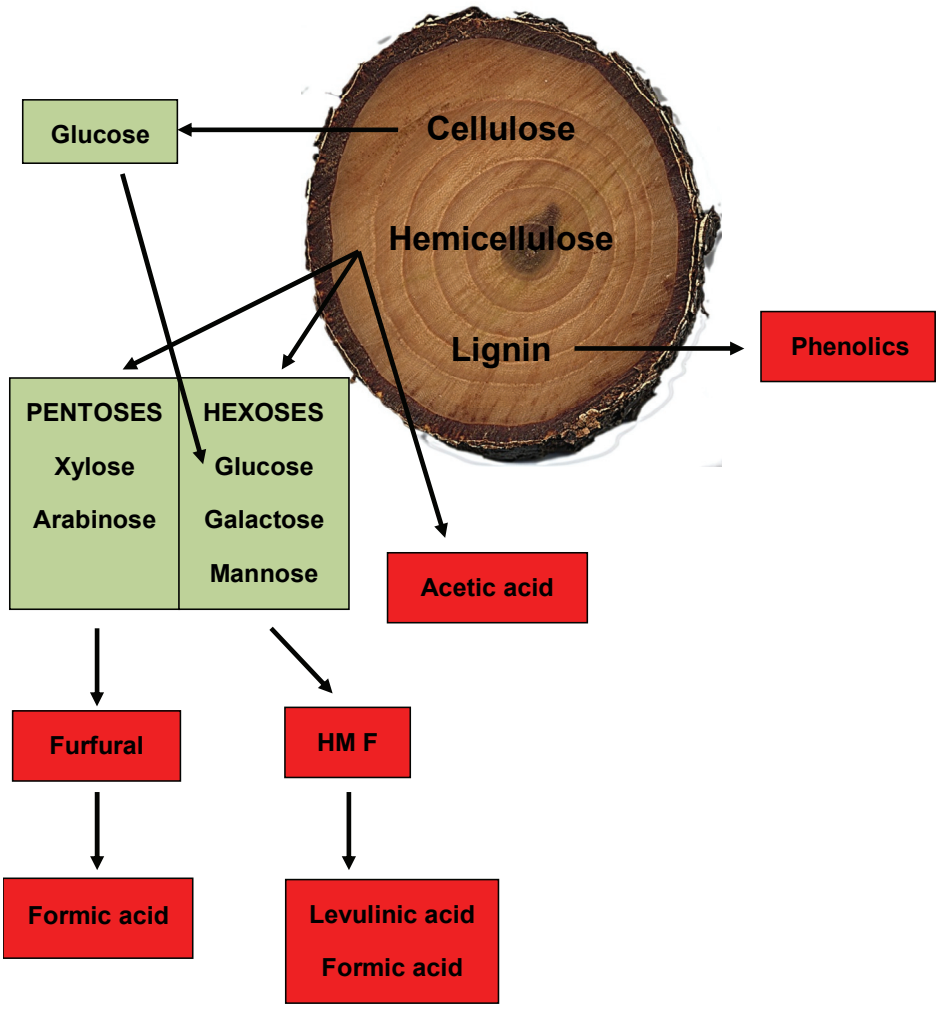


Figure 16 Compounds formed and released during hydrolysis of lignocellulosic materials. The resulting hydrolysate will contain monomeric sugars (green boxes) in addition to inhibitory compounds (red boxes).

4.2.1 Organic acids

The carboxylic acid present at highest levels in lignocellulosic hydrolysates is acetic acid, which is formed when hemicellulose and to some extent lignin is deacetylated. Two additional organic acids occurring most often in hydrolysates are levulinic and formic acid, both of which are produced when furan aldehydes are degraded. Both acids are formed particularly at high temperatures and acidic conditions. Unlike formic acid, which can be produced both from lignin, furfural and 5-hydroxymethylfurfural (HMF) degradation, levulinic acid is only formed from breakdown of HMF [30, 120]. Several different fatty acids originating from wood extractives and branched short-chain organic acids are also present at low concentrations [128].

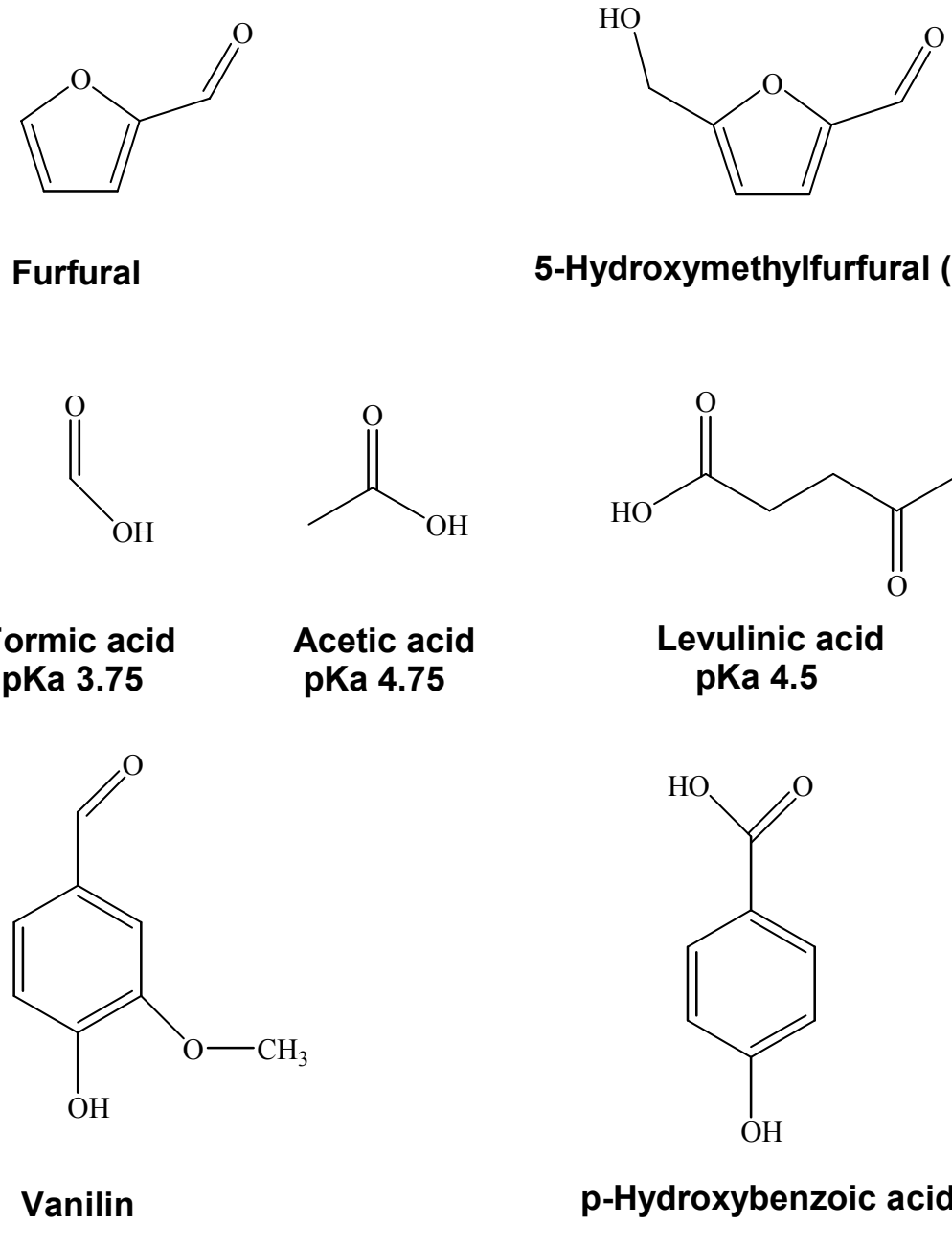


Figure 17 Molecular structure of some toxic compounds found in lignocellulosic hydrolysates.

Organic acids are harmful for the yeast cells and inhibit cell growth. Weak organic acids have therefore long since been used as food preservatives [129]. It is the undissociated acids that cause the inhibition since they are soluble in lipids and can diffuse through the plasma membrane. Due to the concentration gradient of undissociated acid over the cell membrane a constant diffusion of undissociated acid into the cell occurs. In the cytosol where the pH is usually around 7, the undissociated acid can dissociate and release a hydrogen ion and thereby lower the intracellular pH. To avoid a drop in the intracellular pH, the hydrogen ions and the

anions need to be pumped out from the cell by ATP dependent transporters. The process requires ATP, which is why the cell's energy consumption increases. High acid concentrations therefore have an immense negative effect on the biomass and ethanol production. A low acid concentration, on the other hand, has been shown to stimulate ethanol production under anaerobic conditions because of reduced biomass generation and the increased ATP requirement [130].

The toxicity of carboxylic acids is very pH dependent since the pH of the medium and the pK_a value of the acids determine the concentration of undissociated acid. Lignocellulosic hydrolysates usually contain higher levels of acetic acid than formic and levulinic acid [5]. Formic acid has a higher toxicity compared to levulinic acid, and acetic acid shows the lowest toxicity among the three. The higher toxicity of formic acid is explained by its small molecular size and lower pK_a. Levulinic acid is probably more inhibiting than acetic acid because of it higher lipophilicity, which enables it to diffuse more easily into the cell [5, 62].

Even though acetic acid can severely harm the yeast cells, the investigation performed in **Paper IV** demonstrates that continuous cultivation at high dilution rates can be performed even at as high levels of acetic acid as 15 g L^{-1} in the feed medium when a MBR was used. Using 15 g L^{-1} acetic acid, which corresponds to 5.3 g L^{-1} undissociated acetic acid at pH 5.0, ethanol production and sugar utilisation remained high.

4.2.2 Furan aldehydes

Two different types of furan aldehydes are present in relatively large amounts in lignocellulosic derived hydrolysates, namely furfural and HMF. Furfural is a degradation product of pentose sugars. Furfural can also generate formic acid if it is further degraded. In contrast, degradation of hexose sugars generates HMF, which may be further degraded to produce formic and levulinic acid [131]. In dilute acid spruce hydrolysate, HMF concentrations generally vary from 2.0–5.9 g L^{-1} depending on the treatment conditions. Furfural levels are generally lower than for HMF, often around 1 g L^{-1}; however, but this is still enough to be inhibitory for the cells [5] in some situations.

In batch cultivations, furfural has been confirmed to have strong inhibiting effect on *S. cerevisiae*. Furfural will severely inhibit metabolism in the yeast, extend the lag-phase, and reduce the ethanol yield, productivity and specific growth rate [5, 70, 132]. The severity of the

inhibition depends on the applied strain and the furfural concentration. Both HMF and furfural can be converted in to less inhibitory compounds by the yeast, referred to as *in situ* detoxification; however, the ratio of inhibitor to cells has to be kept low [43].

Under anaerobic conditions, less inhibitory alcohol compounds are formed from the furan aldehydes, like in the case of furfural which is converted into the less toxic furfuryl alcohol [70, 133], as shown in Figure 18. Both furfural and HMF can act as electron acceptors. It is generally believed that the cells' *in situ* reduction of furan aldehydes to alcohols is performed by NAD(P)H dependent alcohol dehydrogenases [134]. When yeast is exposed to low levels of furfural (around 6 mM), it has been observed that the cofactor for furfural reduction is NADH [63, 135]. The yeast therefore ceases its glycerol production as a first means to free NADH-coupled reducing capacity. If this reducing capacity is exhausted, the cell can deliver more reducing power by formation of NADPH as a second alternative [136].

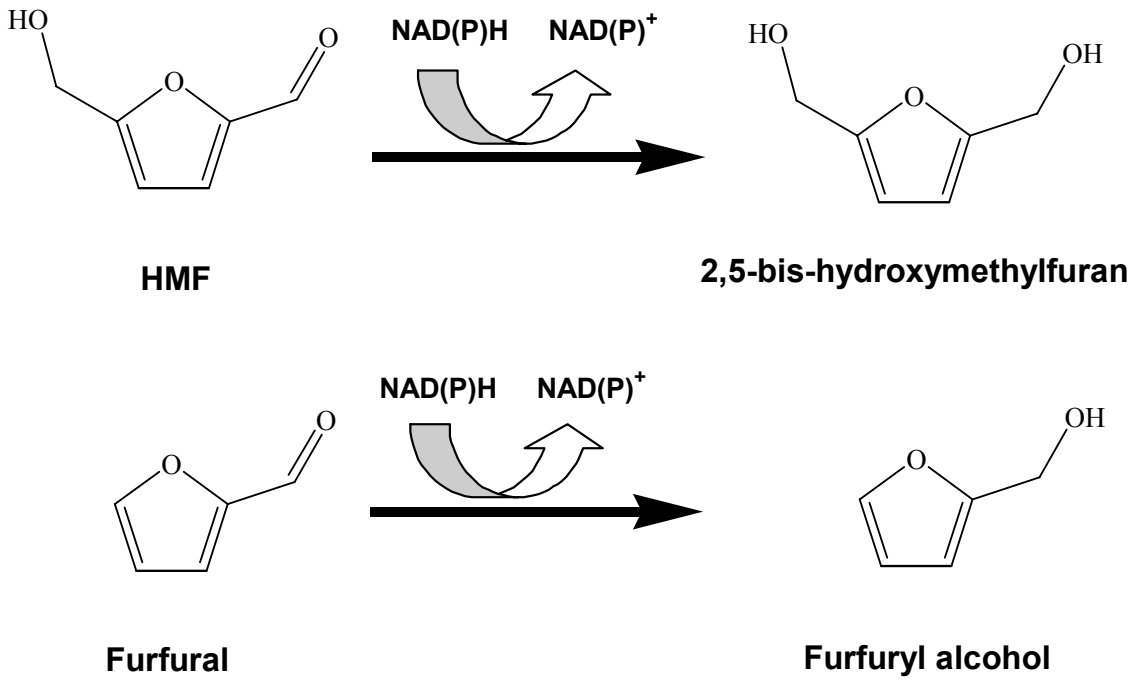


Figure 18 Microbial conversion of HMF and furfural by the action of alcohol dehydrogenase to furfuryl alcohol and 2,5-bis-hydroxymethylfuran, which are less toxic.

HMF can be bio-transformed to 2,5-bis-hydroxymethylfuran [137]. The HMF is generally considered to be less inhibitory than furfural [138]; its conversion rate is yet much slower. Both HMF and furfural will negatively influence the activity of catabolic enzymes such as pyruvate dehydrogenase, acetaldehyde dehydrogenase, alcohol dehydrogenase and triosephosphate dehydrogenase [134].

The yeasts' limits of furfural conversion were evaluated in **Paper III** where furfural was added to a continuous MBR cultivation containing up to 180 g L^{-1} yeast cells. Pulse injections of up to 21.8 g L^{-1} furfural resulted in a high specific conversion rate of 0.35 g g^{-1} h^{-1}. Very high levels of furfural of 17 g L^{-1} could be added to the feed medium without permanent negative effects on the ethanol production. Since the ratio of furfural to yeast biomass can be maintained at a low level in the MBR, very high furfural amounts can be added without any major effects on the ethanol fermentation. By *in situ* detoxification, the large biomass amount in the reactor can keep the furfural concentration very low. No wood hydrolysate has been reported to contain the tested furfural levels used in **Paper III**. It is, however, interesting to illustrate that the limit of high cell density cultivations against convertible inhibitors is very high. This can be advantageous when using more inhibiting wood hydrolyzates, as also indicated in **Paper V**.

4.2.3 Phenolic compounds

Phenolic compounds in wood hydrolysates present a very heterogeneous group, which is difficult to characterize. The soluble phenolic compounds in treated lignocelluloses originate from breakdown of lignin and alkaline extractives [139]. The most common phenolic compounds found in treated lignocellulosic material are 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde and syringic acid [30]. These compounds are inhibitory for yeast even at low concentrations. Some of the phenolic compounds can be converted by the microorganism. It has been shown that *S. cerevisiae* is able to detoxify and transform phenolic compounds by e.g. aldehyde reduction, quinine reduction and double bond saturation [140]. For example, the enzyme phenylacrylic acid decarboxylase, which is an aromatic acid carboxylase, is able to transform cinnamic, p-cumaric and ferulic acids [141, 142] known to be fermentation inhibitors for *S. cerevisiae* [140].

The level and type of phenolic compounds generated during hydrolysis depends largely on the lignocellulosic biomass used because the lignin matrix in various raw materials has different degrees of methoxylation, internal bonding and bonds to hemicellulose and cellulose [136, 143, 144]. Delgenes et al. [145] found over 60 different phenolic derivatives in spruce hydrolysate [5, 145]. Usually, phenolic compounds which are less heavily substituted are more toxic [146].

Lignocellulosic hydrolysates have a very complex composition due to the presence of several lignocellulose-derived inhibitors, making it a difficult substrate for ethanol production. No single compound can be defined as the lone inhibitor responsible for the difficult fermentability. Martín and co-workers tried to mimic lignocellulosic hydrolysates by making cocktails containing defined amounts of inhibitors [147] but did not acquire the same toxicity as in a real hydrolysate. This indicates that some inhibitors present in the hydrolysate at low concentrations can largely impact the fermentability of the hydrolysate. Another possibility is that some compounds create synergetic inhibitory effects. The toxicity of hydrolysates can also be very different depending on the method used to prepare them. It is therefore problematic to compare results from different investigations. Additionally, the inhibitor tolerance between different microorganisms and even between yeast strains within the same species can differ to a large extent [148].

4.3 Strategies to manage inhibition

Section 4.1–4.2 described several problems that occur when utilising lignocellulosic materials as substrate for ethanol fermentation. One of the major problems is the inhibitory effect of the degradation products formed when lignocellulosic material is hydrolysed. In order to reduce the negative effect on the microbial fermentation, various alternative measures can be used (Table 4). In the work performed in this thesis, methods A (fermentation mode = continuous) and F (high yeast cell density) and a short-term microbial adaptation (B) were used to cope with the inhibitors.

Table 4 Strategies to manage inhibition.

Method	Effect
A. Altering fermentation mode – fed-batch or continuous	Lower concentration of inhibitors during the cultivation if the cells can detoxify the inhibitors.
B. Strain adaptation	Adapt the cells to increasing amounts of inhibitors.
C. Isolated or selected inhibitor tolerant strains	The strain is already tolerant of the inhibitors.
D. Metabolic engineering	The strain is engineered by over-expressing genes or introducing new genes to make the strain more inhibitor tolerant.
E. Hydrolysate detoxification	The hydrolysate is treated to lower the concentration of inhibitor.
F. Increased yeast cell density	More cells result in higher volumetric reaction rates which result in higher detoxification rate of convertible inhibitors. The toxic effect of the inhibitors can thereby be reduced or eliminated.

4.3.1 Altering the fermentation process

The fermentation process can be modified in several ways to ease or solve the inhibition problems. If the cultivation is performed in a fed-batch or continuous mode, the level of incoming inhibitors can be controlled and kept at low levels. Accordingly, the yeast's innate ability to convert some of the inhibitors present in the hydrolysate to less toxic ones can be exploited. One example is the microbial conversion of phenolic compounds [140] or conversion of furfural into the less toxic furfuryl alcohol [60, 61, 136].

By controlling the addition of hydrolysate during either a fed-batch or continuous cultivation, the level of inhibitors can be kept low thanks to the simultaneous detoxification. Therefore, toxic levels of inhibitors can be avoided in the cultivation. In **Paper III**, the detoxification capacity of the yeast was put to its limit when furfural containing media was added to continuous MBR cultivations at high yeast density. The concentration of furfural in the inlet media could be increased to as much as 17.0 g L^{-1} without any major changes in the ethanol production. The high furfural conversion and tolerance would not have been possible if the cells were not retained in the MBR.

4.3.2 Cell adaptation

An increased inhibitor tolerance in *S. cerevisiae* has also been successfully achieved by adapting the yeast to either particular inhibitors or complete lignocellulosic hydrolysate [149]. Yeast strains that are exposed to high inhibitor concentrations over a prolonged period of time are known to increase their inhibitor tolerance [150]. Adapting the yeast exploits its innate ability to improve its capability to either resist or degrade toxic compounds present in the hydrolysate. The mode of preparing the yeast has a significant impact on the performance of the yeast, and just a short cultivation period in hydrolysate can drastically improve the yeast's fermentation ability [151]. Adaptation, or directed evolution, can be performed by transferring the yeast stepwise to medium containing higher and higher levels of hydrolysate [150]. Another way is to perform repeated batch cultivations for prolonged periods by using an inhibitor cocktail or real wood hydrolysate to generate strains that are evolved for the particular inhibitory environment [152]. Not only has the inhibitor tolerance been improved by direct evolution, but also the xylose consumption rate [153]. Most of cultivations in **Papers I** and **III–V** were run in continuous mode. When either inhibitors or complete hydrolysate is added into a continuous culture, there is a short period of low but increasing

levels of inhibitors. The yeast has therefore a shorter period to adapt and change its metabolism to cope with the more toxic environment.

4.3.3 Isolated or selected inhibitor tolerant yeast strains

When choosing the specific microorganism and strain, it is crucial to not only search for high sugar utilisation, high ethanol tolerance and production, but also to observe its robustness and inhibitor tolerance. Several investigations have compared the robustness and inhibitor tolerance of yeast strains in inhibitor containing medium [147, 154]. They confirm that the inhibitor tolerance is very strain dependent. Additionally, some strains can have a high resistance for particular inhibitors but be more susceptible to other inhibitors [147]. One problem with adapted strains is, however, how to maintain their improved stability for the inhibitors. More inhibitor tolerant strains can also be isolated; one example the is *S. cerevisiae* TMB3000 isolated from a spent sulphite liquor plant [155]. In the case of *S. cerevisiae* TMB3000, the yeast strain had been put under a long-term selection pressure for maybe several years in the sulphite liquor plant. This had altered the yeast, thus, making it better at coping with the particular environment at the plant.

4.3.4 Metabolic engineering

The growing understanding of microbial metabolism has paved the way to use metabolic engineering to conduct targeted strain improvements. It is today possible to genetically modify a strain to make it more inhibitor tolerant by either introducing new genes or increasing the expression of strain specific genes. One strategy to increase the strain robustness is to improve the inhibitor conversion to less inhibitory compounds e.g. by increasing the production of inhibitor converting enzymes such as the HMF converting alcohol dehydrogenase 6 (ADH6) [156] or the aromatic inhibitor degrading enzyme phenylacryl acid carboxylase (Pad1p) [140]. Other ways are to improve the cells ability to transport and excrete inhibitors out from the cell or to engineer the *S. cerevisiae* strain to e.g. overproduce metabolites with a protective nature, such as glutathione [157].

4.3.5 Hydrolysate detoxification

Detoxifying the hydrolysate (i.e. removal of inhibitors) by methods such as alkaline treatment using $Ca(OH)_2$, NaOH or NH_3 at elevated temperatures, evaporation, ozone, ion-exchange or

enzymatic treatment can remarkably improve the fermentability of inhibitory hydrolysates [158, 159]. The detoxification is, however, costly [160] and results in loss of fermentable sugars [161] and formation of additional waste products.

4.3.6 Increased yeast cell density

The cell performance can be enhanced by increasing the cell density when performing cultivations in inhibitory hydrolysate for example, because it gives a more rapid volumetric detoxification of convertible inhibitors [42] (**Paper III)**. Instead of starting with a high inoculum size [162], cell retention by either immobilisation or cell recirculation has been tested in order to increase the cell density during the cultivation. Several approaches have been used to accomplish a high cell concentration such as cell flocculation [163], sedimentation [163], surface adsorption [164], cross-linking the cells [165], entrapment in a matrix [166], encapsulation [84, 167] (**Papers I–II**) and membrane bioreactors [166] (**Papers III–V**). Cell retention can be utilised if it is simple and the cost of recirculation and reuse is low enough. However, problems arise if the microorganism and cultivation broth include large amounts of solids, making the separation of the cells problematic. This is especially true in SSF processes, where cell recycling in principal is impossible if the process is run in the traditional way.

Most of the cultivations conducted in this thesis work were performed at high yeast cell density, achieved by either encapsulating the yeast (**Papers I** and **II**) or using MBRs (**Papers III–V**). Table 5 shows the maximum yeast cell concentrations during cultivations performed in **Papers I–V**.

Table 5 Yeast cell concentrations during cultivations in **Papers I–V,** with standard deviations.

	Cultivation:	**Yeast density (g L^{-1})**	**Yeast density L $capsules^{-1}$**
Paper I	Consecutive batch	5.2 ± 0.1	46.6 ± 2.9
	Continuous 40 °C	19.7 ± 2.0	118.1 ± 9.2
Paper II	At the end of consecutive batch cultivations (APTES treatment);		
	0.0%	6.3 ± 0.3	48.6 ± 2.6
	0.75%	5.6 ± 0.1	42.9 ± 0.4
	1.5%	4.1 ± 0.7	31.6 ± 5.2
	3.0%	0.4 ± 0.0	2.9 ± 0.2
Paper III	Continuous	180 (max)	
Paper IV	Continuous	210 (max)	
Paper V	Continuous	60 (max)	

4.4 Thermal stress during cultivations

Yeast, in general, grows at relatively low temperatures compared to many prokaryotes, and even thermotolerant and thermophilic yeast have an upper temperature limit of 42 °C and 45 °C [168]. More thermally tolerant yeast would offer many advantages in industrial fermentation processes. Elevated temperatures can seriously alter alcohol production during fermentation. If fermentation processes could be operated at elevated temperatures, significant savings could be made on cooling costs, especially in warm countries. Additional advantages are the reduced risk for contamination and faster ethanol recovery [169].

When cells are exposed to temperatures that are above a critical level, the cells are damaged in several ways, the most serious being membrane disruption, protein denaturation and aggregation [170]. At higher temperatures, the inhibitory effect of ethanol has also been shown to increase, thus, resulting in a lower ethanol production [171]. Screens of mutant *S. cerevisiae* which are able to efficiently produce ethanol at higher temperatures show that only a modest increase in temperature has been obtained, to 40 °C as maximum [172, 173]. It has been suggested that there is a low fermentation efficiency of *S. cerevisiae* at elevated temperatures because of increased membrane fluidity, which changes the composition of fatty acids in the membrane [169]. At elevated temperatures, the cells also start to synthesise heat shock proteins (Hsps). These proteins have an important role in protecting the cell during thermal stress [174].

Enzymatic hydrolysis can only be performed rapidly at temperatures above 45 °C because cellulose enzymes have their highest activity at temperatures of up to 50 °C. However, SSF processes cannot be operated at these high temperatures because of inactivation of the yeast. The SSF therefore needs to be operated at a lower than optimal temperature, which leads to a slower enzymatic hydrolysis. If the SSF could be performed at higher temperature, it could enhance the saccharification rate vastly [169]. Yeast strains capable of producing ethanol above 40 °C have therefore been sought by screening of yeast strains. Sometimes, temperature adaptation has also been tested as a way to increase the thermal tolerance of promising strains [175]. *Kluyveromyces marxianus* is one thermotolerant yeast, which has been reported to produce alcohol at temperatures above 40 °C and is capable of growth at temperatures up to 52 °C [175].

Cell immobilisation has been proven to improve the tolerance towards stress and toxic lignocellulosic hydrolysates [83, 84, 148]. When cells are immobilised and grown in a limited space, they alter their metabolism and growth pattern [81, 176]. **Paper I** describes several experiments performed with encapsulated yeast, which show that encapsulation can increase the yeast's temperature tolerance when compared with normally grown suspended yeast. The used yeast *S. cerevisiae* CBS8066 is not a thermally tolerant strain. The consecutive batch experiments showed that the yeast was able to consume all the glucose during the first 5 batch cultivations at 42 °C, Figure 19. Moreover, the ethanol yield stayed high. However, the rate at which they consumed glucose decreased after each batch. In contrast, the suspended yeast completely failed to consume any glucose already in the second or third batch cultivation.

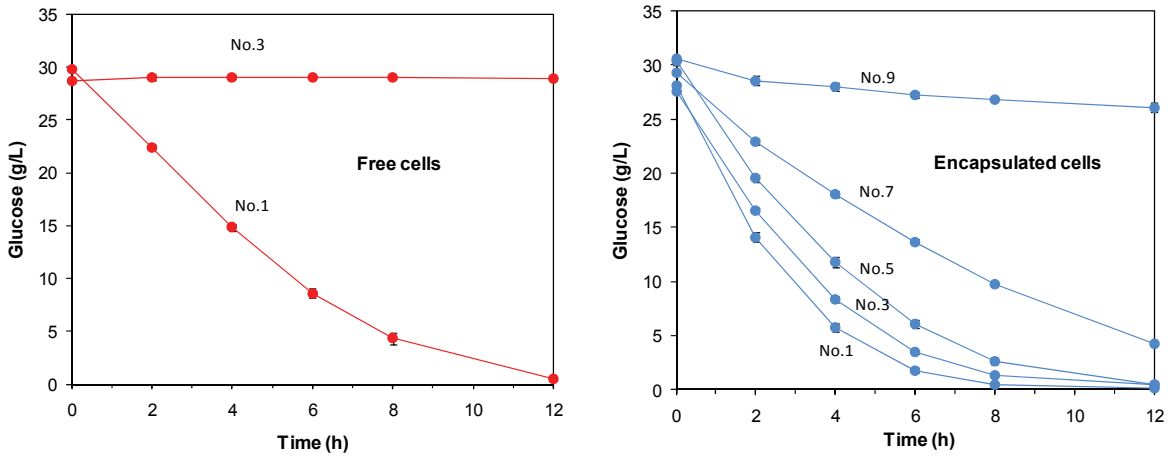


Figure 19 Glucose consumption during consecutive batch cultivations at 42 °C with free cells (left) and encapsulated cells (right). The number and error bar denote the batch number and standard deviation.

If the cellular response upon encapsulation, which allows for the increased tolerance, could be understood in more detail, a more thermally tolerant yeast strain may also be constructed or induced in suspended yeast. Some work has already been done to reveal e.g. the proteomic response of encapsulated compared with suspended yeast. It was shown that three proteins involved in the heat shock response, Glc7p, Hsp12p, and Gre3p, were up-regulated in encapsulated yeast even under normal non-inhibitory conditions [177]. The combination of up-regulated heat response proteins and the increased levels of intracellular trehalose in encapsulated cells [176, 177] can be some of the factors which result in an increased thermal tolerance compared to suspended yeast.

CHAPTER 5

RAPID ETHANOL PRODUCTION

What decides whether a developed ethanol process will be employed in the end is the final price of the produced ethanol. And "time is money" as Benjamin Franklin phrased. For this reason, continuous cultivation has been the main focus throughout this thesis, as this mode has the potential to increase the volumetric productivity and reduce the overall investment cost. Several of the investigations have been performed at a dilution rate of 0.5 h^{-1} and above (**Papers III–V**). Batch cultivations are not suitable for fermentation of lignocellulosic hydrolysates because the yeast is exposed directly to high levels of inhibitors, which results in a long lag phase, a slow rate or a complete failure of the yeast to ferment the hydrolysate. This problem can, however, be solved by using encapsulated cells, which can provide rapid fermentation of hydrolysates even during e.g. consecutive batch cultivations [84], **Paper II**.

The main obstacles for rapid ethanol production from lignocellulosic hydrolysates have been the presence of inhibitors, low ethanol yields and productivity, and incomplete sugar utilisation. This chapter discusses how high cell density cultivations in MBRs were used in this thesis to tackle some of these problems, specifically to use the yeast's own capability to withstand inhibitors and perform *in situ* detoxification at high dilution rates.

5.1 In situ furfural detoxification by *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is able to perform *in situ* detoxification by converting inhibitors such as furfural and HMF present in hydrolysates into less toxic compounds [5]. In **Paper III** furfural was either pulse injected or added to the feed medium at stepwise increasing levels to continuous cultivations (dilution rate of 0.5 h^{-1}) at high yeast density, performed in a MBR. When studying the levels of furfural during pulse injections, it was observed that the yeast was able to convert the furfural very rapidly by *in situ* detoxification, even at a pulse injection of 21.8 g L^{-1} furfural. The ethanol production was affected negatively at the highest pulse injections but recovered quickly when the furfural was depleted, as the yeast converted the compound, as shown in Figure 20. The yeast viability was also stable at approximately 1.5×10^9 CFU ml^{-1}.

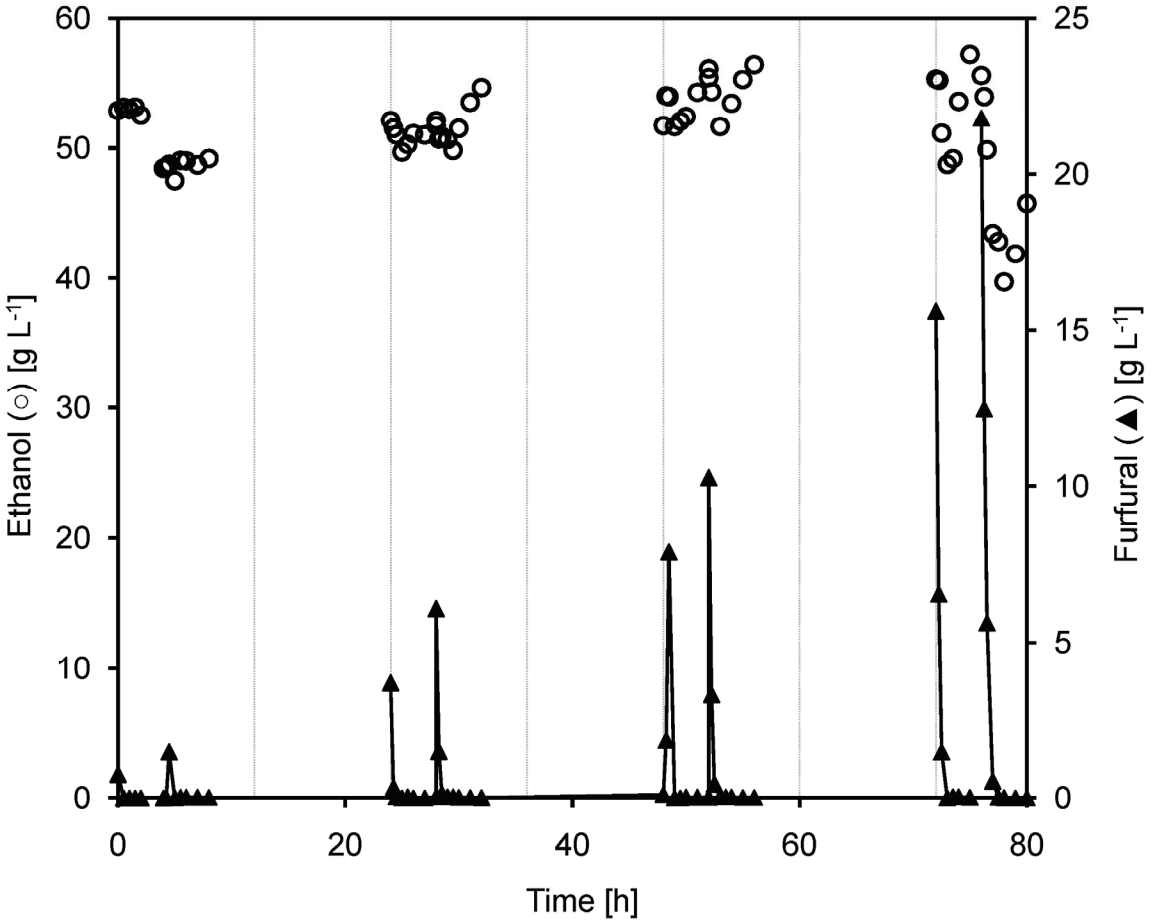


Figure 20 Ethanol and furfural concentrations during furfural pulse injections in continuous MBR cultivations (cultivation A, **Paper III**).

During cultivations with furfural addition to the feed media, ethanol production was stable up to a furfural concentration of 17.0 g L^{-1}, see Figure 21. When the level of furfural was increased to either 18.6 or 20.6 g L^{-1}, the yeast failed to maintain its ethanol production because the furfural reached a critical level inside the fermentor, (**Paper III**).

In a lignocellulosic hydrolyzate, the furfural never reaches such high levels. However, this work illustrates the potential of using the yeast's own detoxification capacity to be able to perform rapid fermentation of a highly toxic medium in continuous mode.

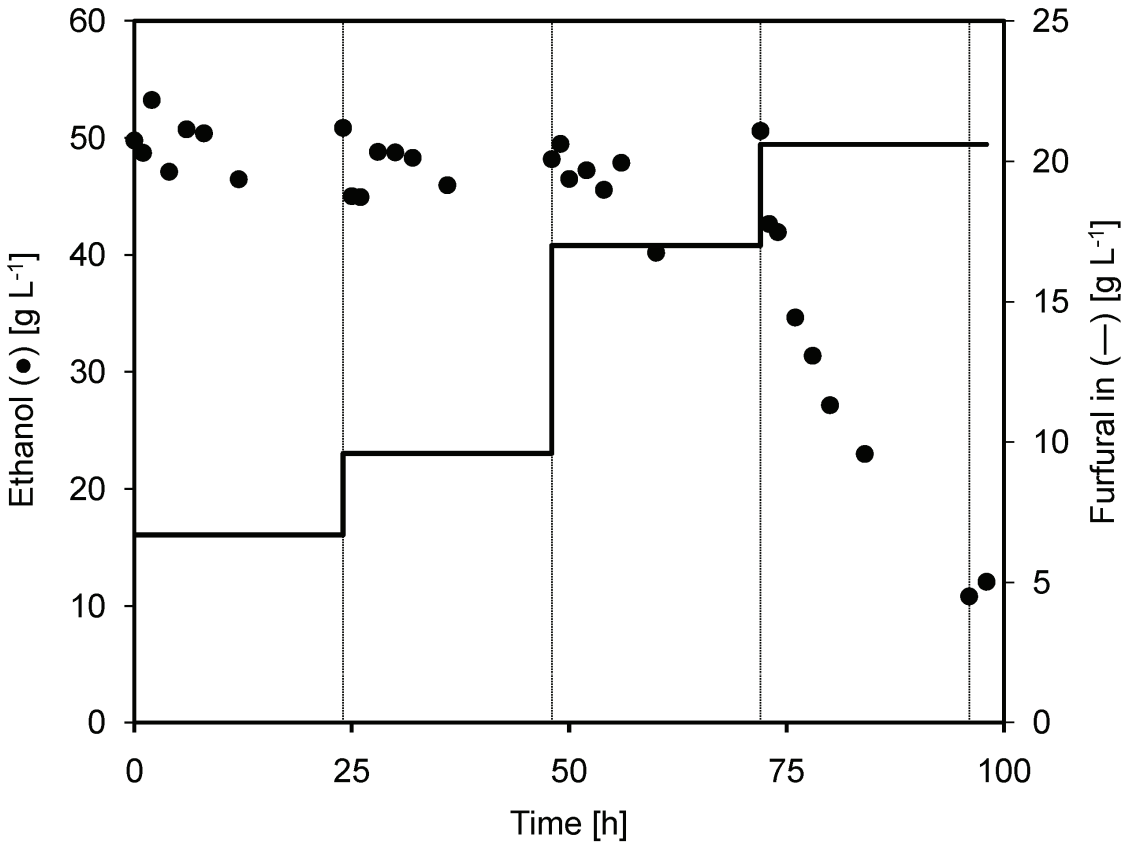


Figure 21 Resulting ethanol concentrations in the permeate and furfural concentration in the feed medium during continuous MBR cultivations (cultivation A, **Paper III**).

5.2 Cultivations at high acetic acid concentration

The negative effects of acetic acid on ethanol production have been thoroughly investigated [130, 178]. However, few investigations have been made in continuous mode, especially at high dilution rates, because acetic acid is known to severely reduce the growth of the yeast. This is problematic in a traditional continuous culture since the cells will be washed out from the bioreactor. **Paper IV** describes how cultivations in a MBR could be performed both at high and low yeast density, even at a high dilution rate of 0.5 h^{-1} and at an increasing level of acetic acid at pH 5.0. The acetic acid concentration was increased from 2.5 up to 20 g L^{-1} in the inlet, and an external cross-flow MBR was used to retain the yeast cell completely inside the bioreactor. The study revealed that the yeast in the MBR was able to withstand acetic acid concentrations up to levels of 15–16 g L^{-1} without any reduction in ethanol production. At acetic acid levels of 17.5 and 20 g L^{-1}, the ethanol production started to decrease (**Paper IV**), as shown in Figure 22.

The volumetric ethanol productivity remained high throughout most of the cultivations and stayed close to 10 g L^{-1} h^{-1} up to a level of 16 g L^{-1} of acetic acid in the cultivations at high yeast concentration. The acetic acid concentration in lignocellulosic hydrolysates seldom reaches such high levels.

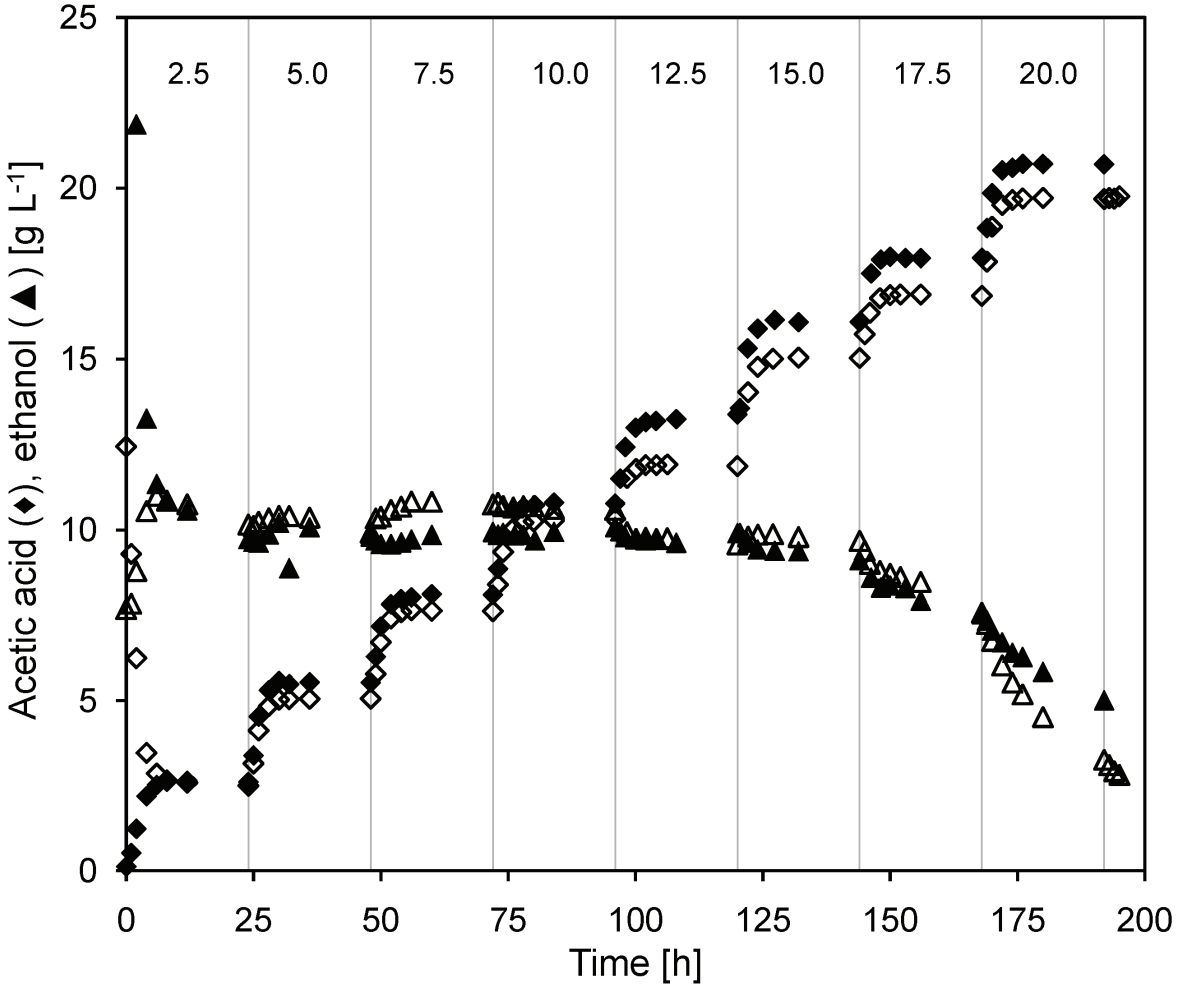


Figure 22 Concentration of ethanol and acetic acid during MBR cultivations at high yeast cell density. Cultivation A is denoted by closed symbols and B by open symbols (**Paper IV**).

5.3 Wood hydrolysate cultivations at high dilution rates

During the final period of the studies presented in this thesis (**Paper V**) a submerged MBR containing a relatively high yeast concentration was constructed and used to ferment an undetoxified wood hydrolysate at different dilution rates. The results illustrate that the yeast in the MBR could efficiently ferment the hydrolysate to ethanol even at the highest tested dilution rate 0.8 h^{-1}. Sugar conversion (mannose and glucose) decreased when the dilution rate was increased. However, it remained at 87% even at a dilution rate of 0.8 h^{-1} (Table 6). The

high sugar utilisation can probably be attributed to the high yeast biomass concentration of about 60 g L^{-1} in the cultivation. When cultivations were performed at a low yeast density of 12.1 ± 1.2 g L^{-1} and a dilution rate of 0.2 h^{-1}, the yeast failed to ferment the hydrolysate.

One problem with the usage of MBR systems is its inherent weakness for particles present in the liquid medium since they can foul the membrane. A possible solution to avoid fouling is to use e.g. cross-flow filtration with small pore size to remove particles in the medium before adding the filtered medium to the MBR. The advantage of this procedure is that the media can be disinfected at the same time by removal of any contaminating bacteria, if a membrane with appropriate pore size is used (≤ 0.22 μm). Another way to reduce fouling due to particles is to have a bleed stream from the bioreactor by which a small amount of fermentation liquid is removed from the bioreactor. Thereby, the concentration of fouling agents can be maintained at controllable levels.

Table 6 Ethanol yield and productivity and conversion of sugars, HMF and furfural during anaerobic MBR fermentation of undetoxified wood hydrolysate (**Paper V**).

			Conversion (%)			
D[a] (h^{-1})	**$Y_{Ethanol/s}$[b] (g g^{-1})**	**Q_p[c] (g L^{-1} h^{-1})**	**Hexoses[d]**	**Mannose and glucose**	**Furfural**	**HMF**
0.2	0.42 ± 0.03	2.16 ± 0.15	85.2 ± 0.7	97.8 ± 0.6	98.3 ± 1.0	89.0 ± 5.9
0.4	0.44 ± 0.01	4.47 ± 0.07	83.5 ± 0.7	96.6 ± 1.4	98.0 ± 1.0	82.2 ± 7.1
0.6	0.45 ± 0.01	6.66 ± 0.16	80.2 ± 0.7	93.7 ± 2.3	98.0 ± 0.9	78.2 ± 7.0
0.8	0.44 ± 0.01	7.94 ± 0.10	74.2 ± 0.4	86.8 ± 1.0	97.4 ± 1.1	70.0 ± 0.3

[a]All results are based on values after 5 retention volumes at the specific dilution rate.
[b]Yields are based on consumed hexoses i.e. glucose, mannose and glucose.
[c]Volumetric ethanol productivity.
[d]Sugar conversion in % of hexoses i.e. glucose, mannose and galactose.

CHAPTER 6

CONCLUDING REMARKS

The main objective of this thesis work was to investigate and develop high cell density yeast cultivations by applying the technologies encapsulation and membrane bioreactors under stressful conditions, for fermentation of lignocellulosic hydrolysates or toxic media. High yeast cell density in the cultivations was shown to be a possible strategy to overcome stress such as elevated temperatures and to manage high levels of inhibitors present in lignocellulosic hydrolysates.

The major outcomes of the projects performed in this thesis can be concluded as follows:

- Both encapsulation and MBRs can be used to retain the yeast cells inside the fermentor and to achieve very high cell concentrations for prolonged periods of about 200 h. A major problem with encapsulations is the robustness of the capsules. One method to make the capsules more robust for long-term use is to treat the capsule membranes with organofunctional silanes to produce polysiloxane ACA capsules.

- The results showed that dilute acid hydrolysed lignocellulosic material can be efficiently fermented, without prior detoxification, in continuous mode by high yeast cell density cultivation, using encapsulation or MBRs. However, at low yeast concentrations of about 12 g L^{-1} and a dilution rate of 0.2 h^{-1}, the yeast in the MBR failed to ferment the dilute acid wood hydrolysate. Further investigations at industrial conditions are, nevertheless, required to investigate how appropriate especially MBRs are at e.g. low nutrient addition and long periods of cultivation.

- Cell retention and high yeast cell density during continuous MBR cultivations in dilute acid wood hydrolysate can result in almost complete sugar utilisation and an efficient *in situ* detoxification of inhibitors such as furfural. At the dilution rate of 0.8 h^{-1}, this resulted in an ethanol productivity of almost 8 g L^{-1} h^{-1}.

- MBRs are beneficial even for fermentation of media containing non-convertible inhibitors, such as acetic acid. In the MBRs, the yeast can be kept inside the fermentor

even if the cells are not growing or if the specific growth rate is low, even at the high dilution rate 0.5 h^{-1}. Yeast can continue to produce ethanol as long as it is metabolically active and the acetic acid concentration does not reach a critically toxic level.

- It was observed that by maintaining a low enough ratio of convertible inhibitors (such as furfural) to yeast biomass, the yeast cells could perform rapid *in situ* detoxification without any larger negative effects on the ethanol production. The high yeast cell density cultivations in the MBR contained up to 180 g L^{-1} yeast and could withstand very high furfural additions of up to 17 g L^{-1} when furfural was added to the feed media. The yeast rapidly detoxified the furfural so the level of furfural inside the bioreactor was only a fraction of the concentration in the feed media.

CHAPTER 7

FUTURE DIRECTIONS

My thesis work contributes to the struggle to find a smart process, by which we hopefully can solve our future transportation fuel problems. Although some industrial plants producing lignocellulosic derived ethanol have already been constructed, it is not on a large commercial scale yet, and process improvements are still needed. The continuation of this work is crucial if we are to make ethanol production from lignocellulosic material possible. Based on the results and knowledge from my work, a few suggestions are made on where to lay the coming efforts in the future:

- Many people have identified that we need to think in a wider concept and not only focus on a single final product such as ethanol, and I agree. We need to think about a biorefinery, where we convert biomass into several products, both fuel, chemicals and power, in order to improve the value of the process. One possible manner is to utilise the liquid fraction from the wood hydrolysis to ferment it in a MBR whereas the solid fraction is used for e.g. biogas or energy production.

- The results from this thesis work are very interesting when it comes to usage of high yeast density cultivations of lignocellulosic hydrolysates by MBR fermentation. However, the system needs to be tested at more industrial like conditions, for example, during long-termcontinuous cultivations to investigate the stability of the system and the membrane module, and by using "real industrial medium" without supplementation of extra nutrients e.g. yeast extract in order to lower the cost of the used medium without altering the performance of the yeast.

- The potential of submerged membrane bioreactors for fermentation of wood hydrolysates has been shown in this thesis. Submerged membrane bioreactors usually require a lower input of energy compared with externally placed cross-flow MBRs. However, cross-flow membranes are less sensitive to fouling at high particulate and cell concentrations. It might therefore be interesting to test the externally placed cross-flow MBRs by running it in fed-batch mode. Consecutive fed-batch cultivations can

then be performed and the membrane module can be operated between each fed-batch to remove the ethanol and consumed broth. No liquid pumping is then required in the membrane module during most of the cultivation, which reduces the energy input.

- Membrane technology can also be used instead of heat treatment to sterilise a liquid substrate stream as long as the substrate does not contain high levels of particles. This can reduce the amount of energy required to kill any present microorganisms in the medium before fermentation.

- Continuous cultivations of wood hydrolysates at dilution rates up to 0.8 h were shown to be possible in MBRs in this thesis. However, dilute acid hydrolysed lignocellulosic materials usually result in liquid media containing low sugar levels. To make distillation economical, the ethanol level needs to be over 4% ethanol in the final effluent. The sugar levels need, therefore, to be higher in the used hydrolysate to reach these values. One possible way to increase the sugar content in wood hydrolysates is to increase the dry weight content of lignocellulosic material during the hydrolysis step since the sugar concentration in the final liquid depends on how much steam is added during the treatment. Even if higher levels of inhibitors are generally produced during this process, the results in this thesis show that MBR cultivation has the potential to handle even more toxic medium. It would therefore be interesting to investigate if it is possible to ferment the liquid fraction from dilute acid hydrolysed spruce prepared at high wood dry weight in a MBR to observe if it is possible to reach 4% ethanol in the final fermentation broth.

- Large investment costs are often required to build an ethanol production plant. If a cheaper construction of e.g. the bioreactor could be used, the motivation to build or test a new system on a larger scale would be more attractive and not as economically risky.

NOMENCLATURE

ACA	Alginate-chitosan-alginate
AC	Alginate-chitosan
APTES	3-aminopropyltriethoxysilane
CBP	Consolidated bioprocessing
CFU	Colony forming units
CMC	Carboxymethylcellulose
Furfural	2-furaldehyde
hAPTES	Hydrolysed 3-aminopropyltriethoxysilane
HMF	5-hydroxymethyl-2-furaldehyde
GDP	Gross domestic product
MBR	Membrane bioreactor
NAD/NADH	Nicotinamide adenine dinucleotide
NADP/NADPH	Nicotinamide adenine dinucleotide phosphate
PS	Polysiloxane
SSF	Simultaneous saccharification and fermentation
SSFF	Simultaneous saccharification filtration and fermentation

ACKNOWLEDGEMENTS

Throughout my post-graduate studies at both School of Engineering at University of Borås and the Industrial Biotechnology group at Chalmers University of Technology, I have met numerous people who have contributed to my studies or laboratory work in one way or another. I would like to express my deepest appreciation to all of them, particularly:

My main supervisor Professor Mohammad Taherzadeh for giving me advice and pushing me forward throughout the course of all projects. I also appreciate the freedom you gave me to complete this work and to find my own path. Calle, my co-supervisor (Associate Professor Carl Johan Franzén), thank you for investing so much time in reading and improving our manuscripts and for always being so positive and encouraging. I wish also to acknowledge Professor Lisbeth Olsson for her never-ending effort to guide all members in the Industrial biotechnology group. I really admire your leadership and manner to manage a large research group. You have all played a crucial role in guiding and letting me develop my own skills even though you have been relatively busy many times.

Thank you Johan, Jorge, Supansa and Adib for being wonderful office mates and for encouraging me to take "fika" breaks when I was too focused on laboratory work. Supansa, I was really happy when you came to work in our group. You came in as a breath of fresh air in the laboratory.

I would also like to express my appreciation to the entire IndBio group, the Biotechnology group and other people at University of Borås for your help and support throughout the years. I will not mention all of you by name, but I have enjoyed and appreciated all of your company, discussions, events and help with laboratory work.

I would also like to thank the Swedish Energy Agency for the financial support of the project.

Finally, I would like to thank my family and especially Hamed for staying by my side throughout my time as a PhD student, which has not always been easy. You have many times wondered what I am really doing in the laboratory and how many samples I need to take this time – thanks for bearing with me.

Päivi Ylitervo
February 2014

REFERENCES

[1] IEA. *http://www.eia.gov/forecasts/steo/report/global_oil.cfm* [cited 2014 January 21].

[2] A.L. Borrion, M.C. McManus, and G.P. Hammond, *Environmental life cycle assessment of lignocellulosic conversion to ethanol: A review,* Renewable and Sustainable Energy Reviews, 16 (2012), pp. 4638-4650.

[3] J. Evans, *Sand banks: If unconventional sources of oil, such as oil sands, could be transformed into crude we could still have a 300-year supply left. The problem is extracting it*, in *Chemistry and Industry* 2006: Ten Alps Publishing. p. 18-36.

[4] D. Arent, F.A. Verrastro, J.L. Bovair, and E.R. Peterson, *Alternative Transportation Fuels and Vehicle Technologies - Challenges and Opportunities, A report of the CSIS Energy and National Security Program*. 2009, CSIS Global Strategy Institute, and National Renewable Energy Laboratory.

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

[6] Rapport från Energimyndigheten, *Kinas växande energibehov - Snabb ekonomisk tillväxt på verkar den globala energimarknaden,* (2005), pp. 1-26.

[7] T. Jackson, *Välfärd utan tillväxt: Så skapar vi ett hållbart samhälle*. 2011: Ordfront Förlag.

[8] Avanza. *https://www.avanza.se/index/om-indexet.html/19005/olja*. [cited 2014 January 23].

[9] *European Commission, Eurostat Pocketbooks, Energy, transportation and environment indicators*. 2013, Publications Office of the European Union: Luxembourg.

[10] *EU Renewable Energy Directive (Dir 2009/28/EC)* 2009.

[11] L. Brennan and P. Owende, *Biofuels from microalgae - A review of technologies for production, processing, and extractions of biofuels and co-products,* Renewable and Sustainable Energy Reviews, 14 (2010), pp. 557-577.

[12] P.V. Attfield, *Stress tolerance: The key to effective strains of industrial baker's yeast,* Nature Biotechnology, 15 (1997), pp. 1351-1357.

[13] J. Zaldivar, J. Nielsen, and L. Olsson, *Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration,* Applied Microbiology and Biotechnology, 56 (2001), pp. 17-34.

[14] A.A. Maris, D. Abbott, E. Bellissimi, J. Brink, M. Kuyper, M.H. Luttik, H.W. Wisselink, W.A. Scheffers, J. Dijken, and J. Pronk, *Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status,* Antonie van Leeuwenhoek, 90 (2006), pp. 391-418.

[15] A.K. Chandel, B.C.M. Gonçalves, J.L. Strap, and S.S. da Silva, *Biodelignification of lignocellulose substrates: An intrinsic and sustainable pretreatment strategy for clean energy production,* Critical Reviews in Biotechnology, (2013), pp. 1-13.

[16] H. Panda, *The Complete Book on Wine Production*. 2011, National Institute of Industrial Research.

[17] K.J. Verstrepen, D. Iserentant, P. Malcorps, G. Derdelinckx, P. Van Dijck, J. Winderickx, I.S. Pretorius, J.M. Thevelein, and F.R. Delvaux, *Glucose and sucrose: hazardous fast-food for industrial yeast?,* Trends in Biotechnology, 22 (2004), pp. 531-537.

[18] F.W. Bai, W.A. Anderson, and M. Moo-Young, *Ethanol fermentation technologies from sugar and starch feedstocks,* Biotechnol. Adv., 26 (2008), pp. 89-105.

[19] B. Petrovska, E. Winkelhausen, and S. Kuzmanova, *Glycerol production by yeasts under osmotic and sulfite stress.,* Canadian Journal of Microbiology, 45 (1999), pp. 695-699.

[20] E. Albers, G. Lidén, C. Larsson, and L. Gustafsson, *Anaerobic redox balance and nitrogen metabolism in Saccharomyces cerevisiae,* Recent Research Developments in in Microbiology, 2 (1998), pp. 253-279.

[21] R. Ansell, K. Granath, S. Hohmann, J.M. Thevelein, and L. Adler, *The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation,* EMBO Journal, 16 (1997), pp. 2171-2544.

[22] J.P. van Dijken and A. Scheffers, *Redox balances in the metabolism of sugars by yeasts,* FEMS Microbiology Reviews, 32 (1986), pp. 199-225.

[23] M.M. Demeke, H. Dietz, Y. Li, M.R. Foulquié-Moreno, S. Mutturi, S. Deprez, T. Den Abt, B.M. Bonini, G. Liden, F. Dumortier, A. Verplaetse, E. Boles, and J.M. Thevelein, *Development of a D-xylose fermenting and inhibitor tolerant industrial Saccharomyces cerevisiae strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering,* Biotechnology for Biofuels 6(2013), pp. 1-24.

[24] L. Diao, Y. Liu, F. Qian, J. Yang, Y. Jiang, and S. Yang, *Construction of fast xylose-fermenting yeast based on industrial ethanol-producing diploid Saccharomyces cerevisiae by rational design and adaptive evolution,* BMC Biotechnology, 13 (2013), pp. 1-9.

[25] T. Ghosh and M. Prelas, *Ethanol*, in *Energy Resources and Systems*. 2011, Springer Netherlands.

[26] M. Vohra, J. Manwar, R. Manmode, S. Padgilwar, and S. Patil, *Bioethanol production: Feedstock and current technologies,* Journal of Environmental Chemical Engineering, (2013), pp. 1-12.
[27] M. Galbe and G. Zacchi, *A review of the production of ethanol from softwood,* Applied Biobiology and Biotechnology, 59 (2002), pp. 618-628.
[28] R. Sims, M. Taylor, J. Saddler, and W. Mabee, *From 1st to 2nd generation biofuel technologies - An overview of current industry and R&D activities*, in *IEA Bioenergy*. 2008, OECD/IEA.
[29] J.O. Westman, *Ethanol production from lignocellulose using high local cell density yeast cultures: Investigations of flocculating and encapsulated Saccharomyces cerevisiae*, in *Department of Chemical and Biological Engineering*. 2014, Chalmers University of Technology: Chalmers Reproservice. p. 91.
[30] H.B. Klinke, A.B. Thomsen, and B.K. Ahring, *Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass,* Applied Microbiology and Biotechnology, 66 (2004), pp. 10-26.
[31] V. Singh and P. Steven, *Sustainable Biotechnology: Sources of Renewable Energy*. 2009: Springer.
[32] C.E. Wyman, *What is (and is not) vital to advancing cellulosic ethanol,* Trends in Biotechnology, 25 (2007), pp. 153-157.
[33] E. Sjöström, *Wood chemistry. Fundamentals and applications*. 1981, San Diego: Academic Press.
[34] D. Dermer and Y. Amor, *Cellulose biosynthesis,* The plant cell, 7 (1995), pp. 987-1000
[35] L. Canilha, W. Carvalho, J. Batista, and A. Silva, *Xylitol bioproduction from wheat straw: hemicellulose hydrolysis and hydrolyzate fermentation,* Journal of the Science of Food and Agriculture, 86 (2006), pp. 1371-1376.
[36] R. Bura, R. Chandra, and J. Saddler, *Influence of xylan on the enzymatic hydrolysis of steam-pretreated corn stover and hybrid poplar,* Biotechnology Progress, 25 (2009), pp. 315-322.
[37] R.O. Moutta, A.K. Chandel, R.C.L.B. Rodrigues, M.B. Silva, G.J.M. Rocha, and S.S. Silva, *Statistical Optimization of Sugarcane Leaves Hydrolysis into Simple Sugars by Dilute Sulfuric Acid Catalyzed Process,* Sugar Tech, 14 (2012), pp. 53-60.
[38] T. Räisänen and D. Athanassiadis, *Basic chemical composition of the biomass components of pine, spruce and birch* in *Forest Refine*. 2013, Finnish Forest Research Institute.
[39] H. Sixta, *Handbook of pulp*. Vol. 1. 2006, Weinheim: WILEY-VCHVerlag GmbH & Co. KGaA.
[40] P. Stenius, *Forest products chemistry In: Paper making science and technology*. 2000, Jyväskylä: Fapet Oy.
[41] F.J. Ruiz-Dueñas and Á.T. Martínez, *Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this,* Microbial Biotechnology, 2 (2009), pp. 164-177.
[42] E. Palmqvist and B. Hahn-Hägerdal, *Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification,* Bioresource Technology, 74 (2000), pp. 17-24.
[43] M.J. Taherzadeh, C. Niklasson, and G. Lidén, *Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation,* Bioresour. Technol., 69 (1999), pp. 59-66.
[44] R.P. Chandra, R. Bura, W.E. Mabee, A. Berlin, X. Pan, and J.N. Saddler, *Substrate Pretreatment: The Key to Effective Enzymatic Hydrolysis of Lignocellulosics?*, in *Biofuels*, L. Olsson, Editor. 2007, Springer Berlin Heidelberg.
[45] M.J. Taherzadeh and K. Karimi, *Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: A review,* BioResources, 2 (2007), pp. 707-738.
[46] P. Alvira, E. Tomás-Pejó, M. Ballesteros, and M. Negro, *Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review,* Bioresource Technology, 101 (2010), pp. 4851-4861.
[47] Y. Sun and J. Cheng, *Hydrolysis of lignocellulosic materials for ethanol production: a review,* Bioresource Technology, 83 (2002), pp. 1-11.
[48] T. Wood, *Properties of cellulolytic enzyme systems,* Biochemical Society Transactions 13 (1985), pp. 407-410.
[49] M. Dashtban, H. Schraft, and W. Qin, *Review Fungal Bioconversion of Lignocellulosic Residues; Opportunities & Perspectives,* International Journal of Biological Sciences, 5 (2009), pp. 578-595.
[50] H. Ding, F. Xu, K.C. McFarland, and J. Cherry, *Enhanced enzymatic biomass conversion to fermentable sugars and fuel ethanol,* ACS National Meeting Book of Abstracts, 230 (2005), pp. 1.
[51] M. Tu, X. Pan, and J.N. Saddler, *Adsorption of Cellulase on Cellulolytic Enzyme Lignin from Lodgepole Pine,* Journal of Agricultural and Food Chemistry, 57 (2009), pp. 7771-7778.
[52] G.P. Philippidis, T.K. Smith, and C.E. Wyman, *Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process,* Biotechnology and Bioengineering, 41 (1993), pp. 846-853.
[53] A. Wingren, M. Galbe, C. Roslander, A. Rudolf, and G. Zacchi, *Effect of reduction in yeast and enzyme concentrations in a simultaneous- saccharification-and-fermentation-based bioethanol process: technical and economic evaluation,* Applied Biochemistry and Biotechnology, 121-124 (2005), pp. 485-499.

[54] M.M. Ishola, A. Jahandideh, B. Haidarian, T. Brandberg, and M.J. Taherzadeh, *Simultaneous saccharification, filtration and fermentation (SSFF): A novel method for bioethanol production from lignocellulosic biomass,* Bioresource Technology, 133 (2013), pp. 68-73.
[55] B.L. Maiorella, *"Ethanol," in Industrial Chemicals, Biochemicals and Fuels*. Vol. Vol. 3, Comprehensive Biotechnology. 1983, Oxford: Pergamon Press.
[56] M.J. Taherzadeh and K. Keikhosro, *Acid-based hydrolysis processes for ethanol from lignocellulosic materials: A review,* BioResources, 2 (2007), pp. 472-499.
[57] Q. Xiang, Y. Lee, and R. Torget, *Kinetics of glucose decomposition during dilute-acid hydrolysis of lignocellulosic biomass,* Applied Biochemistry and Biotechnology, 113-116 (2004), pp. 1127-1138.
[58] M. Finkelstein, B. Davison, Q. Nguyen, M. Tucker, F. Keller, and F. Eddy, *Two-Stage Dilute-Acid Pretreatment of Softwoods*, in *Twenty-First Symposium on Biotechnology for Fuels and Chemicals*. 2000, Humana Press.
[59] M. Gutiérrez-Correa and G.K. Villena, *Chapter 7. Characteristics and Techniques of Fermentation Systems. In "Comprehensive Food Fermentation and Biotechnology"*. Vol. 1. 2010, New Delhi: Asiatech Publisher.
[60] A. Nilsson, M.F. Gorwa-Grauslund, B. Hahn-Hägerdal, and G. Liden, *Cofactor dependence in furan reduction by Saccharomyces cerevisiae in fermentation of acid-hydrolyzed lignocellulose,* Applied and Environmental Microbiology, 71 (2005), pp. 7866-7871.
[61] M.J. Taherzadeh, R. Eklund, L. Gustafsson, N. C., and L. G., *Characterization and fermentation of dilute-acid hydrolyzates from wood,* Ind. Eng. Chem. Res., 36 (1997), pp. 4659-4665.
[62] S. Larsson, E. Palmqvist, B. Hahn-Hägerdal, C. Tengborg, K. Stenberg, G. Zacchi, and N. Nilvebrant, *The generation of fermentation inhibitors during dilute acid hydrolysis of softwood,* Enzyme and Microbial Technology, 24 (1999), pp. 151-159.
[63] I.S. Horváth, M.J. Taherzadeh, C. Niklasson, and G. Lidén, *Effects of furfural on anaerobic continuous cultivation of Saccharomyces cerevisiae,* Biotechnol. Bioeng., 75 (2001), pp. 540-549.
[64] L. Domingues, N. Lima, and J.A. Teixeira, *Contamination of a high-cell-density continuous bioreactor,* Biotechnology and Bioengineering, 68 (2000), pp. 584-587.
[65] M. Taherzadeh, R. Millati, and C. Niklasson, *Continuous cultivation of dilute-acid hydrolysates to ethanol by immobilized Saccharomyces cerevisiae,* Appl. Biochem. Biotechnol., 95 (2001), pp. 45-57.
[66] J.N. Nigam, *Continuous ethanol production from pineapple cannery waste using immobilized yeast cells,* Journal of Biotechnology, 80 (2000), pp. 189-193.
[67] R. Iamuna and S.V. Ramakrohna, *High concentration ethanol production using immobilized yeast cells,* Biomass and Bioenergy, 3 (1992), pp. 117-119.
[68] T. Brandberg, N. Sanandaji, L. Gustafsson, and C. Johan Franzén, *Continuous Fermentation of Undetoxified Dilute Acid Lignocellulose Hydrolysate by Saccharomycescerevisiae ATCC 96581 Using Cell Recirculation,* Biotechnol. Prog., 21 (2005), pp. 1093-1101.
[69] U. Schulze, G. Lidén, J. Nielsen, and J. Villadsen, *Physiological effects of nitrogen starvation in an anaerobic batch culture of Saccharomyces cerevisiae,* Microbiology, 142 (1996), pp. 2299-2310.
[70] M.J. Taherzadeh, L. Gustafsson, C. Niklasson, and G. Lidén, *Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by Saccharomyces cerevisiae,* Journal of Bioscience and Bioengineering, 87 (1999), pp. 169-174.
[71] Ó.J. Sánchez and C.A. Cardona, *Trends in biotechnological production of fuel ethanol from different feedstocks,* Bioresource Technology, 99 (2008), pp. 5270-5295.
[72] A. Fiechter and W. Seghezzi, *Regulation of glucose metabolism in growing yeast cells,* Journal of Biotechnology, 27 (1992), pp. 27-45.
[73] R. AH, *History and scientific basis of large-scale production of microbial biomass. In: Economic microbiology*. Vol. 4. 1979, London: Academic Press.
[74] C.S. Souza, K.F. Oliveira, H.C. Trevisan, and C. Laluce, *A Strategy to Compare Yeast Strains and Improve Cell Survival in Ethanol Production Processes above 30°C.*, in *Communicating Current Research and Educational Topics and Trends in Applied Microbiology* A. Méndez-Vilas, Editor. 2007, FORMATEX.
[75] G. Lidén, *Understanding the bioreactor,* Bioprocess and Biosystems Engineering, 24 (2002), pp. 273-279.
[76] M.J. Taherzadeh, C. Niklasson, and G. Lidén, *On-line control of fed-batch fermentation of dilute-acid hydrolyzates,* Biotechnology and Bioengineering, 69 (2000), pp. 330-338.
[77] C. Webb and G.A. Dervakos, *Studies in viable cell immobilization*. 1996, San Diego: Academic Press.
[78] Y. Kourkoutas, A. Bekatorou, I.M. Banat, R. Marchant, and A.A. Koutinas, *Immobilization technologies and support materials suitable in alcohol beverages production: a review,* Food Microbiology, 21 (2004), pp. 377-397.
[79] G. Najafpour, H. Younesi, and K. Syahidah Ku Ismail, *Ethanol fermentation in an immobilized cell reactor using Saccharomyces cerevisiae,* Bioresource Technology, 92 (2004), pp. 251-260.
[80] Y. Lin and S. Tanaka, *Ethanol fermentation from biomass resources: current state and prospects,* Applied Microbiology and Biotechnology, 69 (2006), pp. 627-642.

[81] F. Talebnia and M.J. Taherzadeh, *In situ detoxification and continuous cultivation of dilute-acid hydrolyzate to ethanol by encapsulated S. cerevisiae,* J. Biotechnol., 125 (2006), pp. 377-384.
[82] R. Purwadi and M.J. Taherzadeh, *The performance of serial bioreactors in rapid continuous production of ethanol from dilute-acid hydrolyzates using immobilized cells,* Bioresour. Technol., 99 (2008), pp. 2226-2233.
[83] M. Pourbafrani, F. Talebnia, C. Niklasson, and M. Taherzadeh, *Protective Effect of Encapsulation in Fermentation of Limonene-contained Media and Orange Peel Hydrolyzate,* International Journal of Molecular Sciences, 8 (2007), pp. 777-787.
[84] F. Talebnia, C. Niklasson, and M.J. Taherzadeh, *Ethanol production from glucose and dilute-acid hydrolyzates by encapsulated S. cerevisiae,* Biotechnology and Bioengineering, 90 (2005), pp. 345-353.
[85] S. Schaffellner, V. Stadlbauer, P. Stiegler, O. Hauser, G. Halwachs, C. Lackner, F. Iberer, and K.H. Tscheliessnigg, *Porcine islet cells microencapsulated in sodium cellulose sulfate,* Transplantation Proceedings, 37 (2005), pp. 248-252.
[86] L. Shi, Z. Li, Z. Zhang, T. Zhang, W. Yu, M. Zhou, and Z. Tang, *Encapsulation of Lactobacillus bulgaricus in carrageenan-locust bean gum coated milk microspheres with double layer structure,* LWT - Food Science and Technology, 54 (2013), pp. 147-151.
[87] B.C. Larisch, D. Poncelet, C.P. Champagne, and R.J. Neufeld, *Microencapsulation of Lactococcus lactis subsp. cremoris,* Journal of Microencapsulation, 11 (1994), pp. 189-195.
[88] K.D. Green, I.S. Gill, J.A. Khan, and E.N. Vulfson, *Microencapsulation of yeast cells and their use as a biocatalyst in organic solvents,* Biotechnology and Bioengineering, 49 (1996), pp. 535-543.
[89] S. Youngsukkasem, S.K. Rakshit, and M.J. Taherzadeh, *Biogas production by encapsulated methane-producing bacteria,* BioResources, 7 (2012), pp. 56-65.
[90] B.M. Brena and F. Batista-Viera, *Immobilization of Enzymes A Literature Survey From: Methods in Biotechnology: Immobilization of Enzymes and Cells* Second Edition ed. 2006, Totowa, NJ: Humana Press Inc.
[91] K.I. Draget, O. Smidsrød, and G. Skjåk-Bræk, *1 Alginate from algae: In:Polysaccharides and Polyamides in the Food Industry. Properties, Production and Patents*. 2005, Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA.
[92] S. Youngsukkasem, H. Barghi, S. Rakshit, and M. Taherzadeh, *Rapid Biogas Production by Compact Multi-Layer Membrane Bioreactor: Efficiency of Synthetic Polymeric Membranes,* Energies, 6 (2013), pp. 6211-6224.
[93] I. Gill and A. Ballesteros, *Bioencapsulation within synthetic polymers (Part 1): sol–gel encapsulated biologicals,* Trends in Biotechnology, 18 (2000), pp. 282-296.
[94] J. Westman, P. Ylitervo, C. Franzén, and M. Taherzadeh, *Effects of encapsulation of microorganisms on product formation during microbial fermentations,* Applied Microbiology and Biotechnology, 96 (2012), pp. 1441-1454.
[95] J.K. Park and H.N. Chang, *Microencapsulation of microbial cells,* Biotechnology Advances, 18 (2000), pp. 303-319.
[96] A. Nussinovitch, *Plant gum exudates of the world - sources, distribution, properties, and application*. 2010: Taylor and Francis Group, LLC.
[97] N.V.N. Jyothi, P.M. Prasanna, S.N. Sakarkar, K.S. Prabha, P.S. Ramaiah, and G.Y. Srawan, *Microencapsulation techniques, factors influencing encapsulation efficiency,* Journal of Microencapsulation, 27 (2010), pp. 187-197.
[98] D. Poncelet and R.J. Neufeld, *Shear breakage of nylon membrane microcapsules in a turbine reactor,* Biotechnology and Bioengineering, 33 (1989), pp. 95-103.
[99] S. DiMari, W. Funke, M.A. Haralson, D. Hunkeler, B. Joos-Müller, A. Matsumoto, O. Okay, T. Otsu, A.C. Powers, A. Prokop, T.G. Wang, and R.R. Whitesell, *Water Soluble Polymers for Immunoisolation II: Evaluation of Multicomponent Microencapsulation Systems*, in *Microencapsulation Microgels Iniferters*. 1998, Springer Berlin Heidelberg.
[100] W. Tong, C. Gao, and H. Möhwald, *Manipulating the Properties of Polyelectrolyte Microcapsules by Glutaraldehyde Cross-Linking,* Chemistry of Materials, 17 (2005), pp. 4610-4616.
[101] F. Kurayama, S. Suzuki, T. Oyamada, T. Furusawa, M. Sato, and N. Suzuki, *Facile method for preparing organic/inorganic hybrid capsules using amino-functional silane coupling agent in aqueous media,* Journal of Colloid and Interface Science, 349 (2010), pp. 70-76.
[102] S. Judd and C. Judd, *The MBR book, principles and applications of membrane bioreactors for water and wastewater treatment.* 2nd ed. 2011, Oxford: Butterworth-Heinemann.
[103] P. Ylitervo, J. Akinbomi, and M.J. Taherzadeh, *Membrane bioreactors' potential for ethanol and biogas production: a review,* Environmental Technology, 34 (2013), pp. 1711-1723.
[104] A. Helble and C.H. Möbius, *Current experience with the use of membrane bioreactor technology for the treatment of paper mill effluent*, in *PTS Water & Environmental Technology Symposium*. 2009: Munich.

[105] W. Guo, H.-H. Ngo, and J. Li, *A mini-review on membrane fouling,* Bioresource Technology, 122 (2012), pp. 27-34.
[106] S. Finnigan and J. Howell, *The effect of pulsed flow on ultrafiltration fluxes in a baffled tubular membrane system.,* Desalination, 79 (1990), pp. 181-202.
[107] S.G. Redkar and R.H. Davis, *Cross-flow microfiltration with high-frequency reverse filtration,* AIChE Journal, 41 (1995), pp. 501-508.
[108] T. Kobayashi, X. Chai, and N. Fujii, *Ultrasound enhanced cross-flow membrane filtration,* Separation and Purification Technology, 17 (1999), pp. 31-40.
[109] M. Mercier, C. Maranges, C. Fonade, and C. Lafforgue-Delorme, *Yeast suspension filtration: Flux enhancement using an upward gas/liquid slug flow—application to continuous alcoholic fermentation with cell recycle,* Biotechnology and Bioengineering, 58 (1998), pp. 47-57.
[110] C. Lafforgue, J. Malinowski, and G. Goma, *High yeast concentration in continuous fermentation with cell recycle obtained by tangential microfiltration,* Biotechnol. Lett., 9 (1987), pp. 347-352.
[111] H.N. Chang, W.G. Lee, and B.S. Kim, *Cell retention culture with an internal filter module: Continuous ethanol fermentation,* Biotechnol. Bioeng., 41 (1993), pp. 677-681.
[112] W. Lee, Y. Lee, H. Chang, and Y. Chang, *A cell retention internal filter reactor for ethanol production using tapioca hydrolysates,* Biotechnology Techniques, 8 (1994), pp. 817-820.
[113] W. Doyen, W. Mues, B. Molenberghs, and B. Cobben, *Spacer fabric supported flat-sheet membranes: A new era of flat-sheet membrane technology,* Desalination, 250 (2010), pp. 1078-1082.
[114] J.E. Hallsworth, *Ethanol-induced water stress in yeast,* Journal of Fermentation and Bioengineering, 85 (1998), pp. 125-137.
[115] M. Ricci, S. Martini, C. Bonechi, L. Trabalzini, A. Santucci, and C. Rossi, *Inhibition effects of ethanol on the kinetics of glucose metabolism by S. cerevisiae: NMR and modelling study,* Chemical Physics Letters, 387 (2004), pp. 377-382.
[116] D. Millar, K. Griffiths-Smith, E. Algar, and R. Scopes, *Activity and stability of glycolytic enzymes in the presence of ethanol,* Biotechnology Letters, 4 (1982), pp. 601-606.
[117] B.L. Maiorella, H.W. Blanch, and C.R. Wilke, *Feed component inhibition in ethanolic fermentation by Saccharomyces cerevisiae,* Biotechnology and Bioengineering, 26 (1984), pp. 1155-1166.
[118] H. Carlsen and D. H, *Effects of alcohols on the respiration and fermentation of aerated suspensions of baker´s yeast,* Journal of General Microbiology, 137 (1991), pp. 2879-2883.
[119] R. Koppram, F. Nielsen, E. Albers, A. Lambert, S. Wännström, L. Welin, G. Zacchi, and L. Olsson, *Simultaneous saccharification and co-fermentation for bioethanol production using corncobs at lab, PDU and demo scales,* Biotechnology for Biofuels, 6 (2013), pp. 1-10.
[120] M.J. Taherzadeh and K. Karimi, *Fermentation Inhibitors in ethanol processes and different strategies to reduce their effects. In: Biofuels, Alternative Feedstocks and Conversion Processes*. 2011: Elsevier Science & Technology.
[121] E. Nevoigt and U. Stahl, *Osmoregulation and glycerol metabolism in the yeast Saccharomyces cerevisiae,* FEMS Microbiology Reviews, 21 (1997), pp. 231-241.
[122] J. Albertyn, S. Hohmann, J.M. Thevelein, and B.A. Prior, *GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway,* Molecular and Cell Biology, 14 (1994), pp. 4135-4144.
[123] C.-S. Shin, D. Damiano, N.-h. Ju, N.-K. Kim, and S.S. Wang, *The effects of nonvolatile toxic substances on the yeast growth during ethyl alcohol fermentations,* Biotechnology Letters, 5 (1983), pp. 831-836.
[124] M. Mota, C. Lafforgue, P. Strehaiano, and G. Goma, *Fermentation coupled with microfiltration: kinetics of ethanol fermentation with cell recycle,* Bioprocess Engineering, 2 (1987), pp. 65-68.
[125] C.W. Lee and H.N. Chang, *Kinetics of ethanol fermentations in membrane cell recycle fermentors,* Biotechnology and Bioengineering, 29 (1987), pp. 1105-1112.
[126] D.S. Inloes, D.P. Taylor, S.N. Cohen, A.S. Michaels, and C.R. Robertson, *Ethanol Production by Saccharomyces cerevisiae Immobilized in Hollow-Fiber Membrane Bioreactors,* Applied Environmental Microbiology, 46 (1983), pp. 264-278.
[127] E. Palmqvist and B. Hahn-Hägerdal, *Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition,* Bioresour. Technol., 74 (2000), pp. 25-33.
[128] C. Luo, D.L. Brink, and H.W. Blanch, *Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol,* Biomass and Bioenergy, 22 (2002), pp. 125-138.
[129] N. Beales, *Adaptation of Microorganisms to Cold Temperatures, Weak Acid Preservatives, Low pH, and Osmotic Stress: A Review,* Comprehensive Reviews in Food Science and Food Safety, 3 (2004), pp. 1-20.
[130] M.J. Taherzadeh, C. Niklasson, and G. Lidén, *Acetic acid—friend or foe in anaerobic batch conversion of glucose to ethanol by Saccharomyces cerevisiae?,* Chem. Eng. Sci., 52 (1997), pp. 2653-2659.

[131] R.J. Ulbricht, S.J. Northup, and J.A. Thomas, *A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions,* Fundamental and Applied Toxicology, 4 (1984), pp. 843-853.
[132] R.L. Fireoved and R. Mutharasan, *Effect of Furfural and Ethanol on the Growth and Energetics of Yeast under Microaerobic Conditions,* Annals of the New York Academy of Sciences, 469 (1986), pp. 433-446.
[133] G.N. Brovenko and T.V. Gusel'nikova, *Chemical Composition of Wood Hydrolyzates as a Substrate for Microbial Protein Synthesis. 1. Furfural and Hydroxymethylfurfural,* Gidroliz. Lesokhim. Prom-st. , 1 (1993), pp. 6-10.
[134] T. Modig, G. Lidén, and M.J. Taherzadeh, *Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase,* Biochemical Journal, 363 (2002), pp. 769-776.
[135] I. Sárvári Horváth, C.J. Franzen, M.J. Taherzadeh, C. Niklasson, and G. Liden, *Effects of furfural on the respiratory metabolism of Saccharomyces cerevisiae in glucose-limited chemostats,* Applied Environmental Microbiology, 69 (2003), pp. 4076-4086.
[136] D. Heer and U. Sauer, *Identification of furfural as the key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain,* Microbial Biotechnology, 1 (2008), pp. 497-506.
[137] Z.L. Liu, 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 and Biotechnology, 31 (2004), pp. 345-352.
[138] B. Sanchez and J. Bautista, *Effects of furfural and 5-hydroxymethylfurfural on the fermentation of Saccharomyces cerevisiae and biomass production from Candida guilliermondii,* Enzyme and Microbial Technology, 10 (1988), pp. 315-318.
[139] G.T. Tsao, A.P. Brainard, H.R. Bungay, N.J. Cao, P. Cen, Z. Chen, J. Du, B. Foody, C.S. Gong, P. Hall, N.W.Y. Ho, D.C. Irwin, P. Iyer, T.W. Jeffries, C.M. Ladisch, M.R. Ladisch, Y.Y. Lee, N.S. Mosier, H.M. Mühlemann, M. Sedlak, N.Q. Shi, J.S. Tolan, R.W. Torget, D.B. Wilson, and L. Xia, *Ethanol Production from Renewable Resources*, in *Recent Progress in Bioconversion of Lignocellulosics*. 1999, Springer Berlin Heidelberg.
[140] S. Larsson, A. Quintana-Sainz, A. Reimann, N. Nilvebrant, and L. Jönsson, *Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae,* Applied Biochemistry and Biotechnology, (2000), pp. 617-632.
[141] A.R. Goodey and R.S. Tubb, *Genetic and Biochemical Analysis of the Ability of Saccharomyces cerevisiae to Decarboxylate Cinnamic Acids,* Journal of General Microbiology, 128 (1982), pp. 2615-2620.
[142] M. Clausen, C.J. Lamb, R. Megnet, and P.W. Doerner, *PAD1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in Saccharomyces cerevisiae,* Gene, 142 (1994), pp. 107-112.
[143] M. García-Aparicio, I. Ballesteros, A. González, J. Oliva, M. Ballesteros, and M. Negro, *Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis,* Applied Biochemistry and Biotechnology, 129 (2006), pp. 278-288.
[144] J. Pérez, J. Muñoz-Dorado, T. de la Rubia, and J. Martínez, *Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview,* International Microbiology, 5 (2002), pp. 53-63.
[145] J.P. Delgenes, 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, 19 (1996), pp. 220-225.
[146] N. Nishikawa, R. Sutcliffe, and J. Saddler, *The influence of lignin degradation products on xylose fermentation by Klebsiella pneumoniae,* Applied Microbiology and Biotechnology, 27 (1988), pp. 549-552.
[147] C. Martín and L.J. Jönsson, *Comparison of the resistance of industrial and laboratory strains of Saccharomyces and Zygosaccharomyces to lignocellulose-derived fermentation inhibitors,* Enzyme and Microbial Technology, 32 (2003), pp. 386-395.
[148] M. Desimone, J. Degrossi, M. D'Aquino, and L. Diaz, *Ethanol tolerance in free and sol-gel immobilised Saccharomyces cerevisiae,* Biotechnology Letters, 24 (2002), pp. 1557-1559.
[149] C. Martín, M. Marcet, O. Almazán, and L.J. Jönsson, *Adaptation of a recombinant xylose-utilizing Saccharomyces cerevisiae strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors,* Bioresource Technology, 98 (2007), pp. 1767-1773.
[150] F. Keller, D. Bates, R. Ruiz, and Q. Nguyen, *Yeast adaptation on softwood prehydrolysate,* Applied Biochemistry and Biotechnology, 70-72 (1998), pp. 137-148.
[151] M. Alkasrawi, A. Rudolf, G. Lidén, and G. Zacchi, *Influence of strain and cultivation procedure on the performance of simultaneous saccharification and fermentation of steam pretreated spruce,* Enzyme and Microbial Technology, 38 (2006), pp. 279-286.
[152] R. Koppram, E. Albers, and L. Olsson, *Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass,* Biotechnology for Biofuels, 5 (2012), pp. 1-12.

[153] E. Tomás-Pejó, M. Ballesteros, J.M. Oliva, and L. Olsson, *Adaptation of the xylose fermenting yeast Saccharomyces cerevisiae F12 for improving ethanol production in different fed-batch SSF processes,* Journal of Industrial Microbiology & Biotechnology, 37 (2010), pp. 1211-1220.

[154] T. Brandberg, C.J. Franzén, and L. Gustafsson, *The fermentation performance of nine strains of Saccharomyces cerevisiae in batch and fed-batch cultures in dilute-acid wood hydrolysate,* Journal of Bioscience and Bioengineering, 98 (2004), pp. 122-125.

[155] T. Lindén, J. Peetre, and B. Hahn-Hägerdal, *Isolation and characterization of acetic acid-tolerant galactose-fermenting strains of Saccharomyces cerevisiae from a spent sulfite liquor fermentation plant,* Applied Environmental Microbiology, 58 (1992), pp. 1661-1669.

[156] A. Petersson, J.R.M. Almeida, T. Modig, K. Karhumaa, B. Hahn-Hägerdal, M.F. Gorwa-Grauslund, and G. Lidén, *A 5-hydroxymethyl furfural reducing enzyme encoded by the Saccharomyces cerevisiae ADH6 gene conveys HMF tolerance,* Yeast, 23 (2006), pp. 455-464.

[157] M. Ask, V. Mapelli, H. Hock, L. Olsson, and M. Bettiga, *Engineering glutathione biosynthesis of Saccharomyces cerevisiae increases robustness to inhibitors in pretreated lignocellulosic materials,* Microbial Cell Factories, 12 (2013), pp. 87.

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

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

[160] M.V. Sivers, G. Zacchi, L. Olsson, and B. Hahn-Hügerdal, *Cost Analysis of Ethanol Production from Willow Using Recombinant Escherichia coli,* Biotechnology Progress, 10 (1994), pp. 555-560.

[161] P. Persson, J. Andersson, L. Gorton, S. Larsson, N. Nilvebrant, and L.J. Jönsson, *Effect of Different Forms of Alkali Treatment on Specific Fermentation Inhibitors and on the Fermentability of Lignocellulose Hydrolysates for Production of Fuel Ethanol,* Journal of Agricultural and Food Chemistry, 50 (2002), pp. 5318-5325.

[162] I.S. Chung and Y.Y. Lee, *Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula,* Biotechnol. Bioeng., 27 (1985), pp. 308-315.

[163] R. Purwadi, T. Brandberg, and M. Taherzadeh, *A Possible Industrial Solution to Ferment Lignocellulosic Hydrolyzate to Ethanol: Continuous Cultivation with Flocculating Yeast,* Int. J. Mol. Sci., 8 (2007), pp. 920-932.

[164] V.V. Samonin and E.E. Elikova, *A study of the adsorption of bacterial cells on porous materials,* Microbiology, 73 (2004), pp. 696-701.

[165] V.A. Abelyan, *A new method for immobilization of microbial cells by cross-linking,* Applied Biochemistry and Microbiology, 36 (2000), pp. 310-314.

[166] T. Brandberg, K. Karimi, M.J. Taherzadeh, C.J. Franzén, and L. Gustafsson, *Continuous fermentation of wheat-supplemented lignocellulose hydrolysate with different types of cell retention,* Biotechnology and Bioengineering, 98 (2007), pp. 80-90.

[167] J. Westman, O. , M. Taherzadeh, J., and C.J. Franzén, *Inhibitor tolerance and flocculation of a yeast strain suitable for second generation bioethanol production,* Electronic Journal of Biotechnology, 15 (2012), pp. 1-14.

[168] H. Arthur and K. Watson, *Thermal adaptation in yeast: growth temperatures, membrane lipid, and cytochrome composition of psychrophilic, mesophilic, and thermophilic yeasts* Journal of Bacteriology, 128 (1976), pp. 56-68.

[169] I.M. Banat, P. Nigam, D. Singh, R. Marchant, and A.P. McHale, *Review: Ethanol production at elevated temperatures and alcohol concentrations: Part I – Yeasts in general,* World Journal of Microbiology and Biotechnology, 14 (1998), pp. 809-821.

[170] M.A. Singer and S. Lindquist, *Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose,* Trends in Biotechnology, 16 (1998), pp. 460-468.

[171] T. D'Amore, G. Celotto, I. Russell, and G.G. Stewart, *Selection and optimization of yeast suitable for ethanol production at 40°C,* Enzyme and Microbial Technology, 11 (1989), pp. 411-416.

[172] C. Shimoda, A. Itadani, A. Sugino, and M. Furusawa, *Isolation of thermotolerant mutants by using proofreading-deficient DNA polymerase delta as an effective mutator in Saccharomyces cerevisiae,* Genes & Genetic Systems, 81 (2006), pp. 391-397.

[173] M.I. Rajoka, M. Ferhan, and A.M. Khalid, *Kinetics and thermodynamics of ethanol production by a thermotolerant mutant of Saccharomyces cerevisiae in a microprocessor-controlled bioreactor,* Letters in Applied Microbiology, 40 (2005), pp. 316-321.

[174] J. Verghese, J. Abrams, Y. Wang, and K.A. Morano, *Biology of the Heat Shock Response and Protein Chaperones: Budding Yeast (Saccharomyces cerevisiae) as a Model System,* Microbiology and Molecular Biology Reviews, 76 (2012), pp. 115-158.

[175] I.M. Banat, P. Nigam, and R. Marchant, *Isolation of thermotolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C,* World Journal of Microbiology and Biotechnology, 8 (1992), pp. 259-263.
[176] F. Talebnia and M.J. Taherzadeh, *Physiological and morphological study of encapsulated Saccharomyces cerevisiae,* Enzyme and Microbial Technology, 41 (2007), pp. 683-688.
[177] J.O. Westman, M.J. Taherzadeh, and C.J. Franzén, *Proteomic Analysis of the Increased Stress Tolerance of Saccharomyces cerevisiae Encapsulated in Liquid Core Alginate-Chitosan Capsules,* PLoS One, 7 (2012), pp. e49335.
[178] L. Lindberg, A.X.S. Santos, H. Riezman, L. Olsson, and M. Bettiga, *Lipidomic Profiling of Saccharomyces cerevisiae and Zygosaccharomyces bailii Reveals Critical Changes in Lipid Composition in Response to Acetic Acid Stress,* PLoS One, 8 (2013), pp. 1-12.