

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Ethanol production from lignocellulose using high local cell density yeast cultures

Investigations of flocculating and encapsulated *Saccharomyces cerevisiae*

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Department of Chemical and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2014



UNIVERSITY OF BORÅS

SCHOOL OF ENGINEERING

Borås, Sweden 2014

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ISBN 978-91-7385-950-9

Doktorsavhandlingar vid Chalmers tekniska högskola

Ny serie nr 3632

ISSN 0346-718X

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Skrifter från Högskolan i Borås, nr. 46

ISSN 0280-381X

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Cover illustration: Cross-section of an alginate-chitosan capsule filled with yeast cells.

Photographs by Johan O Westman

Printed by Chalmers Reproservice

Gothenburg, Sweden 2014

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ABSTRACT

Efforts are made to change from 1st to 2nd generation bioethanol production, using lignocellulosics as raw materials rather than using raw materials that alternatively can be used as food sources. An issue with lignocellulosics is that a harsh pretreatment step is required in the process of converting them into fermentable sugars. In this step, inhibitory compounds such as furan aldehydes and carboxylic acids are formed, leading to suboptimal fermentation rates. Another issue is that lignocellulosics may contain a large portion of pentoses, which cannot be fermented simultaneously with glucose by *Saccharomyces cerevisiae*. In this thesis, high local cell density has been investigated as a means of overcoming these two issues.

Encapsulation of yeast in semi-permeable alginate-chitosan capsules increased the tolerance towards furan aldehydes, but not towards carboxylic acids. The selective tolerance can be explained by differences in the concentration of compounds radially through the cell pellet inside the capsule. For inhibitors, gradients will only be formed if the compounds are readily convertible, like the furan aldehydes. Conversion of inhibitors by cells close to the membrane leads to decreased concentrations radially through the cell pellet. Thus, cells closer to the core experience subinhibitory levels of inhibitors and can ferment sugars. Carbohydrate gradients also give rise to nutrient limitations, which in turn trigger a stress response in the yeast, as was observed on mRNA and protein level. The stress response is believed to increase the robustness of the yeast and lead to improved tolerance towards additional stress.

Glucose and xylose co-consumption by a recombinant strain, CEN.PK XXX, was also improved by encapsulation. Differences in affinity of the sugar transporters normally result in that glucose is taken up preferentially to xylose. However, when encapsulated, cells in different parts of the capsule experienced high and low glucose concentrations simultaneously. Xylose and glucose could thus be taken up concurrently. This improved the co-utilisation of the sugars by the system and led to 50% higher xylose consumption and 15% higher final ethanol titres.

A protective effect by the capsule membrane itself could not be shown. Hence, the interest in flocculation was triggered, as a more convenient way to keep the cells together. To investigate whether flocculation increases the tolerance, like encapsulation, recombinant flocculating yeast strains were constructed and compared with the non-flocculating parental strain. Experiments showed that strong flocculation did not increase the tolerance towards carboxylic acids. However, the tolerance towards a spruce hydrolysate and especially against furfural was indeed increased. The results of this thesis show that high local cell density yeast cultures have the potential to aid against two of the major problems for 2nd generation bioethanol production: inhibitors and simultaneous hexose and pentose utilisation.

Keywords: Yeast, encapsulation, lignocellulose, ethanol, fermentation, flocculation, inhibitors, tolerance, xylose, co-utilisation

LIST OF PUBLICATIONS

The thesis is mainly based on results presented in the following articles:

- I. **Westman, Johan O**; Taherzadeh, Mohammad J; Franzén, Carl Johan, (2012), Inhibitor tolerance and flocculation of a yeast strain suitable for second generation bioethanol production. **Electronic Journal of Biotechnology, 15(3).**
- II. **Westman, Johan O**; Manikondur, Ramesh Babu; Franzén, Carl Johan; Taherzadeh, Mohammad J, (2012), Encapsulation-induced stress helps *Saccharomyces cerevisiae* resist convertible lignocellulose derived inhibitors, **International Journal of Molecular Science, 13, 11881-11894.**
- III. **Westman, Johan O**; Taherzadeh, Mohammad J; Franzén, Carl Johan, (2012), Proteomic analysis of the increased stress tolerance of *Saccharomyces cerevisiae* encapsulated in liquid core alginate-chitosan capsules, **PLOS ONE, 7(11): e49335. doi:10.1371/journal.pone.0049335**
- IV. **Westman, Johan O**; Coelho, Aires; Da Costa Pereira, Joana Paula; Carvalho Esteves, Gonçalo; Bonander, Nicklas; Taherzadeh, Mohammad J; Franzén, Carl Johan, Improved sugar co-utilisation by encapsulation of a recombinant *Saccharomyces cerevisiae* strain in alginate-chitosan capsules. **Submitted**
- V. **Westman, Johan O**; Mapelli, Valeria; Taherzadeh, Mohammad J; Franzén Carl Johan, Flocculation causes inhibitor tolerance in *Saccharomyces cerevisiae* during 2nd generation bioethanol production. **Submitted**

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

- VI. **Westman, Johan O**; Ylitervo, Päivi; Franzén, Carl Johan.; Taherzadeh, Mohammad J, (2013), Effects of encapsulation of microorganisms on product formation during microbial fermentations. **Applied Microbiology and Biotechnology, 96, 1441-1454.**
- VII. Nyman, Jonas; Lacintra, Michael; **Westman, Johan O**; Berglin, Mattias; Lundin, Magnus; Lennartsson, Patrik; Taherzadeh, Mohammad J, (2013), Pellet formation of zygomycetes and immobilization of yeast. **New Biotechnology, 30, 516-522.**

STATEMENT OF CONTRIBUTION

My contributions to the papers included in this thesis are as follows:

Paper I: Responsible for most of the idea and design of the experimental work. Performed all experimental work and wrote the manuscript.

Paper II: Responsible for most of the idea, the design of the experimental work and all experimental work, except for the fermentations. Responsible for supervision of the remaining experimental work and wrote the manuscript.

Paper III: Responsible for part of the idea and all cultivations and samplings. Responsible for a major part of the analysis and the interpretation of the proteome data and wrote the manuscript.

Paper IV: Responsible for part of the idea, all cultivations and the writing of the corresponding parts of the manuscript.

Paper V: Responsible for most of the idea and design of the experiments. Performed all experimental work and wrote the manuscript.

'But then science is nothing but a series of questions that lead to more questions'.

– Terry Pratchett

PREFACE

This PhD dissertation serves as a partial fulfilment of the requirements for a PhD degree at the Department of Chemical and Biological Engineering, Chalmers University of Technology, Sweden. The PhD project was initiated in April 2009. The research was carried out under the supervision of Associate Professor Carl Johan Franzén, Chalmers University of Technology, and Professor Mohammad Taherzadeh, University of Borås.

The main part of the work performed concerns the impact of high local cell density growth of *Saccharomyces cerevisiae*, in the form of encapsulated and flocculating cells, on second generation bioethanol production.

My PhD project was funded by the Swedish Energy Agency, the Swedish Research Council and the University of Borås.

Johan Westman

January 2014

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CHAPTER 1

INTRODUCTION

Although unclear and debated as to when, it is an undisputed fact that fossil fuel reserves will be depleted. This, as well as an increased environmental awareness, has made it necessary to come up with alternatives that are renewable and environmentally neutral or even beneficial. For electricity generation, solar-, wind- or hydro-power is gaining a lot of attention. However, for vehicles it will probably be necessary to, at least in part, use a liquid fuel. Of the various liquid renewable fuels available, the 1st generation bioethanol from sugary or starchy raw materials is presently the most widely used [1]. There are also thousands of years of accumulated knowledge of the process, with traditional wine and beer production from sucrose and starch containing raw materials like fruit and grain.

Nonetheless, the utilisation of sucrose- and starch-containing raw materials, mainly sugar cane in Brazil and corn in the USA, for fuel ethanol production has some drawbacks [2]. The main drawback is that the raw materials in the process could instead be utilised as food or animal feed. A less controversial choice of raw materials is the so called 2nd generation lignocellulosic materials that build up e.g. trees and corn cobs. These materials are for example abundant in wastes from forestry and agricultural industries, and its utilisation would be significantly less controversial [3].

However, due to the inherent recalcitrance of lignocellulosic materials, it is not as easy to produce the 2nd generation bioethanol in comparison to the 1st [4]. The production process of 2nd generation bioethanol contains a number of necessary steps (Figure 1), including a rather harsh initial pretreatment of the material to make the cellulose accessible to the subsequent hydrolysis step [3]. This adds complexity and cost to the process and, not least, inhibitors of the subsequent fermentation are easily formed during this pretreatment [5]. The fermentation of lignocellulosic hydrolysates is therefore not as straightforward as that of starch or sugar.

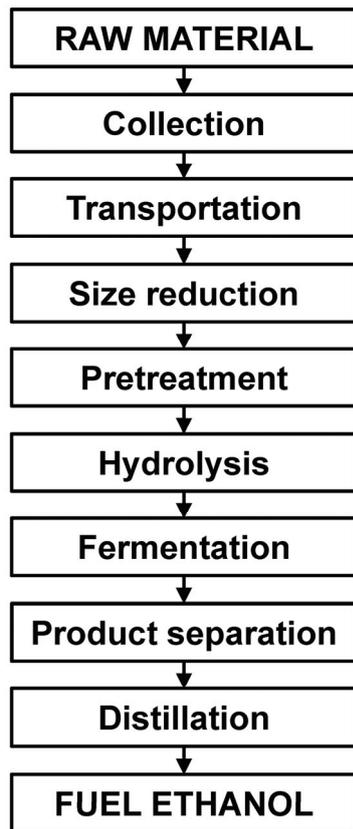


Figure 1 Subprocesses in bioethanol production. A number of subprocesses are necessary in the production of ethanol from lignocellulosic raw materials. All of the steps need to be optimised for economical feasibility. The raw materials have to be produced close to the ethanol plant to minimise the cost of collection and transportation. To minimise the energy utilisation, the size reduction should be kept at the minimum level required for an efficient pretreatment and hydrolysis. The harshness of the pretreatment should be low in order to not produce too much fermentation inhibitors. The hydrolysis has to reach a high yield so that all of the raw material can be utilised for ethanol production. In the fermentation step, all carbohydrates, hexoses and pentoses, have to be fermented into ethanol. The separation of the ethanol from the cells is an energy demanding process. By retention of the cells in the reactor, this step can be simplified. Since the distillation is also energy demanding, the ethanol concentration in the fermentation broth should be at a concentration of at least 4–5% (w/w) for an economically viable process [6].

Another issue with 2nd generation bioethanol production is that the lignocellulosic hydrolysates contain significant amounts of both hexoses and pentoses, while in the 1st generation materials there are mainly hexoses. Unfortunately, the microorganism commonly used in bioethanol production, *Saccharomyces cerevisiae*, is unable to utilise pentoses. As a result, a large portion of the carbohydrates in lignocelluloses cannot be used to produce ethanol by the same yeast, as is generally used in the 1st generation production. To overcome this problem the yeast has to be genetically modified to express the enzymes required for conversion of pentoses into ethanol [7]. However, the yeast still has a strong preference for hexoses, which is why simultaneous utilisation of the carbohydrates is not efficient. A thorough background, describing the challenges in the transition from the 1st generation bioethanol production of today to 2nd generation bioethanol production, is given in **Chapter 2** of this thesis. Furthermore, the fermentation by *S. cerevisiae* and physiological aspects of special importance for bioethanol production from lignocellulose is described in **Chapter 3**.

It has previously been shown that yeast encapsulated in semi-permeable gel capsules could tolerate hydrolysates that were too inhibitory for freely suspended yeast to ferment [8]. (The process of yeast encapsulation is described in **Chapter 4**.) It has also been

observed that flocculating strains often are tolerant to inhibitory hydrolysates. Flocculation is a natural way for the yeast cells to attach to each other. The basis of yeast flocculation and construction of recombinant flocculating strains is presented in **Chapter 5**. However, the reason why these ‘high local cell density’ cultures are more tolerant towards toxic hydrolysates was not known when this thesis project was initiated.

At the beginning of the project, there were three hypotheses as to why encapsulated yeast tolerated inhibitory hydrolysates better than freely suspended cells.

- First, diffusion limitations through the membrane or cell pellet were hypothesised to decrease the concentration of inhibitors locally. This hypothesis was addressed in **Papers II and IV** and further elaborated on in **Paper V**.
- Secondly, it was hypothesised that stress responses might be triggered in the yeast by the encapsulation. This hypothesis was addressed in **Papers II–III**.
- Lastly, it was hypothesised that by using a data-driven approach based on genome wide data collection, further physiological effects triggered by encapsulation could be identified. This hypothesis was addressed in **Paper III**.

Having these hypotheses as the starting point, the main goal of my thesis project was to investigate the physiological changes that occur in the yeast cells upon encapsulation and the reasons for its increased inhibitor tolerance. The results from these investigations are presented in **Papers II–III**.

A new hypothesis that arose from these subprojects was that encapsulation of a pentose utilising yeast strain would promote simultaneous co-utilisation of hexoses and pentoses, which led to the investigations presented in **Paper III**.

The first task during my PhD project also involved investigations of an interesting, inhibitor tolerant, flocculating strain. These investigations are presented in **Paper I**. The results from that subproject and the results from the investigations of the encapsulated yeast led to investigations of whether flocculation in itself could increase the inhibitor tolerance of the yeast. The results of this subproject are presented in **Paper V**. The results of the research performed as part of this thesis work are presented in **Chapter 6** and conclusions are presented in **Chapter 7**. Future directions for research in the area are presented in **Chapter 8**.

CHAPTER 2

BIOETHANOL FROM LIGNOCELLULOSE

Although the main focus of the work performed in this thesis has been on fermentation, a thorough background overview is necessary in order to understand the difficulties regarding 2nd generation bioethanol production. Therefore lignocellulosic materials as well as their route towards ethanol are described in this and the following chapter.

2.1 Biofuels and the bioethanol market

There are a number of different biofuels on the market and under development, e.g. biogas, biodiesel, biohydrogen and biobutanol [1]. However, the predominant one is without doubt ethanol. Ethanol, or ethyl alcohol, has a long tradition as a vehicle fuel and was, for example, one of the options in Henry Ford's Model T cars [9]. Henry Ford was also slightly before his time when he expressed his thought in 1925 that ethanol was 'the fuel of the future' [10]. As a blend with gasoline, ethanol can be used efficiently in existing gasoline engines and it can even boost the octane rating. In this way, the fuel usage with blended ethanol is more efficient than without [9].

Countries in different parts of the world have set high goals for utilisation of biofuels. For example, the European Union have set a goal that by 2020, 10% of the fuel used for transportation should be biofuels. The US Department of Energy Office also have a scenario that by 2030, 30% of the gasoline demand should be replaced by biofuels [11]. The two main producers of bioethanol are the United States and Brazil. During 2012, these two countries produced 87% of the fuel ethanol in the world with 50.3 and 21.1 billion litres, respectively [12]. However, the raw materials used for production of this ethanol are mainly corn and sugar cane in the two countries, respectively. The utilisation of biofuels in the US is categorised into four classes: total renewable fuel, advanced biofuel, cellulosic biofuel and biomass-based diesel. All classes have been able to reach its mandate in part or fully, except the cellulosic biofuels [13]. It is therefore clear that more research is necessary in order to increase the economic feasibility of lignocellulosic biofuels production.

2.2 Production of bioethanol

There are a number of different microorganisms able to produce ethanol as a by-product when they utilise sugars for growth [14]. Examples can be found among both bacteria and fungi, like *Escherichia coli*, *Zymomonas mobilis*, *Clostridium thermocellum* and *Pichia stipitis*, among others. However, there are issues with these microorganisms, such as low ethanol productivity or low ethanol tolerance. These problems are less pronounced with the yeast *S. cerevisiae*, which is why it is often utilised for ethanol production.

S. cerevisiae is preferred due to its long history of utilisation for both ethanol production and baking, and the fact that it has GRAS (Generally Recognised As Safe) status. It also possesses many of the traits important for production of 2nd generation bioethanol, such as high ethanol yields and productivity and relatively high inhibitor tolerance. However, it lacks the ability to utilise all sugars present in lignocellulosic hydrolysates, due to an, in the best case, extremely poor pentose utilisation. Its inhibitor tolerance can also be a limiting factor, as will be discussed in the following chapter. For production of 1st generation bioethanol, from sugar and starch-rich materials, these shortcomings are not major drawbacks. In sugar and starch-rich materials the carbohydrates are mainly readily fermentable hexoses. They are also extracted from the raw materials without the need for harsh pretreatment. Therefore, significant amounts of inhibitors are usually not formed [1]. The case is completely different when it comes to 2nd generation bioethanol, which explains why its breakthrough as a serious competitor on the liquid biofuel market has still not come.

2.3 Lignocellulose as raw material – 2nd generation bioethanol

Production of ethanol from sugars by fermentation is a process that has been utilised by humans for at least 6,000 years [15]. However, the production from grain and fruit has mainly been for the purpose of beverage production: consequently, the utilisation of these raw materials for fuel production is rather controversial [2]. For this and other reasons, such as land use issues [16], the production of fuel ethanol from lignocellulosic materials is an interesting option. The raw materials available for 2nd generation biofuel production are abundant. The annual production is estimated to be $1.8 \cdot 10^{11}$ tonnes

lignocellulosic biomass per year, of which 10% is considered as potentially accessible [17]. However, since utilisation of all available biomass for ethanol production is not a likely scenario, liquid biofuels will most probably not be the sole replacement of fossil fuels. Other alternatives such as biomethane, hydrogen and electricity will surely also be needed [18]. Furthermore, to obtain profitability in the production of 2nd generation biofuels, a biorefinery is likely necessary. In a biorefinery, value added products are produced in addition to the low cost fuel [19]. However, there are still drawbacks that need to be overcome for 2nd generation bioethanol production, mainly related to the highly recalcitrant structure of the raw material [1,4,11,20].

Lignocellulosic materials are mainly composed of cellulose, hemicellulose and lignin (Figure 2), in varying proportions depending on the source.

The major constituent of lignocellulose is cellulose, the most abundant biopolymer on earth. Cellulose is a polymer of cellobiose units, which in turn is a dimer of glucose [21]. In wood, it has approximately 10,000 glucose units per chain [22]. Multiple cellulose chains are hydrogen bonded together to create a robust structure. Due to the many hydrogen bonds, the cellulose fibres are said to be highly crystalline. This high crystallinity makes cellulose fibres very difficult to degrade, since accessibility by the enzymes is restricted, thus limiting their ability to hydrolyse the bonds [4].

The second polysaccharide present in lignocellulosic materials, hemicellulose, is not a homopolymer like cellulose. It is instead made up of a heterogeneous group of polysaccharides [23]. Hemicellulose contains both hexoses and pentoses and its structure is considerably more irregular than cellulose. The composition of hemicellulose also differs considerably depending on the source [23]. As examples, a hemicellulose hydrolysate from birch contains a significant portion of xylose, and one from spruce contains a high proportion of mannose [24]. The irregular structure of hemicellulose, with short and branched polymers, makes it more amorphous and in that aspect easier to degrade than cellulose [23,25]. However, the presence of significantly more types of bonds in its structure necessitates a wider variety of degrading enzymes when compared to cellulose. To facilitate an economically feasible production of 2nd generation bioethanol, it is important that all sugars in the raw material can be used, including the different pentoses present in the hemicellulose fraction [26].

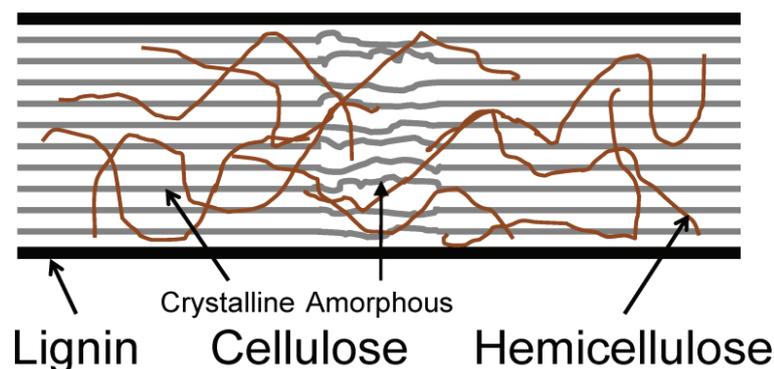


Figure 2 Schematic structure of lignocellulose. Three major constituents build up lignocellulose: cellulose, hemicellulose and lignin. The cellulose is surrounded by hemicellulose and lignin, forming a highly recalcitrant structure. Figure adapted from [27].

Lignin, the last of the major polymeric constituents of lignocellulose, is made up of non-carbohydrate monomers that cannot be used for fermentative production of ethanol. Hence, lignin is often simply burnt as a fuel for production of heat or electricity that can be utilised for operation of the ethanol production plant [28]. Lignin polymers are mainly made up of three building blocks: syringyl (S), *p*-hydroxyphenyl (H) and guaiacyl (G) [29]. The ratio of these building blocks vary among different plant species, with softwood containing mostly G units and hardwood G and S units [5]. Incorporated lignin in the cell walls, intertwined with the cellulose and hemicellulose, is what gives the main structural stability of large plants such as trees [25].

2.4 Pretreatment of lignocellulosic material

The recalcitrant nature of lignocellulosic materials makes a pretreatment step necessary in order to open up the structure for subsequent hydrolysis of the carbohydrate polymers, as mentioned in many reviews, e.g. [3,27,30]. The purpose of the pretreatment is mainly to delignify the biomass and decrease the cellulose crystallinity. This leads to an increased accessible surface area and increased porosity and thus enables easier enzyme access. The pretreatment step also releases sugar monomers, in particular, from the hemicellulose fraction [31]. Enzymatic hydrolysis of the cellulose in lignocellulosic materials is significantly less efficient without the pretreatment step. Generally, the hydrolysis yield is less than 20% of the theoretical without pretreatment, while more than 90% can be reached with pretreatment [32].

The pretreatment can be performed in numerous different ways, usually divided into physical, physico-chemical, chemical and biological methods [3]. Ideally, the pretreatment should be applicable to a range of raw materials with low energy demand at low capital and operating costs. Furthermore, the sugar recovery should be high with low or no degradation into inhibitory substances [30]. However, the most common practice is that different pretreatment methods are combined in a cost effective way. The methods of choice are also highly dependent on the specific raw material [3,30]. As an example, a common treatment of spruce, *Picea abies*, can be mentioned. The raw material first undergoes a physical pretreatment, chipping or milling for size reduction. The chips are impregnated with SO₂ or H₂SO₄ and pretreated with steam for a few minutes at high pressure and temperature, followed by a rapid pressure release [33]. This degrades the hemicellulose and decreases the crystallinity of the cellulose. Thereafter, enzymatic hydrolysis of the cellulose can follow. Acid catalysed steam explosion was used to create the hydrolysates used in this thesis and is referred to when pretreated biomass or hydrolysates are discussed in the following chapters of this thesis. Steam explosion can also be performed without added catalyst. In this case, acids released or formed from the material at high temperatures will cause autohydrolysis. However, this process works significantly better for hardwood, with more highly acetylated hemicellulose, than for softwood [34].

2.5 Hydrolysis of cellulose

In the hydrolysis step, the cellulose is hydrolysed into fermentable glucose monomers. There are mainly two ways to hydrolyse cellulose: chemically by acid catalysis, including hydrolysis of the hemicellulose, and biologically through treatment with a mixture of hydrolysing enzymes.

2.5.1 Acid hydrolysis

Acid hydrolysis can be carried out with either concentrated or dilute acid, with distinct differences between the methods. The concentrated acid process operates at a low temperature of around 30–40°C, usually utilising H₂SO₄ because of its low cost, but HCl and HF have also been used [34,35]. The hydrolysis yields are good, with up to 90% of the theoretical hexoses and pentoses released. In addition to this, the degradation of

sugars into inhibitory compounds is low [36]. However, drawbacks of the method include the requirement of an efficient acid recovery process as well as the corrosiveness of the acid used [34,35]. It is, therefore, difficult to make the process economically feasible and there is nowadays little focus on this process.

The dilute acid process operates with significantly lower concentrations of acid, usually less than 2%. For this reason the hydrolysis has to be operated at a higher temperature (180–230°C) than the concentrated process in order to achieve high reaction rates [35]. The high temperature and low pH causes degradation of the sugar monomers into toxic compounds, as further discussed in the next chapter of this thesis. Accordingly, the process is often divided into two stages. First, the more easily hydrolysed hemicellulose is broken down and removed. Thereafter, the remaining solids are treated under harsher conditions [35]. The sugar yields from this process are around 50–60% and up to 90% of the theoretical, for the cellulosic part and the hemicellulosic part, respectively [34]. A mild version of the dilute acid hydrolysis can also be considered as a pretreatment step, since it opens up the material and reduces the crystallinity of the cellulose. It is thereafter followed by enzymatic hydrolysis of the remaining solids.

2.5.2 Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose is performed by a class of enzymes called cellulases. These enzymes are produced naturally by microorganisms that derive their energy from cellulose degradation in nature, such as fungi that grows on rotting wood or microorganisms in the rumen of cows, but also by various insects [37]. The most famous producer of cellulases is the fungus *Hypocrina jecorina*, formerly known as *Trichoderma reesei*. It was first isolated from the US army's cotton canvas tents on the Solomon Islands during the Second World War, after it was found to be the cause of severe deterioration of the cotton [38]. This fungus is used in commercial production of cellulases, with the ability to produce enzyme titres in excess of 100 g/l [39].

Three major classes of cellulases are known to be necessary for complete hydrolysis of cellulose into glucose monomers: endo-1,4- β -D-glucanases, exo-1,4- β -D-glucanases and 1,4- β -D-glucosidases [40]. However, another enzyme family, GH61, has recently been shown to be of importance for degradation of crystalline cellulose. Enzymes from this family catalyse oxidative cleavage of cellulose chains on the surface of the cellulose

fibre, including even their crystalline form [21]. Endo-glucanases cleave β -1,4-glycosidic bonds randomly inside the cellulose polymer. Thereby, two chains with twice as many ends are created. This is utilised by the exo-glucanases, or cellobiohydrolases as they are also called, which cleave off cellobiose units from both ends of cellulose fibres. The cellobiose units are in turn hydrolysed into two glucose molecules by the 1,4- β -glucosidases [40].

2.6 Process configurations for enzymatic hydrolysis

There are two main ways to perform the enzymatic hydrolysis of the cellulose for bioethanol production (Figure 3). Either it is done separately from the fermentation, called separate hydrolysis and fermentation – SHF, or in the same reactor, called simultaneous saccharification and fermentation – SSF. SSF is termed SSCF if co-fermentation of hexoses and pentoses is performed by a microorganism that ferments pentoses [6]. Which route is best is highly debated and probably dependent on the specific raw material, as well as the enzymes and microorganism used.

Separate hydrolysis and fermentation has certain advantages in that it enables optimisation of the process parameters in both reactors where the hydrolysis and fermentation take place [36]. This can speed up the process, but the high concentration of sugar reached in the hydrolysis reactor may lead to end-product inhibition of the enzymes [41]. Another advantage of the separated processes is easier reuse of the microorganisms used in the fermentation. Since they are only mixed with the liquid part of the hydrolysate, they can easily be separated from the product, as compared to the SSF process where the cells are mixed with the lignin residue [36].

In simultaneous saccharification and fermentation, the process parameters have to be a compromise between the optimal for the enzymes and the optimal for the fermenting microorganism. This usually means that the temperature is held at or below 37°C, when *S. cerevisiae* is used. This is slightly higher than what is preferred for optimal fermentative performance [42]. However, it is significantly lower than the optimal temperature for cellulases, typically 45–50°C [36]. Plenty of work has been and is being done to increase the thermotolerance of the fermenting microorganism and to find new strains with better heat tolerance [43-46]. Engineering of and search for new cellulases

has also been conducted [47]. A benefit of the SSF process is the need for a single reactor, which lowers the capital investment costs of a plant [6]. However, the greatest advantage of the process is that the product of the saccharification, the glucose, can be immediately consumed by the fermenting microorganism. This decreases the effect of product inhibition of the cellulases, increasing the hydrolysis rate [42].

A third way to perform the enzymatic hydrolysis is by so called consolidated bioprocessing, CBP, which is actually a form of SSF. In CBP, the microorganisms that convert the sugar monomers into ethanol also produce the enzymes necessary for hydrolysis of the cellulose [48]. There are two different options that can be chosen in CBP, the recombinant and the native. In the recombinant option, cellulases are expressed recombinantly in a good ethanol producer, such as *S. cerevisiae* [49]. In the native option, improvements in e.g. the ethanol tolerance of native cellulase producers such as *Clostridium thermocellum* have to be made [50]. The advantages of a successful CBP are obvious, since the cost of enzymes needed for cellulose hydrolysis constitutes a major portion of the cost of the final product in SHF and SSF processes [11]. However, the same challenges as for SSF often apply, with different temperature and other parameter optima for the cellulases and the fermenting microorganisms. Although there has been some progress in the field, successful fermentations of industrial substrates has not yet been demonstrated, as most research has been done on amorphous cellulose [51].

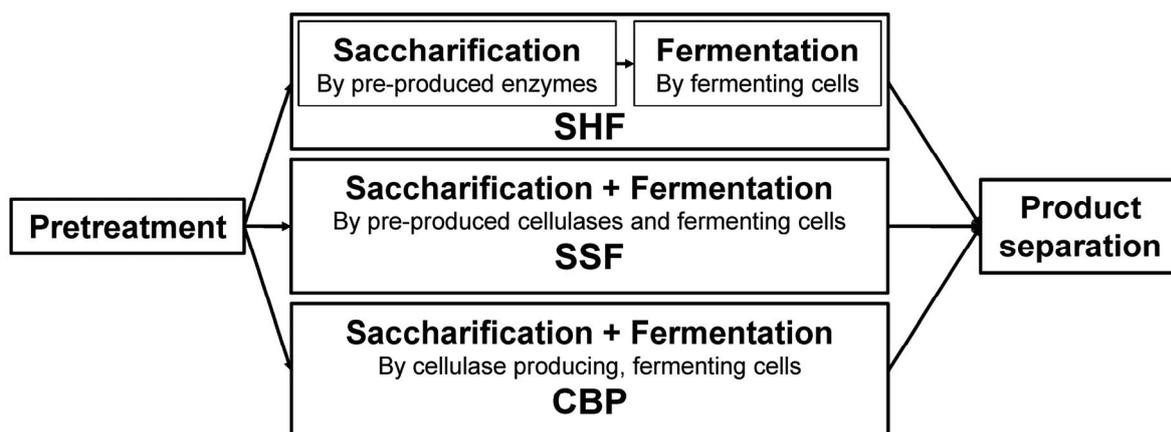


Figure 3 Process configurations in 2nd generation bioethanol production. Three different process configurations are commonly mentioned in bioethanol production. In SHF, the hydrolysis, by pre-produced enzymes and fermentation are done in two separate stages. In SSF, the two steps are performed in a single reactor. In CBP, the fermenting microorganisms also produce the enzymes for cellulose hydrolysis.

CHAPTER 3

YEAST IN 2ND GENERATION BIOETHANOL PRODUCTION

As mentioned in the previous chapter, ethanol is produced through fermentation of sugars by the yeast *S. cerevisiae*. A number of physiological traits are of fundamental importance to understand when it comes to the performance of microorganisms and the influence of the external factors prevailing in second generation ethanol production. Stress responses and resistance to various fermentation inhibitors are of major importance. In this chapter, the problems with fermentation inhibitors formed during the pretreatment step of lignocellulosic materials, as well as different stress responses, are presented. Methods used to study the physiology of the yeast cells are discussed and the fermentation process and different process modes are described.

3.1 Hexose utilisation

The most important part of the ethanol production process is the fermentation of sugars into ethanol. The yeast *S. cerevisiae* is a facultative anaerobe, which means that it can grow both in the absence and presence of oxygen. However, unsaturated fatty acids and ergosterol have to be supplied during anaerobic growth, as they cannot be produced without oxygen [52,53]. To sustain growth, the yeast has to produce ATP from breakdown of energy rich materials, with glucose as the preferred substrate (Figure 4). Under aerobic conditions, in the presence of O₂, the pyruvate and NADH produced in the glycolysis can be completely oxidised in the tricarboxylic acid (TCA) cycle and respiration chain into CO₂ and H₂O. Under fermentative, anaerobic, conditions on the other hand, the pyruvate is converted into ethanol via acetaldehyde. Ethanol production yields a net total of only two ATP per glucose molecule by substrate level phosphorylation in glycolysis. This is significantly lower than the ATP production per glucose in the respiratory route [54]. Ethanol production regenerates the NAD⁺ from the NADH and makes the route redox neutral, just like the respiratory route. Despite the fact that fermentation does not result in additional ATP production, it is still preferred over the respiratory route for *S. cerevisiae*. Even under fully aerobic conditions, the fermentative pathway is chosen when the yeast is transferred to a medium with excess

glucose [55]. This phenomenon of fermentation in the presence of oxygen is known as the Crabtree effect, discovered in cancer cells by Crabtree in the 1920s [56,57]. The short-term Crabtree effect, fermentation after transition to sugar excess in glucose-limited chemostats, is believed to be an effect of the limited rate of the respiratory pathway. This leads to ethanol formation due to overflow at the pyruvate branch point, as the rate through glycolysis is faster than the carbon flux that can be processed through respiration [58]. There is also a long-term Crabtree effect, appearing e.g. in a batch with glucose excess or a glucose-limited chemostat operated over a certain dilution rate [59]. This effect involves adaptation of the cellular metabolism, with e.g. glucose repression of genes involved in respiration [60]. The need for a high rate of energy generation rather than full energy utilisation is tentatively an evolutionary adaptation. As a result of its fast glucose consumption, *S. cerevisiae* could outcompete other yeasts by 'eating the food first'. The production of significant amounts of ethanol, which it tolerates better than many other yeasts and bacteria, can subsequently also be utilised as food [61]. However, the yeast cells do not simply want to produce energy and ethanol, they mainly want to produce biomass – more yeast cells. The biomass formation does not result in complete regeneration of NAD⁺. Thus, there will be a surplus of NADH that has to be oxidised to maintain an internal redox balance. The cells therefore produce glycerol, which is why glycerol and biomass formation are naturally linked.

What is described above is valid not only for glucose, but also for other hexoses present in plant material. Mannose, which is a C-2 epimer of glucose, can also be easily utilised by *S. cerevisiae*. The utilisation is performed simultaneously with glucose and it is taken up by the same family of transporters, the Hxt family. However, the mannose uptake has a slightly lower affinity [62]. Before mannose can enter the glycolysis pathway, it has to be converted into mannose-6-phosphate and subsequently isomerised into fructose-6-phosphate. Thereafter, the same route as for glucose is followed [63]. Another hexose commonly present in lignocellulosic hydrolysates, galactose, is a C-4 epimer of glucose. However it is not as easily utilised as glucose and mannose. Galactose enters the glycolysis pathway as glucose-6-phosphate via the action of the enzymes in the Leloir pathway: galactokinase, galactose-1-P uridylyltransferase and UDP-galactose 4-epimerase [64]. The expression of these enzymes is repressed by high levels of glucose, which leads to a sequential utilisation of the two hexoses. Galactose is thus not utilised until glucose is almost depleted and the pathway has been induced by the presence of

galactose [65]. Galactose is taken up by the galactose permease Gal2p [66]. Gal2p can also transport other hexoses, while the Hxt family transporters do not transport galactose [67]. The uptake of sugars is further discussed in section 6.5.

To produce biomass, reducing power in the form of NADPH is also needed. It is utilised in the production of lipids as well as nucleic and amino acids [68]. NADPH is generated in the Pentose Phosphate Pathway (PPP), which starts as a branch at glucose-6-phosphate in the glycolysis. Some of the compounds in the PPP pathway, such as erythrose-4-phosphate and ribose-5-phosphate, are also used as building blocks for further production of amino and nucleic acids. As will be discussed in the following section, the PPP pathway is also important for xylose metabolism by *S. cerevisiae*.

3.2 Pentose uptake and fermentation

A major drawback of using *S. cerevisiae* for 2nd generation bioethanol production is that most strains do not naturally possess the ability to utilise pentoses, which are abundant in the hemicellulose fraction of the lignocellulose. However, there are *S. cerevisiae* strains that show slow utilisation [69]. Other microorganisms, both bacteria and yeasts, do however possess this ability. An example is *Scheffersomyces stipitis*, formerly known as *Pichia stipitis* [70]. However, the bacteria and yeasts naturally able to ferment pentoses are not optimal for ethanol production for other reasons, such as ethanol sensitivity [71]. Therefore, strategies of recombinant expression of genes necessary for xylose utilisation in *S. cerevisiae* predominate [7].

The two genes that enable xylose utilisation, usually taken from *S. stipitis*, are *XYL1* and *XYL2*, which encode a xylose reductase (XR) and a xylitol dehydrogenase (XDH), respectively. These enzymes convert the xylose into D-xylulose, which will be converted into D-xylulose 5-phosphate by the endogenous *S. cerevisiae* xylulokinase (Figure 4). There is also an alternative route to D-xylulose used by some microorganisms, primarily bacteria, where a single enzyme, xylose isomerase, directly converts the xylose into xylulose. The isomerase route does not require the cofactors NAD(P)H and NAD⁺, which are needed in the oxidoreductive XR/XDH pathway [72-74]. The fact that no cofactors are needed is an advantage, as xylose utilisation can otherwise lead to a redox imbalance. The redox imbalance in the XR/XDH pathway can to some extent be overcome by

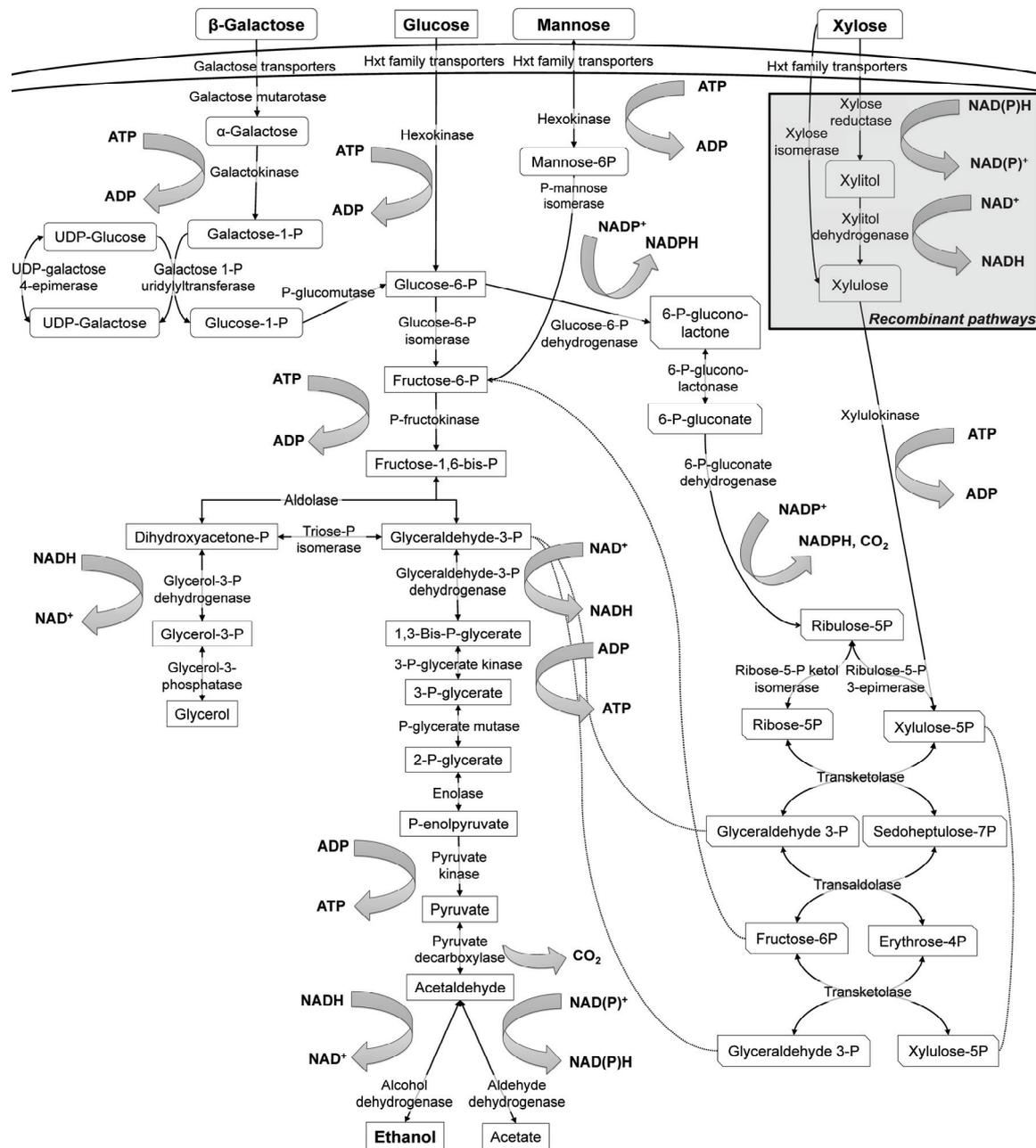


Figure 4 Sugar catabolism in *S. cerevisiae* and possible routes for xylose utilisation. The utilisation of the major monomeric carbohydrates present in lignocellulosic hydrolysates by *S. cerevisiae* all go through the glycolysis, although they go through different steps on their way there. Glucose and mannose are preferred by the yeast and the utilisation is mostly simultaneous. Galactose requires the action of several additional enzymes, whose expression, most importantly the transporters, and hence the utilisation, is repressed by the presence of glucose. Xylose metabolism requires expression of one or two recombinant proteins. Furthermore, the uptake of xylose into the cell is hindered by high concentrations of glucose, since the same transporters, which prefer glucose, are used.

using a mutated xylose reductase that prefers NADH [75]. D-xylulose 5-phosphate produced by either route enters the native *S. cerevisiae* PPP pathway, as mentioned in the previous section. However, the xylulokinase has been observed as becoming a rate-limiting step in *S. cerevisiae* strains that carry *XYL1* and *XYL2*. Therefore, it is often overexpressed in recombinant xylose utilising *S. cerevisiae* strains [76]. Once D-xylulose 5-phosphate has entered the PPP pathway, it can be converted into fructose-6-phosphate and glyceraldehyde-3-phosphate that are both intermediates in the glycolysis pathway (Figure 4). In addition to the three above-mentioned genes, genes that encode enzymes in the non-oxidative PPP can also be overexpressed to ensure fast conversion of xylulose into glycolytic intermediates [77-80]. Furthermore, in order to decrease the amount of xylitol accumulation by improving the redox balance, the native aldose reductase *GRE3* can be deleted [79,80]. The aldose reductase encoded by *GRE3* is NADPH-specific and will thus cause an imbalance when used together with the NAD-specific xylitol dehydrogenase (Figure 4). Attempts to overexpress *GRE3* together with xylitol dehydrogenase and xylulokinase showed that the resulting strain fermented xylose. However, it had severe redox imbalance problems under anaerobic conditions [81]. Recently, specific xylose transporters have been isolated and shown to improve the xylose utilisation when overexpressed in *S. cerevisiae* [82]. The strategy of utilising specific pentose transporters has the potential to allow simultaneous xylose and glucose utilisation, as there would be no competition in their uptake.

Arabinose, the second most abundant pentose in lignocellulosic materials, can also be utilised in *S. cerevisiae* for ethanol production with expression of recombinant genes. This can be done by either expression of a bacterial pathway including an isomerase, or a fungal pathway based on a reductase and a dehydrogenase [83,84].

3.3 Protection against stress

For yeast cells to grow and function, homeostasis has to be maintained. Enzymes require certain pH and temperature for optimal activity, and cellular structures can be destabilised by the same perturbations. Perturbations can also disturb chemical gradients, thus disrupting the metabolic fluxes in the cells. Furthermore, the cells have to be able to withstand osmotic shocks and oxidative damage. In order to counteract these kinds of perturbations, yeast cells have a number of defence mechanisms at their disposal.

A common stress response includes production and accumulation of molecules that act as protectants of the cells. An example is trehalose, which is also a storage carbohydrate. Trehalose stabilises proteins and membranes in the cells. It is accumulated, for example, during heat shock and starvation [85,86]. Another example is glycerol, which is important in the response to osmotic stress. Glycerol is accumulated in yeast cells upon hyperosmotic stress, by closing the Fps1p channel that otherwise lets produced glycerol exit the cells [87]. In this way, the osmotic stress is counteracted by an increase in the intracellular osmolarity.

Oxidative stress is caused by too high concentrations of reactive oxygen species (ROS). This stress can damage membranes, DNA and proteins in the cells by oxidation. Oxidised proteins can lose their function, sometimes irreversibly, which is why protection against ROS is extremely important [88]. Yeast cells have both enzymatic protection systems against ROS and the ability to produce small antioxidant molecules such as glutathione. Glutathione is a tripeptide with two thiol groups that can be oxidised reversibly while the ROS are neutralised [88]. An increased production of glutathione can thus make cells more robust [89].

Another stress response that is common to many stresses is the expression of heat shock proteins, hsp [90]. Many of these proteins are chaperones that assist in the refolding of misfolded proteins, and prevent polypeptide chains from aggregating [91]. Many stresses lead to accumulation of misfolded proteins in the ER. If this problem is not solved by the cells, it can lead to severe cellular damage. Therefore, the hsp help to decrease the effect of various unfavourable conditions. In addition to the hsp, many other responses to stresses are often the same, for seemingly unrelated stresses. Furthermore, cells can become better prepared to handle a second stress after being subjected to a first. This is referred to as cross-tolerance and is further discussed in section 6.4.

As will be described in the following section, certain inhibitory compounds can also be detoxified *in situ* by the cells, by conversion into less inhibitory substances. Other compounds can be pumped out of the cells, commonly facilitated by active transporters that require energy for their operation [92,93]. However, if the perturbation by the compounds is not transient, pumping out the compounds can lead to a futile cycle. The ATP demand to keep the compound out of the cells will then lead to ATP depletion. Therefore, for the cells to survive it is necessary that they adapt to the compound, by

changes in the membrane composition for example, which may make the cells less permeable to the compound.

3.4 Lignocellulose derived inhibitors and their effects on the cells

During the pretreatment and non-enzymatic hydrolysis of lignocellulosic material, with sometimes extreme pH, temperature and pressure, the material is not only broken down to the sought after sugar monomers. Degradation products that are inhibitory to the subsequent fermentation are also formed. Furthermore, the degradation into the inhibitory compounds leads to a loss of fermentable sugars. Therefore, the pretreatment should be performed as mildly as possible to obtain high sugar yields with low inhibitor concentrations in the hydrolysate.

Differences in composition between different raw materials, as well as different pretreatment methods, cause the concentrations of different inhibitors to vary widely between different lignocellulosic hydrolysates [24,94]. The lignocellulose derived inhibitors are commonly divided into three classes: furan aldehydes, carboxylic acids and phenolic compounds [5]. It has however been argued that a more suitable classification would be based on the functional groups of the inhibitory compounds [95]. For the work in this thesis, another categorisation of the inhibitors has also been done, specifically, into the anaerobically readily convertible and not readily convertible inhibitors. The former class, including e.g. the furan aldehydes, can rapidly be converted into less inhibitory compounds, whereas those in the latter class are not converted, such as the carboxylic acids, as mentioned further on in this chapter. Among the phenolic compounds, there are both those that are converted and those that are not [96].

3.4.1 Furan aldehydes

The furan aldehydes class is generally said to consist of two inhibitory compounds: 2-furaldehyde, or furfural, and 5-hydroxymethyl-2-furaldehyde (HMF) that are derived from pentoses and hexoses, respectively [5]. They are formed at high temperatures and low pH by dehydration of the sugars [97]. Considering the conditions often used for pretreatment of lignocellulosic materials, it is easy to understand that the furan aldehydes can be found in significant amounts in pretreated lignocellulose. The inhibitory effect of

furfural, in particular, has been thoroughly studied, although everything is not yet elucidated [98-106]. The effect can easily be observed in that there is usually a lag phase where the furfural is converted into less inhibitory compounds prior to the fermentation of glucose. The fermentation rate also decreases; moreover, too high concentrations lead to an inability to fully detoxify the medium and thus an incomplete fermentation (Figure 5). Since the furan aldehydes can be converted *in situ*, the inhibitor to cell ratio is of importance for the fermentative performance of the yeast [105]. Successful fermentations can therefore be achieved with e.g. fed batch or membrane bioreactors. Here, the cells can be held at a high concentration compared to the inhibitors, resulting in a maintained efficient conversion [106,107]. The furan aldehydes are mainly converted into their less inhibitory corresponding alcohols under anaerobic conditions [99,108].

The adversary effects of furan aldehydes on microbial cells are numerous. In addition to the mentioned lag phase and slower fermentation rate, the viability of the cells is also decreased. This is likely an effect as a result of the induction of ROS that damages the membranes and DNA of the cells [109]. Several enzymes involved in the production of ethanol are also inhibited by the furan aldehydes. An example is the alcohol dehydrogenases that are thought to be involved in the detoxification to the corresponding

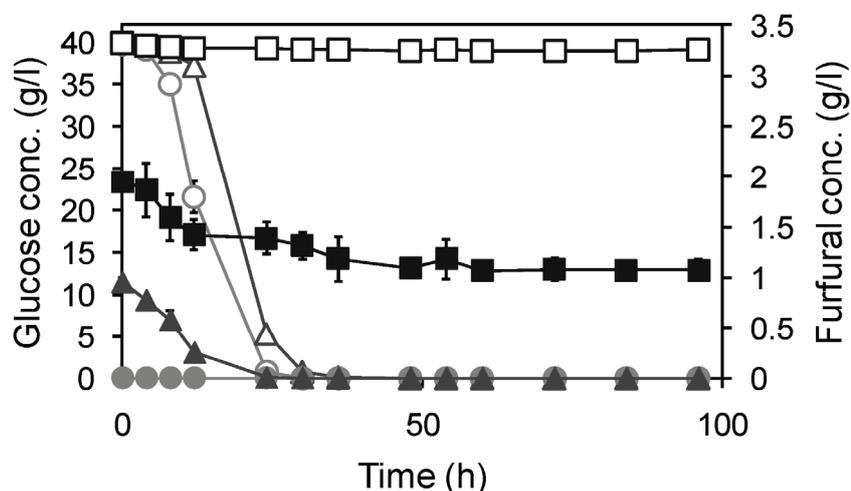


Figure 5 Furfural inhibition of fermentation. The inhibitory effect of furfural on the glucose (open symbols) consumption can be observed in anaerobic batch experiments with 0 g/l (circles), 1 g/l (triangles) and 2 g/l (squares) of furfural (closed symbols) in the medium. At the lower furfural concentration, a lag phase during which the furfural was converted was observed, compared to the medium without furfural. At the highest concentration of furfural, virtually no glucose consumption occurred and less than half of the furfural was converted. [Data from Paper IV.]

alcohols [99,109,110]. Furfural can also act as a redox sink. Thus, in the presence of furfural the glycerol production decreases, which can be beneficial for the ethanol production process [102]. The redox sink effect can also be beneficial for xylose fermenting yeasts, where furfural can act as an electron acceptor during reoxidation of the excess NADH produced. Xylitol accumulation can thereby be decreased [111].

3.4.2 Weak carboxylic acids

The fact that weak acids are potent inhibitors of microorganisms has been 'known' for centuries, as food is believed to have been pickled since 2400 BC [112]. The food can either be stored in vinegar, acetic acid, or be anaerobically fermented to produce lactic acid. The weak acids relevant for 2nd generation bioethanol are: acetic, formic and levulinic acid, which are derived from deacetylation of hemicellulose and degradation of furan aldehydes, respectively [113]. Some phenolic compounds are also weak acids, but are usually not placed in this class. At high concentrations the acids are strongly inhibitory to the cells, due to intracellular acidification and accumulation of anions [109]. The pH of the medium is very important for the toxicity of acids, as the undissociated forms can easily pass through the membrane from the medium of lower pH to the cytosol with higher pH. The undissociated forms of the acids, and not only the H⁺, are therefore toxic [114]. With pK_a values in the range of 3.75–4.75, the weak acids will dissociate once they are inside the cells. This leads to decreased pH values and necessitates the H⁺-ATPase pumps to pump out the excess protons [115]. Acetic acid can, therefore, at low concentrations be beneficial for the fermentation, since the pumps require energy in the form of ATP. This is obtained by production of more ethanol, which increases the ethanol yield while the biomass and glycerol yields are reduced [33,116]. However, if the concentration of the acid is too high, the pH of the cells will inevitably be lowered beyond the capacity of the cells. This leads to disrupted fermentation and cell death. The toxicity of the acids has been determined to be in the following order of increased toxicity: acetic acid < levulinic acid < formic acid [33]. Accumulation of the anions inside the cells has also been brought forward as the main effect of toxicity, at least in the case of bacteria [117]. The anionic form of the acids gets trapped inside the cells and accumulates until there is equilibrium of the undissociated form over the membrane. This will eventually lead to toxic anion concentrations. However, anions of small carboxylic acids can also be transported out of the cells. The transporter proteins responsible for this

act by active transport [92]. Hence, this process, just as the process of maintaining the intracellular pH by transporting H⁺, requires ATP and can lead to ATP depletion.

3.4.3 Phenolic compounds

The inhibitor class termed phenolic compounds is not surprisingly the most heterogeneous, since it is derived from lignin, the most heterogeneous polymeric constituent of the raw material [5,29]. For this reason, the nature of the phenolic compounds in different hydrolysates is clearly mostly affected by the source of the raw material, as the composition of the building blocks vary between different plants [5]. Spruce, as an example, contains mainly phenolic compounds derived from guaiacyl – coniferyl alcohol [118]. The toxic effect of phenolic inhibitors often increases in the order: alcohols < acids < aldehydes [109]. This might also be a reason for the lower inhibitory effect of the furan alcohols compared to the aldehydes. Smaller phenolic compounds are also generally more toxic than high molecular weight compounds [5]. The hydrophobicity of the compounds also affects the toxicity, with more hydrophobic compounds being more toxic [96,119]. This is related to their interactions with biological membranes. The function of the membranes is disturbed through disturbance of their integrity [120].

3.5 Investigations of cellular physiology

In order to understand the changes in fermentation performance that are observed upon changes in e.g. operational parameters or differences in media composition, it is necessary to study the physiology of the fermenting cells. Cellular responses often involve many different, connected, processes in the cells, which are regulated on the level of gene transcription. To study these responses, genome-wide analyses are often performed. Genome-wide studies of the cellular physiology can be made either on gene transcript level, mRNA, on protein level or on metabolite level. The most commonly used method is to investigate gene expression using microarray or qPCR experiments, comparing mRNA levels of different samples [121,122]. With a microarray, the expression levels of all genes in an organism can be compared over different samples. A microarray contains short DNA probes from each gene in the genome of the microorganism. By hybridisation of labelled cDNA from the samples onto the chips and

detection of the labels from different samples, a relative quantification can be performed. In qPCR, fewer, specific genes are usually investigated. Here, the cDNA is amplified using PCR and quantified using fluorescence.

However, it is on the protein level that actual cellular events take place and there is not always a linear relationship between mRNA levels and protein levels [123,124]. Unfortunately, it is not as straightforward to measure the levels of all proteins in a cell, as it is to measure mRNA levels using a chip. The first challenge is that all proteins are not easily and equally isolated from the cells. Membrane bound proteins can be especially difficult to isolate. Furthermore, the proteins have to be individually identified and the expression levels compared between different samples. Thus, it is easy to understand that a genome wide coverage on the protein level is not easily attainable. Two commonly used proteomic methods, 2D-DIGE and nLC-MS/MS, are briefly described in section 6.3 and also in Paper III. Adding an additional level of information, post-translational modifications (PTMs) of proteins can also occur. These can be detected with proteomics and be of great importance for the cellular physiology [125]. Changes in abundance are slower on the protein level than on the mRNA level, as DNA is transcribed into mRNA, which is thereafter translated to the corresponding protein. This can be of importance in the design of a genome-wide study, as the sampling of the cells should be considered. For example, cells encapsulated in membrane capsules have to be mechanically released from the capsules. This increases the sampling time somewhat compared to what can be achieved for freely suspended cells. However, it is important to remember that the levels of both mRNA and proteins are not necessarily of importance for the physiology. It is ultimately the activity of the proteins that determines the function of the cells.

A measure of the activity of the cells can be determined by investigations of the metabolome – metabolomics. In metabolomics, as many metabolites in a cell as possible are measured, using enzymatic reactions or various chromatographic methods (e.g. HPLC, GC), usually coupled to mass spectrometry. Since metabolites are generally not very stable, a fast and efficient extraction and quenching protocol is a prerequisite for successful metabolomics [126].

The large data sets generated by the methods mentioned above enable a deep understanding of cellular physiology. However, the vast amount of data also creates challenges for the interpreter, as it may be difficult to discern which of the changes that

are important. Specifically, to identify a single gene, gene product or pathway that has important effects on the phenotype of an organism, as hypothetically may be the case for encapsulated yeast, is not necessarily easy with these approaches. The levels of transcripts or products of many other genes may also change upon changes in conditions, potentially concealing the most important changes.

3.6 Modes of cell cultivation/fermentation

There are a number of different modes of system configurations during fermentations [127]. These include different reactor configurations as well as modes to operate the reactors. The easiest system is a batch reactor. Here, all substrates and cells are added to the reactor at the start of the batch and taken out in the end. A system that is of high importance for fundamental studies, is the continuous system. In such a system, substrates are added in a continuous flow into the reactor, where the cells perform the fermentation, and products are continuously taken out of the reactor. This system, at stable conditions, reaches a steady state and becomes a chemostat. The cells then grow at the same specific growth rate as the dilution rate of the reactor [127]. This is of great importance for studies of cell physiology, as the growth rate can be kept the same while e.g. the medium composition is changed. However, this is the case only for cells freely suspended in the medium. If the cells are retained inside the reactor, a steady state may not be achieved. The third common reactor configuration is the fed-batch system. It brings together benefits from both the batch and the continuous system. It is started as a batch, with cells in a small volume of medium. After an initial start-up period, more medium is added continuously, without an outflow. In this way, inhibitors in the medium, if they can be converted, can be kept at a low level throughout the fermentation [128,129]. Fed-batch is also the best way to produce yeast biomass, as the glucose level can be kept low [130]. Thereby, the respiratory route of *S. cerevisiae* is favoured, as described in section 3.1. Accordingly, more than five times as much biomass can be produced compared to the fermentative, anaerobic, route [131].

In the current thesis project, batch cultivations with shake flasks have been the choice for cell cultivations. The reason was mainly due to their ease of use when performing a large number of experiments and easier comparisons between ‘freely suspended’ and, by different means, immobilised cells.

3.7 Cell retention

Retention of biologically active material, in the form of cells or enzymes, is a cost effective way of increasing the volumetric productivity of a bioreactor, by increasing the amount of biocatalyst present [132]. In addition to increased productivity of the system, it also enables higher dilution rates of continuous systems [133-136]. This is a significant benefit in industrial settings. However, it is of less interest to study experimentally, since more cells will obviously perform a task faster than fewer cells. In addition to this, cell retention also enables easier separation of the product from the catalyst [137]. The separation of cells from the produced ethanol is otherwise an energy demanding process. In many cases, cell retention will also lead to a lower growth rate. Implications of lower growth rate will be further discussed in Chapter 6.

Cell retention can be performed in a number of ways, for example, adsorption to a surface, chemical cross-linking, immobilisation in a matrix, encapsulation inside a membrane, or by self-flocculation (Figure 6) [138]. Encapsulation in a semi-permeable membrane and self-flocculation are further discussed in Chapters 4 and 5, respectively.

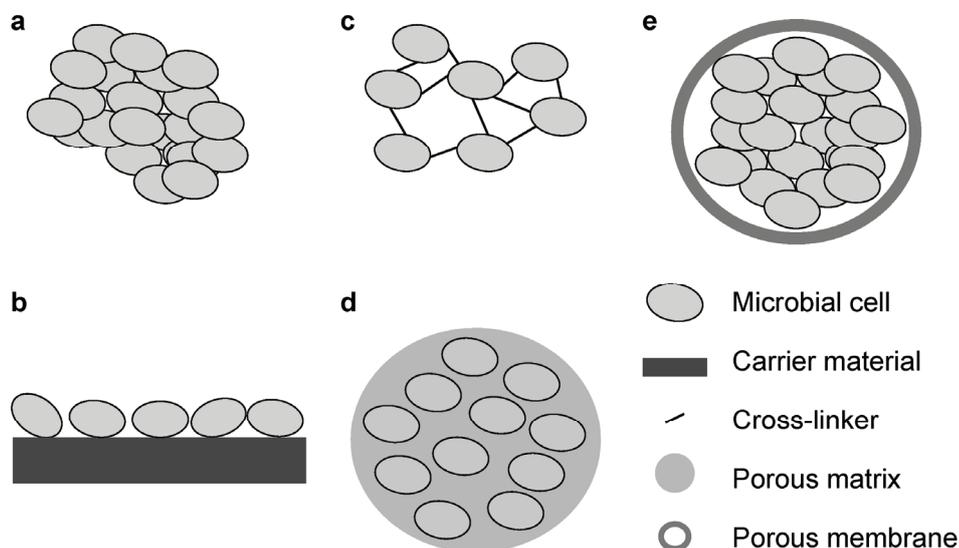


Figure 6 Different methods of cell retention. Different ways of cell retention or immobilisation are e.g. self-flocculation (a), adsorption to a surface (b), cross-linking (c), immobilisation in a matrix (d) and encapsulation inside a semi-permeable membrane (e). Figure from [138], reprinted with permission.

3.8 Inhibitor tolerance by high cell density cultures

Many strains of *S. cerevisiae* are capable of *in situ* detoxification of toxic hydrolysates, as described in section 3.4. However, for the cells to be able to detoxify the medium fast enough, the ratio of inhibitors per cells must be rather low. This can be achieved in different ways. First, the inhibitor concentration can be kept low by a constant slow inflow of medium, by the use of fed-batch [107,129] or continuous cultivations [101]. Secondly, a high capacity of the system to detoxify a medium can be achieved, for example, through a high inoculum concentration in a batch [139]. This decreases the lag phase by an increased rate of furfural conversion. In continuous processes, the cell concentration can be increased by cell retention. In addition to the types of cell retention shown in Figure 6, it can also be achieved through cell recirculation [139,140]. By recirculating the cells in a continuous system, the cells can continue to convert inhibitors even though their growth rate does not match the dilution rate of the system. In fermentations with cell retention by membrane bioreactors, the concentration of furfural could be increased to as high as 17 g/l in the feed, with maintained performance [106]. However, in this case the ratio of inhibitors per cells was rather low, since the cell concentration reached up to around 170 g DW/l. In this perspective, 17 g/l furfural in the inlet medium is not necessarily experienced as a very high concentration for the cells. At a high cell concentration, the cells simply convert the furfural rapidly enough to make the toxic effect almost negligible. Furthermore, as a result of this, the concentration experienced by the cells will be low in a balanced continuous system, as the inlet feed is instantaneously mixed with the entire reactor volume. In order for this to work, it is also important that the furfural does not have an immediate toxic effect. If it is rapidly converted, the cells will survive even high concentration pulses of furfural [106]. In this thesis, however, I have investigated what happens when the cells have a low concentration relative to the inhibitors in the reactor, but a high cell concentration *locally*.

3.9 Improved hydrolysate fermentation by high *local* cell density cultures

Yeast, at high local cell density in the form of flocculating strains, has been reported to possess tolerance towards toxic hydrolysates (Table 1). However, the good performance of the flocculating strains has often been attributed to isolation from industrial locations, rather than being linked to the flocculation. Industrial conditions lead to requirements for higher robustness compared to the laboratory environment. Encapsulation of yeast in semi-permeable gel membranes has been observed to improve the fermentation performance of a non-tolerant strain in lignocellulosic hydrolysates (Table 1). A macromolecular characterisation of the encapsulated yeast has been performed, revealing changes upon prolonged encapsulation [141]. However, no conclusive explanation for the improved performance in hydrolysates has been given.

Table 1 Examples of high local cell density cultures showing tolerance to toxic media.

Mode of high local cell density	Yeast strain used	Medium fermented	Mode of operation	Ref
Flocculation	CCUG53310	Spruce hydrolysate	Continuous & batch	[136]
Flocculation	KF-7	Wood hydrolysate	Continuous	[142]
Flocculation	ATCC96581	Spent sulphite liquor	Batch	[143]
Flocculation	ATCC96581	Synthetic medium with inhibitor cocktail	Batch	[144]
Flocculation	ATCC96581	Spruce hydrolysate	Batch & fed-batch	[128]
Flocculation	TMB3720	Spent sulphite liquor	Batch	[145]
Encapsulation	CBS8066	Spruce hydrolysate & Synthetic medium with 5 g/l furfural	Batch	[8]
Encapsulation	CBS8066	Spruce hydrolysate	Continuous	[134]

3.10 Challenges for commercialisation of 2nd generation bioethanol

As described above, there are a number of issues related to the production of 2nd generation compared to the 1st generation bioethanol, which explains why successful commercialisation has not been realised yet. Two of these issues are addressed in this thesis (Figure 7). The first issue is the tolerance towards inhibitory compounds formed during the harsh pretreatment and hydrolysis of the recalcitrant raw materials used for 2nd generation bioethanol. The second issue is the fact that the preferred microorganism for production, *S. cerevisiae*, even with expression of recombinant pathways, cannot simultaneously utilise all sugars present in the produced hydrolysate. The results in this thesis show that there may be a common solution to these two problems: *High local cell density*.

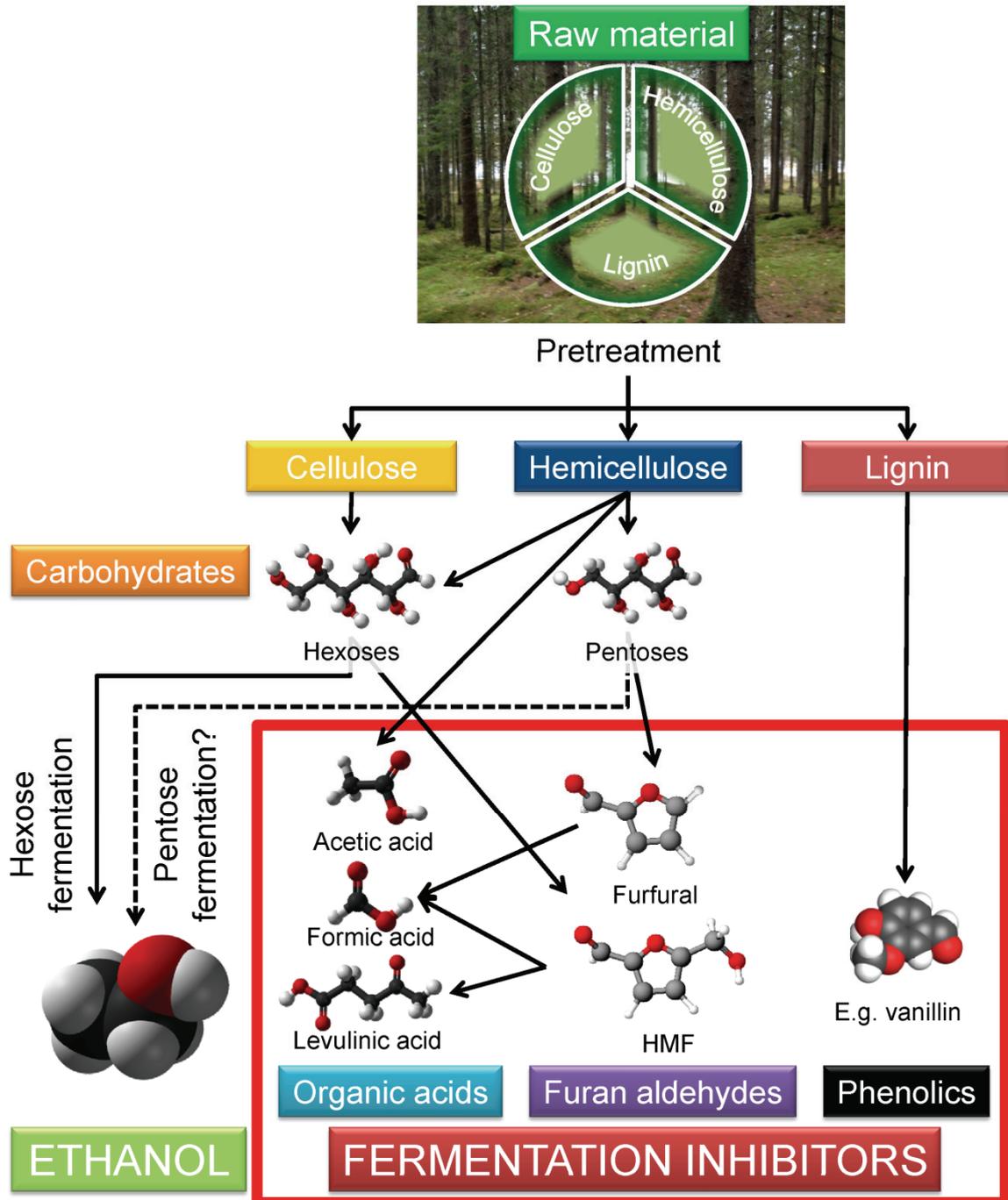


Figure 7 Challenges in 2nd generation bioethanol production addressed in the thesis. Lignocellulosic materials contain both pentoses and hexoses with the potential of being converted into ethanol through fermentation. However, most strains of *S. cerevisiae* do not naturally possess the ability to utilise the pentoses present. This necessitates genetic modifications of the strains to be used in the ethanol production process. For these strains, simultaneous utilisation of hexoses and pentoses remains a problem. Furthermore, the pretreatment step necessary to break up the recalcitrant structure of the raw material often creates a number of inhibitors that the fermenting microorganism has to cope with. These two obstacles have to be solved in order to obtain an economically feasible 2nd generation bioethanol production process.

CHAPTER 4

ENCAPSULATION

In the literature, there are different definitions of cell encapsulation. It can be used to describe both immobilisation/entrapment in a gel matrix, as well as encapsulation within a semi-permeable membrane capsule (Figure 6 D & E). The latter definition, with a liquid core holding the encapsulated cells, is used in the current thesis. The cells inside the capsules can obtain nutrients through the membrane, as well as excrete products through it, but they cannot themselves leave the capsules. The technique for yeast encapsulation for ethanol production has been around for two decades [146]. A number of improvements to the capsules and production process have been made over the years, as will be briefly reviewed in the following section.

4.1 Methods and materials of encapsulation

The most commonly used materials for encapsulation of cells are natural organic polyelectrolytes such as alginate (Figure 8 A) [138]. Alginate is a negatively charged polymer that consists of two different building blocks, α -L-guluronic acid and β -D-mannuronic acid [147]. Alginate forms gels when mixed with divalent cations, e.g. calcium ions, whereby guluronic acid residues are linked together by the cations (Figure 8 A) [148]. When a cell suspension with a high concentration of calcium ions (usually in the form of CaCl_2) is dripped into a sodium alginate solution, the cells in the drops will be encapsulated. The encapsulation happens as the alginate forms a gel with the outer layer of Ca^{2+} in the drop. Thereafter, the membrane becomes thicker by diffusion of the internal Ca^{2+} out of the newly formed capsule. The cores of the capsules remain as a liquid because the alginate molecules cannot diffuse through the membrane. This capsule production method is known as the one-step method, or the liquid droplet formation method [149]. Another way to produce liquid core capsules is by the two-step method. In this method, beads are first formed when a cell solution mixed with alginate is dripped into a solution with high Ca^{2+} concentration. The beads are thereafter coated, for example, with chitosan (Figure 8 B), a polyvalent cationic polymer of 1,4-linked-2-amino-2-deoxy- β -D-glucan [150]. The chitosan molecules replace some of the Ca^{2+} used

in the membrane. It can thereafter be followed by another, optional, layer of alginate [151,152]. However, the chitosan molecules will not reach the capsule core, which consists of Ca-alginate, because of its large molecular size. If the coated beads are thereafter treated with a chelating agent, such as citrate, the core will be liquefied as the Ca^{2+} are sequestered. Dissolving the core will lead to capsules with a liquid alginate core [151,152]. The capsules made by the one-step method can also be coated by chitosan, to create a stronger membrane (Figure 8 C) [153]. If this is not done, and Ca^{2+} is not added to the medium in excess, the capsules are weakened by a slow release of Ca^{2+} to the surrounding medium [146,154].

Non-ionic surfactants such as Nonoxynol₉₅ [146] or Tween 20 [154] are added to the alginate solution during capsule formation in order to improve the permeability of the capsules. The capsules are otherwise prone to accumulate bubbles of CO_2 during fermentation using encapsulated yeast [146,154]. Adding these surfactants, however, may not be enough to ensure good gas permeability. If the yeast inside the capsules ferments a medium too fast, too much CO_2 will almost inevitably be accumulated inside

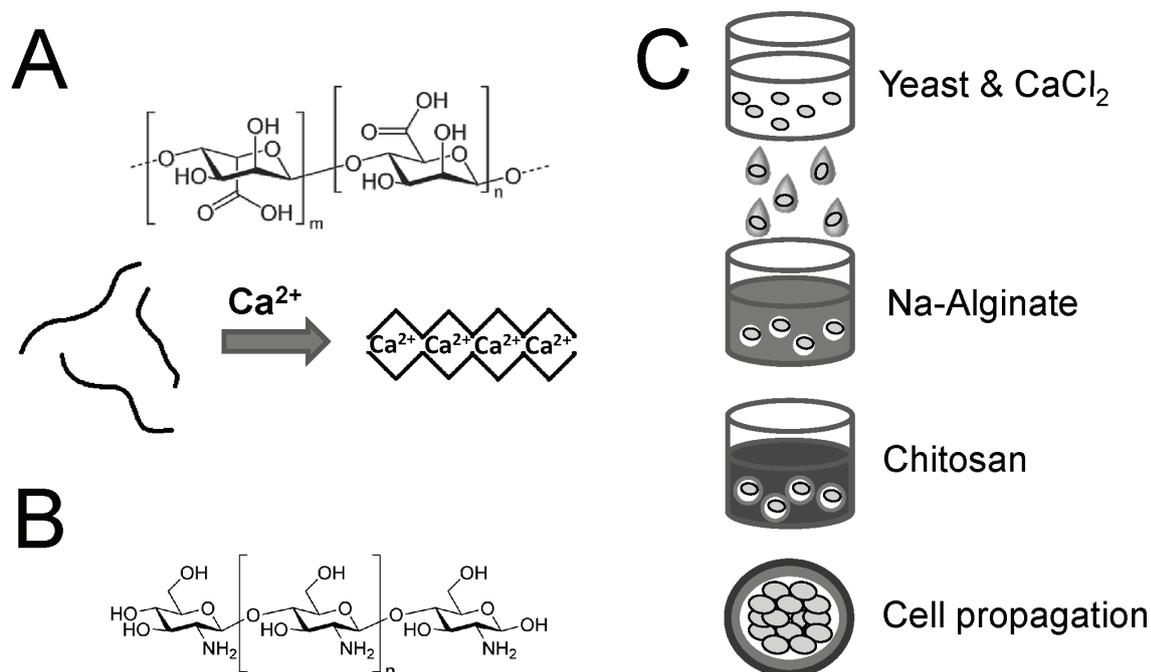


Figure 8 Encapsulation procedure and structures of alginate and chitosan. A) Structure of alginate and the Ca-alginate gel. B) Structure of chitosan. C) Encapsulation procedure used in this thesis – a cell suspension containing calcium ions is dripped into a solution of Na-alginate, whereby thin capsules are formed instantaneously around the yeast cells. The capsules are thereafter treated with chitosan, which replaces some of the calcium ions and makes the capsules stronger. [Method used in Papers II, III & IV.]

the capsules. The capsules then float up to the surface of the medium and thereafter often burst from the high internal pressure.

When encapsulated, the cells tend to grow from one point inside the membrane. This results in the formation of a cell pellet already before the capsules are full with cells (Figure 9) [141]. This growth pattern is of importance for the benefits of encapsulated cells, discussed in Chapter 6.

A newly reported method for improving the strength of the capsules is to treat them with hydrolysed 2-aminopropyltriethoxysilane, hAPTES [155]. This treatment had a somewhat detrimental effect on the cell viability. However, by optimising the concentration of hAPTES used in the treatment, a balance could be found between the cell viability and improvement in capsule strength. The hAPTES has amino groups that react with the carboxylate groups on the alginate. Thereafter, silanol molecules undergo a condensation reaction that leads to the formation of a polysiloxane coating of the capsules. However, the problem with capsules rupturing due to rapid CO₂ formation was observed also with these capsules.

One critical aspect of encapsulation, which remains and can likely not be solved, is that if the cells are not fermenting a perfectly fine tuned inhibitory medium, the cells will eventually grow until they totally fill up the capsules and start to leak out. If the medium is inhibitory at the right level, there can theoretically be a balance between the cells that divide and those that die, so that there is no net growth inside the capsules.

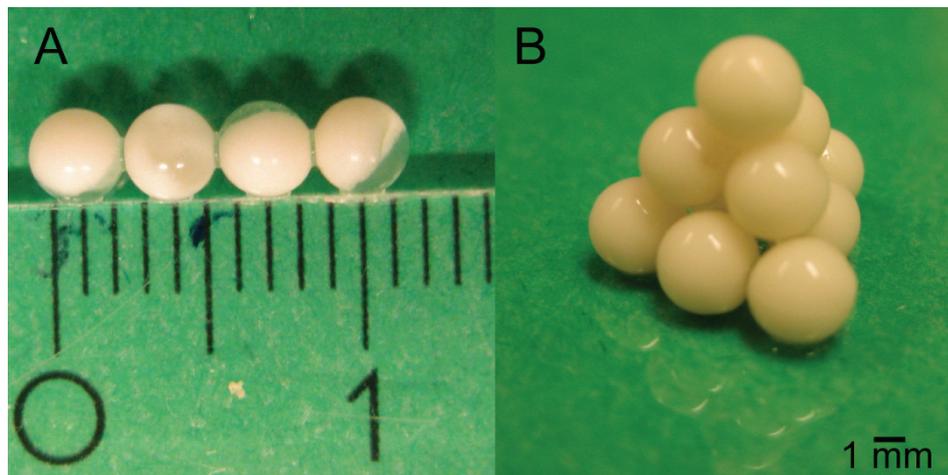


Figure 9 Alginite-chitosan capsules. A) Capsules roughly half full with cells. B) Capsules filled up with cells.

CHAPTER 5

FLOCCULATION OF *S. CEREVISIAE*

In biology, flocculation is the process by which microorganisms asexually aggregate. This is of great benefit in many biotechnological applications, as it provides a simple and natural way of cell retention or separation from the product. The most commonly known application is probably beer production. Flocculation of brewery strains of yeast naturally occurs towards the end of the fermentation process, as the sugars in the wort are depleted. Flocculation thus provides an easy and energy efficient method of separating the yeast from the beer. In the case of beverage ethanol production, however, it is important that the flocculation does not start prematurely, as this can lead to off-flavours in the product [156]. In fuel ethanol production, this is not an issue and constitutive yeast flocculation can be used as a means of increasing the amount of cells in a continuous reactor at high dilution rates [136]. Other examples of biotechnological applications where flocculation can be used are the harvesting of microalgae [157] or retainment of digesting bacteria in anaerobic treatment of wastewater [158].

5.1 Mechanism of yeast flocculation

Yeast flocculation is controlled by a number of genes called the *FLO* gene family [159]. The *FLO* genes, with the exception of *FLO8* which is a transcription factor [160], encode cell surface proteins anchored in the cell wall. These cell wall proteins, also called flocculins, have the ability to bind to carbohydrates present in the cell wall of neighbouring yeast cells with a lectin-like mechanism [161]. In this way, complexes of several millimetres can be obtained, even with constant agitation of the medium containing the cells (Figure 10). The fact that the proteins bind to carbohydrates also means that the flocculation can be inhibited by competitive binding of free sugars present in the medium [162]. The flocculation is also dependent on Ca^{2+} [163]. Depending on the sources of inhibition, flocculating yeast strains are divided into two different phenotypes, the Flo1 and the NewFlo phenotypes [164]. The flocculation of Flo1 strains is only affected by mannose, whereas the flocculation of NewFlo strains is also inhibited by e.g. glucose, maltose and sucrose. Industrial brewery strains are commonly of the NewFlo

type and hence do not flocculate until all of the sugars in the medium are fermented. This facilitates an efficient fermentation and thereafter easy separation of the beer from the cells [165]. The major flocculation gene is *FLO1*, encoding a 1537 amino acid long protein with an interesting domain structure. The N-terminal domain of at least Flo5p and by homology also Flo1p and Flo9p, a so called PA14/Flo5-type domain, is responsible for sugar binding with two carbohydrate-binding loops [166]. The middle region of the protein consists of repeated sequences of 45 amino acids. This region makes up the largest difference between Flo1p and Flo5p and Flo9p, the other two flocculation proteins that give rise to strong flocculation in *S. cerevisiae* [167]. The C-terminal part of the protein contains a GPI modification site, which facilitates the anchoring of the protein in the cell wall of the yeast cells [168]. The transcription factor *FLO8* controls the expression of the *FLO* gene family [160], which also contains the genes *FLO10* and *FLO11*. *FLO10* gives rise to weak flocculation, whereas *FLO11*, or *MUC1* as it is also called, is responsible for invasive and pseudohyphal growth [169-171].

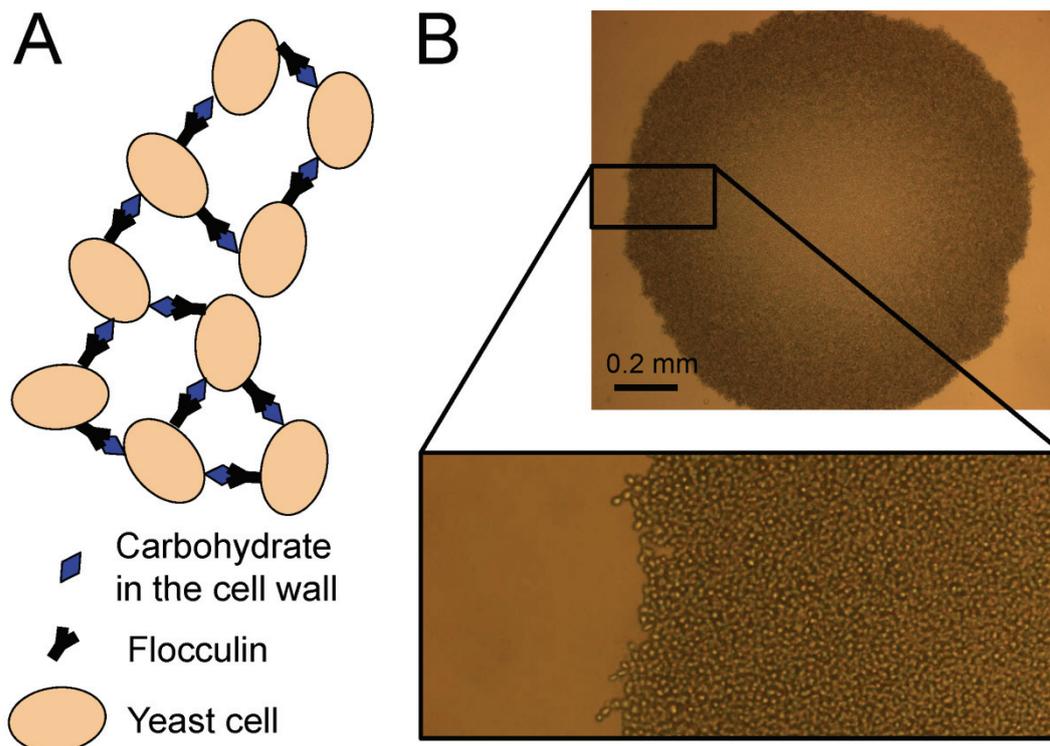


Figure 10 Flocculation creates large cell communities. **A)** Flocculating yeast cells attach to each other using cell wall proteins that bind to carbohydrates in the cell wall of neighbouring cells. This binding also requires calcium ions [166]. **B)** Through these bindings, strong cell complexes of several millimetres in diameter can be formed.

The natural variability of flocculation and flocculation genes in wild yeast strains has been addressed in several studies, e.g. [169,172,173]. The genes responsible for flocculation are known to be controlled by three signalling pathways, responding to stimuli from the environment, e.g. stress or nutrient limitation: the cAMP/PKA pathway, the MAPK-pathway and the glucose-repression pathway [165,171]. For industrial strains, however, the contribution of each pathway might be different, since this has been elucidated mainly for laboratory yeast strains. The initiation of flocculation during yeast cultivations has been shown to be affected by e.g. medium composition, cell age and anaerobic conditions, as well as by the hydrophobicity of the cells [169,174-179].

Most strains used for research in laboratories around the world have been selected for their lack of flocculation, since it enables easier experimental work. For example, flocculating cells are not equally distributed throughout the medium they are in. Hence, they cannot be quantified in the same way as freely suspended yeast, by turbidimetry. Sampling during cultivations also presents problems in that cell removal might be disproportionate to the medium removal. Since more or less cells can be removed, not necessarily related to the total cell concentration, the cell concentration will change when a sample of the medium is taken. This is an issue during batch cultivations, which nonetheless is the best way of comparing flocculating and non-flocculating strains. During continuous cultivations, significantly more biomass will be retained in the reactor for flocculating cells compared to non-flocculating. For strongly flocculating cells it is also evident that there will be cells exhibiting significantly different cell physiology in the same cultivation, which is normally not desired in the laboratory.

5.2 Construction of recombinant flocculating yeast strains

In Paper V, a set of flocculating yeast strains was constructed by recombinant expression of variants of the *FLO1* gene isolated from *S. cerevisiae* S288C in *S. cerevisiae* CEN.PK 113–7D. *S. cerevisiae* CEN.PK 113–7D lacks the major flocculation genes in its genome and does not flocculate [180]. The lack of the flocculation genes makes it a perfect reference strain for investigations of the effects of flocculation. *S. cerevisiae* S288C is also a non-flocculating strain. However, the reason is not lack of the flocculation genes, but a nonsense mutation in *FLO8*, the transcriptional regulator [181]. Initially, the strategy for the construction of a flocculating strain was to insert the *FLO1* gene into a

vector after the *TDH3* promoter sequence. However, amplification of the plasmid in *E. coli* proved impossible, since the size of the *FLO1* gene was decreased after transformation. This has also been observed in other studies [182]. The amplified gene was therefore merged with a cassette containing the *kanMX* gene and the *TDH3* promoter sequence for control of the *FLO1* expression, using PCR. However, an optimal PCR program could not be found, despite numerous attempts to optimise using a number of different polymerases. The PCR products always appeared smeared when analysed on gels, with a range from the expected size towards smaller size. However, an attempt to transform the CEN.PK strain with the PCR-product, followed by selection on YPD plates with 200 µg/ml G418 (Sigma), did give rise to transformants. A number of transformants were chosen and grown overnight in liquid medium, whereby some showed flocculation of different strengths. Three flocculating mutants were isolated and the inserted gene product was investigated by PCR and sequencing. The mutants were named after their strength of flocculation and are shown in Table 2 together with the other yeast strains used in this thesis.

Table 2 Yeast strains used in this thesis.

Yeast strain	Reference/Source
CBS8066	CBS ¹
CCUG53310	[136]/CCUG ²
S288C	[183]
CEN.PK 113–7D	[184]/SR&D ³
CEN.PK 113–7D Weakly flocculating	This study, [Paper V]
CEN.PK 113–7D Intermediately flocculating	This study, [Paper V]
CEN.PK 113–7D Strongly flocculating	This study, [Paper V]
CEN.PK XXX	This study, [Paper IV]

¹CBS – Centraalbureau voor Schimmelcultures (Delft, the Netherlands),
²CCUG – Culture Collection at University of Gothenburg (Gothenburg, Sweden),
³Scientific Research & Development (Oberursel, Germany)

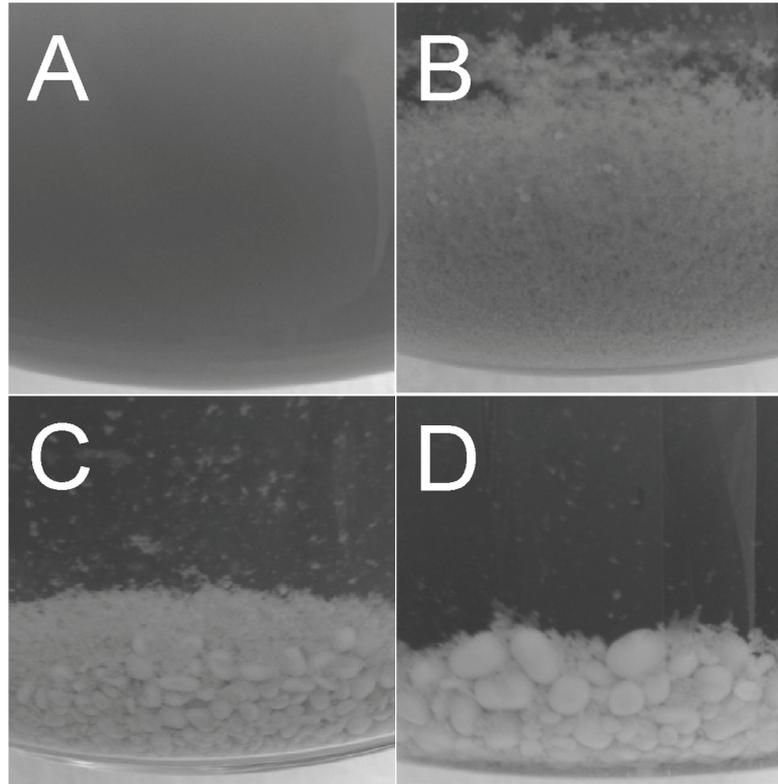


Figure 11 Flocculation strength depends on the size of the *FLOI* gene variant. Different strength of flocculation could be observed in mutants with different length variants of the *FLOI* gene inserted into their genome. A) Non-transformed CEN.PK 113-7D, B) weakly, C) intermediately and D) strongly flocculating cells, photographed after shaking at 125 rpm in 100 ml YPD medium. [Adapted from Paper V.]

Analysis of the inserted genes by PCR showed that the different mutants had *FLO* genes of varying lengths inserted into their genomes. Furthermore, the longer the *FLOI* variant, the stronger the flocculation of the mutant (Figure 11). Sequencing of the amplified and purified genes followed by sequence analysis showed that the difference between the mutants lay mainly in the number of repeat units in the middle of the *FLOI* gene. The strongly flocculating mutant missed 7 repeats of 45 amino acids compared to that of the native *FLOI*, and the intermediately and weakly flocculating mutants lacked 9 and 13 repeats respectively. The *TDH3* promoter sequence was identical in all mutants. This resulted in constitutive expression, independent of the environmental conditions that are important for native flocculation.

In addition to the binding properties of the flocculins, increased cell wall hydrophobicity arising from their expression is believed to increase the flocculation capacity [169,178]. However, increased hydrophobicity of the cell wall is not a major determinant of flocculation strength. Overexpression of *FLO11* has been shown to lead to an equal or

even more hydrophobic cell surface compared to both *FLO1* and *FLO5* overexpression, but does not result in flocculation [169,185]. Significant increases in the hydrophobicity of the cells could also be observed for the strains constructed in this study. A correlation between the length of the *FLO1* gene variant and the hydrophobicity could be observed, with higher cell wall hydrophobicity for longer gene variants (Figure 12). The reasons for stronger flocculation as well as higher hydrophobicity might be that the larger the protein, the farther it can extend from the surface of the cell. If the binding part of the protein extends farther from the surface, more simultaneous bindings between adjacent cells are enabled. It is also likely to give a more hydrophobic cell surface. The theory of extension from the surface is supported by the fact that the central domain of *FLO1* contains a large proportion of serine and threonine [182]. Post-translational modifications in the form of O-glycosylation of these amino acids are believed to lead to a rod-like, semi-rigid structure, extending the active domain further from the cell surface the longer the central domain [165,167,186].

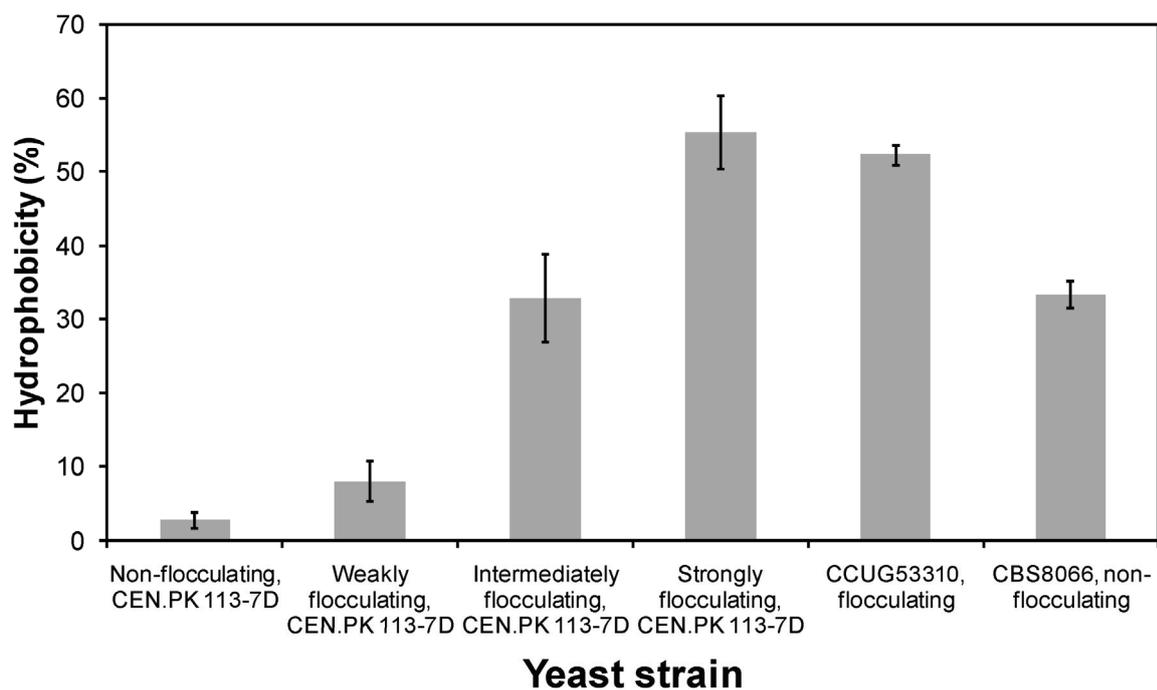


Figure 12 The hydrophobicity of the cell surface was affected by expression of *FLO1* variants. The larger the size of the inserted *FLO1* gene variant, the higher the measured cell wall hydrophobicity. The hydrophobicity is also dependent on the strain background. This can be seen in that the non-flocculating CBS8066 had a cell surface as hydrophobic as the intermediately flocculating CEN.PK 113–7D. [Data from Papers I & V.]

CHAPTER 6

HIGH *LOCAL* CELL DENSITY FOR SUCCESSFUL FERMENTATION OF LIGNOCELLULOSE HYDROLYSATES

6.1 A naturally occurring high local cell density strain

Numerous attempts have been made to increase the inhibitor tolerance of *S. cerevisiae* strains towards various compounds present in lignocellulosic hydrolysates, for example, by gene over-expression approaches and evolutionary engineering [89,110,187,188]. However, there are also naturally occurring strains that have been shown to possess superior qualities compared to most so called laboratory strains. One example is *S. cerevisiae* CCUG53310, which has been isolated from an ethanol plant (Domsjö Fabriker AB, Örnsköldsvik, Sweden), and has been demonstrated to have a high tolerance towards lignocellulosic hydrolysate [136,189].

The performance of *S. cerevisiae* CCUG53310 in media containing inhibitors from the different classes of lignocellulose derived inhibitors was investigated and compared to the common laboratory strain *S. cerevisiae* CBS8066 [Paper I]. It was observed that CCUG53310 had clear advantages in media containing the furan aldehydes: HMF and furfural, or the carboxylic acids: formic, acetic and levulinic acid (Figure 13A–D). However, when the strain was subjected to a medium containing phenolic compounds, in the form of vanillin, guaiacol and catechol, the strain was significantly more sensitive than the laboratory strain (Figure 13 E).

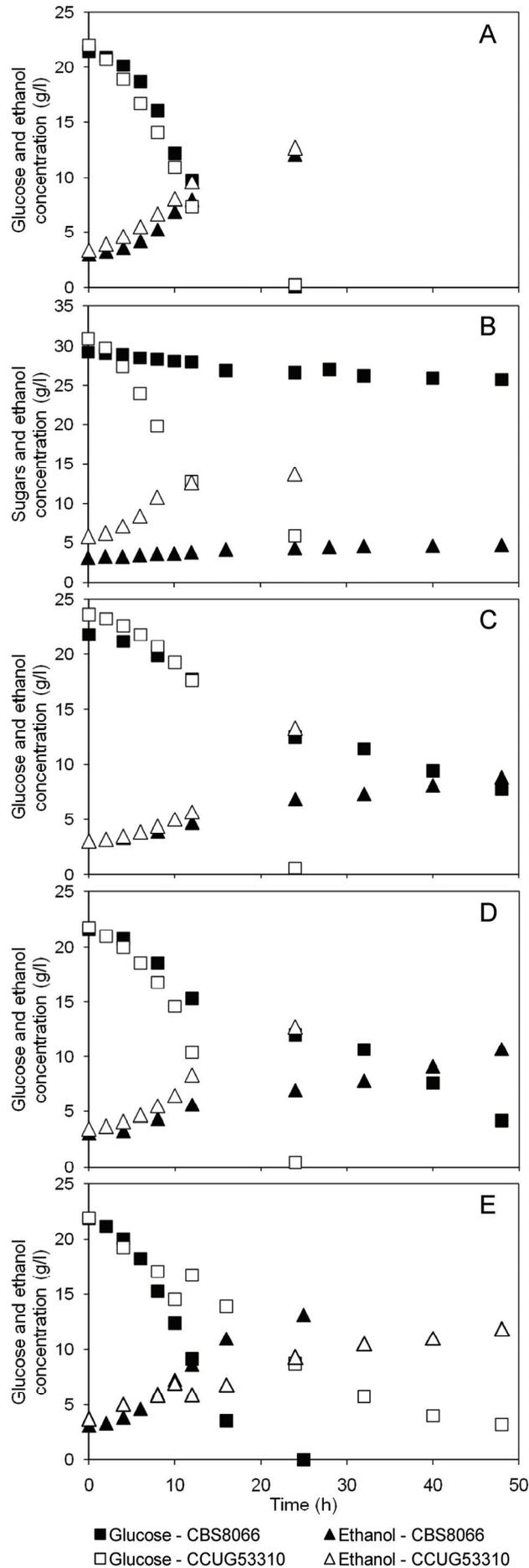


Figure 13 *S. cerevisiae* CCUG53310 and CBS8066 are affected differently by different inhibitors. CCUG53310 could successfully ferment the hexoses in spruce hydrolysate (B), defined medium with carboxylic acids (C) and defined medium with furan aldehydes (D) in 24 hours, without being severely inhibited compared to the fermentation of the non-inhibitory medium (A). CBS8066 was clearly more affected by these inhibitory media, while the performance in non-inhibitory medium was similar between the strains. However, the defined medium with phenolic compounds (E) was more easily fermented by CBS8066 than CCUG53310. [Figure adapted from Paper I.]

These intriguing results led to a further analysis of the physiological state of the cells in the different media. The gene expression levels of three genes known to be important for the inhibitor tolerance towards inhibitors present in lignocellulosic hydrolysates was investigated [190]. The genes investigated were *ATRI*, *FLRI* and *YAPI*. *ATRI* and *FLRI* are transporter proteins, known to confer resistance towards components in lignocellulosic hydrolysates when overexpressed, especially towards HMF and coniferyl alcohol [187]. *YAPI* is a transcription factor involved in the transcription of *ATRI* [191] and *FLRI* [192]. It is also known to be active in the response to various stresses, e.g. carbon starvation [193] as well as oxidative stress [88,194]. Moreover, it has also been described as the main responsible regulator of oxidoreductases involved in conversion of aldehyde inhibitors [95]. Furthermore, *YAPI* has been shown to confer resistance to spruce hydrolysate when overexpressed [187].

The gene expression analysis performed by qPCR showed that the expression of *YAPI* was 50% higher already in the non-inhibitory medium for *S. cerevisiae* CCUG53310 compared to CBS8066. When subjected to an inhibitory medium, however, the level generally increased more for CBS8066 (see Figure 2, Paper I). This indicated that CCUG53310 did not experience the change to an inhibitory medium to be quite as stressful as the reference strain did. CCUG53310 thus required a smaller adaptation, and could tolerate the inhibitory medium much easier, since the cells were better prepared initially. However, increased expression of *YAPI* was not observed for the medium with carboxylic acids. Thus, it is plausible that *YAPI* does not play a major role in the tolerance towards the acids.

The expression of *ATRI* correlated rather well with the fermentation performance in the various media, except for the medium with carboxylic acids. For CBS8066, its expression was increased in the furan aldehydes, hydrolysate and phenolics media, whereas for CCUG53310 the expression was only increased in the phenolics medium, which was inhibitory to the strain. Carboxylic acids in the medium led to decreased expression of *ATRI* in both strains. The expression of *FLRI* was strongly induced by furan aldehydes, observed in both the furan aldehydes and hydrolysate media. This indicated that Flr1p was involved in the transport of these compounds over the membrane in some way. This is possibly true also for some phenolic compounds, as the expression of *FLRI* was also increased in the medium with phenolic compounds. As was

the case for *YAPI*, the expression of *FLR1* was higher in non-inhibitory medium for CCUG53310, with smaller increases in the expression level compared to CBS8066 when subjected to inhibitory media.

In addition to the inhibitor tolerance, the strain *S. cerevisiae* CCUG53310 has another remarkable feature. It is also strongly and constitutively flocculating, forming dense cell flocs of several millimetres in diameter during cultivations in shake flasks. This led to the hypothesis that the flocculation in itself was one of the reasons for the superior qualities observed in this yeast strain. However, the comparison with the non-flocculating CBS8066, which has a different genetic background, did not allow conclusions to be drawn about the role of flocculation for increased inhibitor tolerance. The flocculation of *S. cerevisiae* CCUG53310 was also investigated at the transcriptional level, looking at the occurrence of flocculation genes. It was found that *FLO8* and *FLO10* were active in CCUG53310. However, none of the major flocculation genes, *FLO1*, *FLO5* and *FLO9* could be detected with certainty [Paper I]. Specific probes have to be used for their detection since the high similarity between these genes makes the use of general SYBR green chemistry difficult [169]. However, the probes used in Paper I did not enable detection of any genes. The probes were designed from the sequences of the *S. cerevisiae* S288C genes, which most likely differed too much from the genes in CCUG53310. Experiments using SYBR green chemistry detected significant amounts of gene product using the primers for *FLO5*. However, more than one PCR product was obtained in this case, as observed on the dissociation curve.

In further experiments, a novel flocculation gene was isolated from the genome of CCUG53310 using primers designed for *FLO1* [Westman et al., unpublished data]. Sequencing and phylogenetic analysis showed similarities between the gene and *FLO1*. However, it did not show a Flo1 phenotype when overexpressed in *S. cerevisiae* CEN.PK 113–7D. Instead, the yeast displayed a NewFlo flocculation phenotype, inhibited by several sugars (Figure 14). These results showed that *S. cerevisiae* CCUG53310 must have additional flocculation genes at its disposal, since CCUG53310 itself displayed a Flo1 flocculation phenotype, inhibited by only mannose.

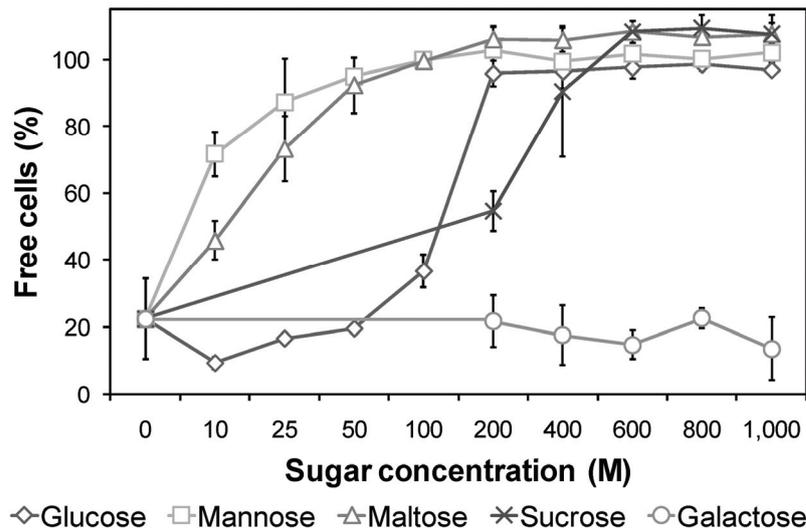


Figure 14 A novel NewFlo type flocculation gene. The flocculation gene isolated from *S. cerevisiae* CCUG53310 was expressed constitutively in *S. cerevisiae* CEN.PK 113–7D. The resulting flocculation was inhibited by mannose, maltose, glucose and sucrose, but not galactose. This means that it exhibited a NewFlo phenotype. *S. cerevisiae* CCUG53310 on the other hand, shows a Flo1 phenotype, with inhibition of flocculation only by mannose. [Westman et al., unpublished data.]

6.2 Inhibitor tolerance of encapsulated cells

Encapsulated yeast has been shown to have certain advantages over yeast suspended freely in the medium they ferment. For example, it has been shown that encapsulated yeast could successfully ferment lignocellulosic hydrolysates that were too toxic for suspended cells to ferment, in both batch and continuous systems [8,134]. However, the reasons for the inhibitor tolerance and its specificity were not elucidated. Talebnia and Taherzadeh [141] investigated the macromolecular composition of the encapsulated yeast during sequential batches. They observed an increased accumulation of trehalose and glycogen in the cells, as well as decreased protein and RNA levels. These are indications of decreased growth, which can be expected for encapsulated yeast, where both the space and availability of nutrients may be limited.

To further elucidate the source of the inhibitor tolerance obtained through encapsulation of yeast, batch fermentations with encapsulated yeast in different media were performed and compared with data from suspended yeast at similar initial cell concentration [Papers I & II]. The chosen levels of inhibitors gave similar inhibition levels for the suspended cells in the medium with furan aldehydes and carboxylic acids [Paper I]. While

consuming the first 12 g/l glucose, the cells could reach approximately 40% of the glucose consumption rate obtained in the non-inhibitory defined glucose medium (DGM) (Figure 15). It could be observed from the results of the fermentation that encapsulation of the cells increased the tolerance against the furan aldehyde inhibitors (Figure 15). The glucose consumption rate was close to that in the non-inhibitory medium, 80% as fast, compared to 40% as fast for the suspended cells. In the medium with carboxylic acids, on the other hand, the cells were equally inhibited as the suspended cells. These results showed that encapsulation did not induce a general inhibitor tolerance, but was somehow specific with regard to which inhibitors it aided against.

The most likely reason for the specific increase in tolerance towards inhibitors upon encapsulation is differences in diffusion properties of the compounds over the membrane. An example where the capsule membrane was the reason for the increased tolerance towards a compound is the fermentation of orange peel hydrolysates. The strongly inhibitory compound limonene is too hydrophobic to diffuse through the hydrophilic capsule membrane [195]. However, the carboxylic acids: formic, acetic and levulinic acid and the furan aldehydes: furfural and HMF, as well as glucose, all displayed similar diffusivities through the capsule membrane (Figure 16). The slight differences could tentatively be linked to the molecular size of the compounds, with faster diffusion for

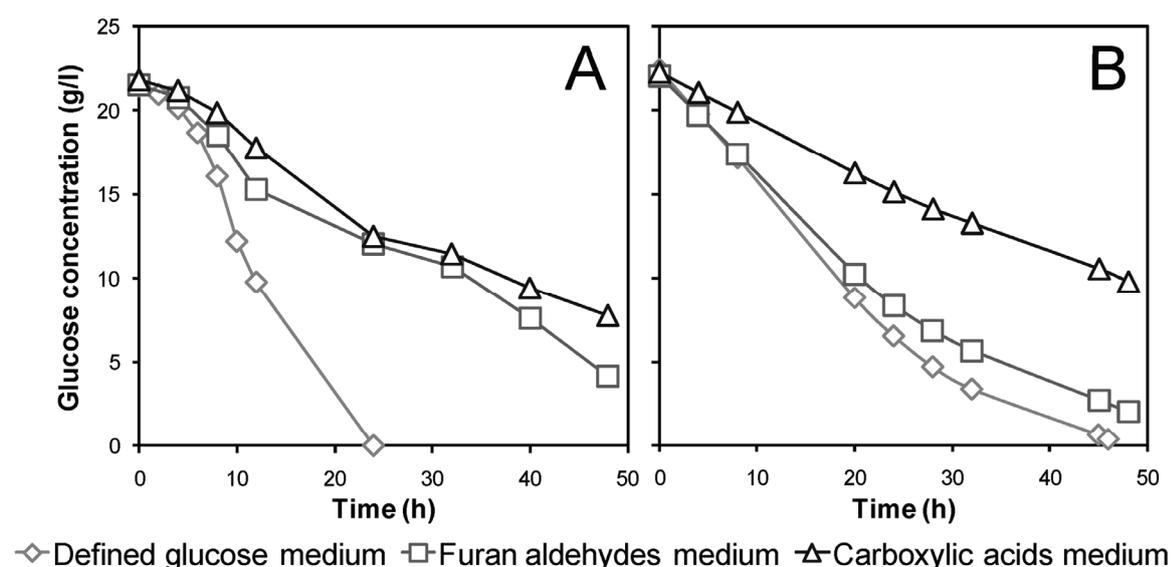


Figure 15 Comparison of suspended and encapsulated yeast. Glucose consumption profiles of **A)** suspended and **B)** encapsulated *S. cerevisiae* CBS8066 in different media showed that encapsulation increased the tolerance towards the furan aldehydes, but not against the carboxylic acids. [Data from Papers I & II.]

smaller molecules.

The small differences in diffusion of the inhibitors through the membrane made it possible to rule that out as the reason for the differences in acquired tolerance upon encapsulation. Instead, the properties of the two different classes of inhibitors were considered. The furan aldehydes can be easily and rapidly converted into less inhibitory compounds *in situ* by *S. cerevisiae*. Anaerobically, this is done to its respective alcohol [99,108]. The acids, on the other hand, are not readily converted anaerobically [196].

If one imagines a cell pellet in the capsule, one can visualise how the cells close to the membrane may convert an inhibitor into less inhibitory compounds. Thus, cells closer to the core of the cell pellet will be left with subinhibitory levels, since a gradient of the inhibitor concentration will appear (Figure 17). A reaction-diffusion system will be formed in this case, where the conversion is tentatively limited by diffusion into the capsules and through the cell pellet. Due to diffusion limitations, cells closer to the core of the cell pellet will face subinhibitory concentrations of the inhibitors and thus be protected. A prerequisite for this is that the inhibitor in question is converted rapidly enough. An inhibitor that is not converted rapidly will give rise to a concentration gradient only initially, due to mass transfer limitations. When a non-convertible inhibitor

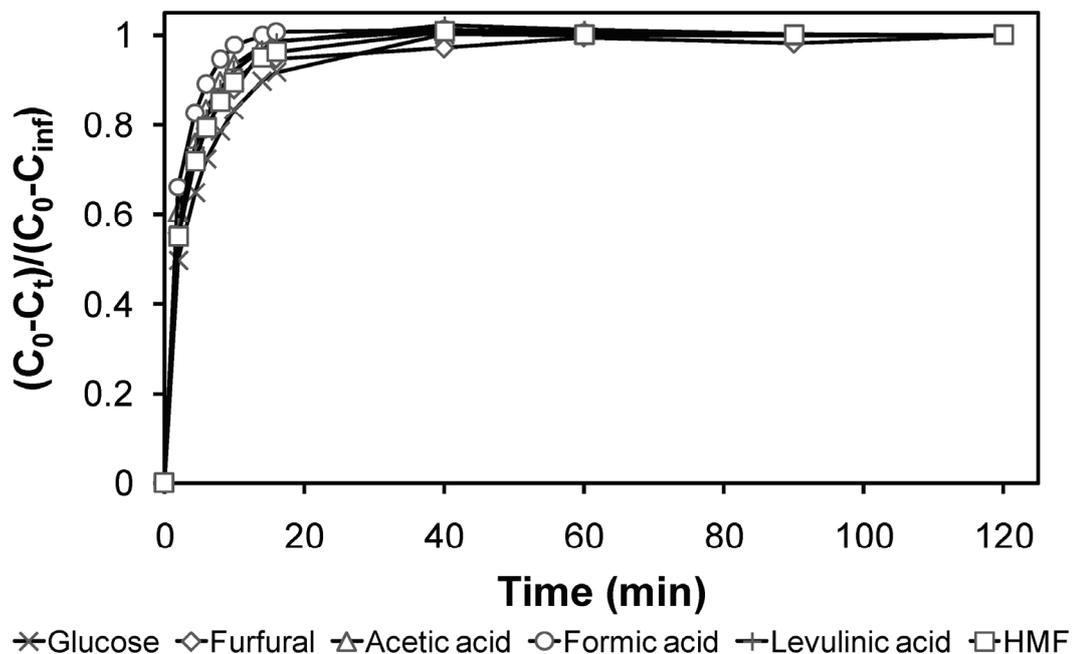


Figure 16 Diffusion of inhibitors and glucose through the capsules' membranes. The diffusion of glucose and various inhibitors over the capsule membrane was followed over time and shown to have similar profiles. [Data from Paper II.]

has penetrated the whole cell pellet, it will be present at a constant concentration and thus be equally inhibitory for all cells in the capsule. Obviously, no significant increase in tolerance can be obtained from the encapsulation in this case.

To further investigate the protective effect of encapsulation, the expression of the same three stress-responsive genes investigated in Paper I were analysed in the encapsulated yeast. The expression profiles, when compared to the data from the free *S. cerevisiae* CBS8066 cells from Paper I, showed clearly that encapsulated yeast had higher transcript levels of the genes already in the non-inhibitory medium (Figure 18). *YAPI*, in particular, showed a rather similar level of expression in all different media for the encapsulated

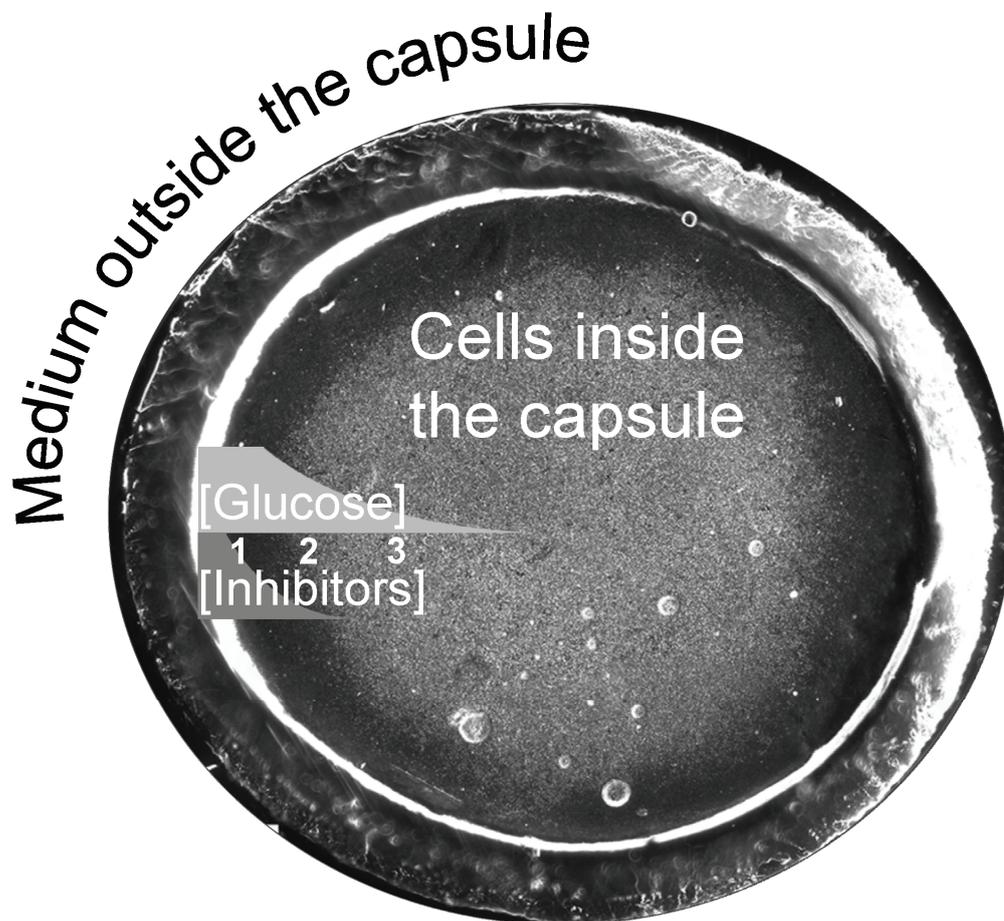


Figure 17 Schematic representation of the hypothesised concentration profiles in the cell pellet of the capsules. Radial concentration gradients through the cell pellet in a medium with convertible inhibitors, such as furan aldehydes, give rise to differences in cell physiology inside the capsules. The numbers along the radius in the cross-section of the capsule indicate different cell ‘populations’: 1. Non-fermenting cells that convert inhibitors; 2. Cells with triggered stress response, which ferment, grow and convert inhibitors; 3. Slightly starvation-stressed cells that ferment the medium unaffected by the inhibitors. [Figure adapted from Paper II.]

cells. The level was slightly higher in the hydrolysate medium, but significantly higher in the non-inhibitory medium compared to the suspended cells. The higher initial expression of *YAPI* showed that the cells sensed a stress from the encapsulation, which was then beneficial for the cells as they were subjected to a more inhibitory medium. This indicated that the Environmental Stress Response (ESR) was activated in the encapsulated yeast [197,198]. The ESR is common for many stresses, and through stress cross-tolerance, the cells with the ESR activated will cope better with a second stress. Stress cross-tolerance is further discussed in section 6.4. The increased readiness through activation of the ESR can only do so much to help the cells in coping with increased stress. A constant carboxylic acids stress, which cannot be relieved through conversion of the acids to less inhibitory compounds, was obviously too harsh for the cells to cope with.

The largest difference between the suspended and encapsulated yeast was found in the expression of *FLRI* in the furan aldehydes medium. The transcript level of *FLRI* was only slightly increased in encapsulated yeast, whereas the level was vastly increased for the suspended yeast. This difference can be explained by the fact that cells that do not experience higher levels of furan aldehydes will not increase the transcription of the *FLRI* gene. Due to the concentration gradients of furan aldehydes through the cell pellet, there will be cells that express *FLRI* at different levels. Only the cells closest to the membrane will experience a similar concentration of furfural as the freely suspended cells do. The average cell in the community of encapsulated cells will thus not show as large changes in the transcription as the suspended cells, where all cells face the same levels of the inhibitor.

Most likely, the gradients of furan aldehyde concentrations through the cell pellet were less steep in the hydrolysate medium compared to the furan aldehydes medium, due to additional inhibitory compounds affecting the cells. Less steep gradients would lead to higher expression of *FLRI* in a larger proportion of the cells inside the capsules. Indeed, the difference in *FLRI* expression between the encapsulated and the freely suspended cells was less pronounced in the hydrolysate medium (Figure 18 E, F).

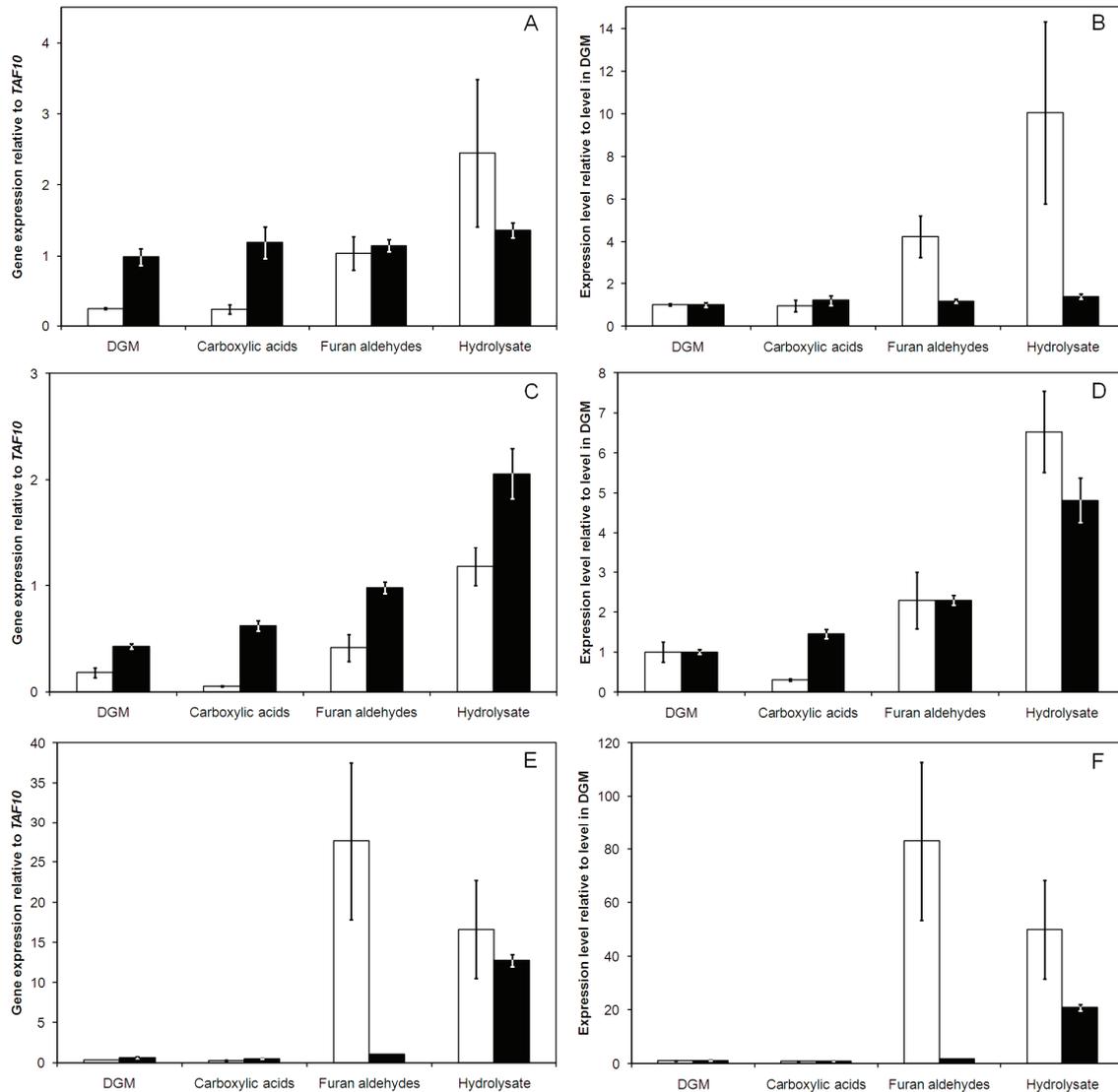


Figure 18 Gene expression analysis showed encapsulation induced stress. Gene expression in different media after two hours of anaerobic batch cultivations for free yeast (white) and encapsulated yeast (black). Expression of *YAPI* (A), *ATR1* (C) and *FLR1* (E) relative to the level of the reference gene *TAF10* show that the transcript levels were higher in encapsulated yeast in non inhibitory medium (DGM). The expression changes of *YAPI* (B), *ATR1* (D) and *FLR1* (F) in inhibitory media relative to the expression level in DGM show that the changes were generally larger for the suspended yeast. [Figure adapted from Paper II.]

The source of the slight stress level that the cells sensed from being encapsulated was at this point unknown. However, it was hypothesised to come from starvation of cells closer to the core of the cell pellet inside the capsules. Starvation would be caused by nutrient limitations arising from mass transfer limitations, as well as consumption of nutrients by cells closer to the membrane of the capsule. However, in order to verify this theory, a more in depth analysis of the physiology of the encapsulated cells had to be performed.

6.3 Proteomic analysis reveals starvation-induced stress

As described in section 3.5, genome wide investigations can be performed on transcript, mRNA, or protein level. When it came to performing a genome wide physiological investigation of encapsulated yeast, proteomics was chosen [Paper III]. However, no analysis of post-translational modifications (PTMs) was performed. A comparison of two different proteomic methods was performed: a chromatography and mass spectrometric method (nLC-MS/MS) and a gel based two dimensional difference gel electrophoresis method (2D-DIGE).

In the chromatographic method, the proteins in a sample are first digested into peptides by an enzyme. The peptides are thereafter labelled with molecules of a specific molecular weight, that is, mass tags. Different samples are labelled with molecules of slightly different weight. Thereafter, the samples are pooled and the peptides fractionated using chromatographic methods. Using mass spectrometry and data base searches, the proteins that the peptides are derived from are subsequently identified. By comparing the signals from the different mass tags, the relative abundance of the proteins in different samples can be determined.

In the gel based method, proteins in different samples are first labelled with different fluorescent dyes. The samples are thereafter mixed and the proteins are separated on a gel in two dimensions. In the first dimension, the proteins are separated according to their charge, and in the second dimension according to their molecular weight. By scanning with the excitation wavelength of each fluorescent dye, the signal from each protein spot can thereafter be detected and compared. The differences in strength between the different fluorescent signals correspond to differences in abundance of the proteins in the samples. However, in order to identify the proteins on the gel, the spots have to be individually picked and analysed by mass spectrometry and database searches.

The two methods used in Paper III showed similar results, with the number of identified proteins constituting the major difference. The method utilising chromatographic separation, with online mass spectrometric measurements, resulted in the quantification of a significantly larger number of proteins than the gel based method. The chromatographic method is also significantly more sensitive than the gel based method. The gel based method mainly detects highly expressed glycolytic proteins, while the

chromatographic method also detects proteins with low expression. An advantage with the 2D-gels is that they facilitate an easier analysis of PTMs. Proteins with changes in pI due to PTMs will be located next to each other on a gel, since the changes in molecular weight are small, while the changes in pI can be significant [125]. However, this analysis lay outside the scope of Paper III and this thesis, and the comparison of the two methods will not be further discussed in the thesis.

Cultivations for the proteome analysis were performed anaerobically in defined medium without inhibitors. Fifty capsules were used as inoculum in cultivations with 100 ml medium, with 40 g/l glucose as carbon source. Freely suspended yeast was precultivated in a similar way as the encapsulated, and the anaerobic batches were started with the same amount of initial biomass.

From the glucose consumption profiles, it was clear that the encapsulated cells were significantly slower at consuming the sugar present in the medium. Therefore, it was judged that a specific glucose level would be more appropriate for sampling of the cells for proteome analysis, rather than a specific time point. At a specific time point, the cells would be in different states due to different glucose availabilities in the medium. The proteome analysis was therefore performed on cells taken at very different time points. The glucose levels were, however, similar at 12.6 ± 0.7 and 15.8 ± 0.1 g/l for the encapsulated and freely suspended cells, respectively. Thus, a compromise between the residual glucose level and the total cell amount in the cultivations was made. The measured biomass yield was significantly lower for the encapsulated compared to the free cells at 41 ± 3 and 74 ± 2 mg DW produced per gram glucose consumed, respectively.

Out of the 842 identified proteins, the proteome analysis using nLC-MS/MS yielded 116 significantly up-regulated and 95 significantly down-regulated proteins in encapsulated compared to freely suspended yeast. It was not unexpected that such a large number of the identified proteins (25%) would show an altered expression, considering the significantly different environments experienced by the cells, although the medium and other experimental variables were the same in both cases.

The most prominent change detected in the encapsulated yeast was the increased expression of glucose repressed proteins. The strongest up-regulated proteins in the

encapsulated yeast were the high affinity hexose transporters Hxt6p and Hxt7p (Figure 19). It is well known that these transporters are normally highly expressed only at extracellular concentrations of glucose of less than 5 g/l, i.e. at glucose concentrations below those where the cells were sampled [199-201]. Certain enzymes, such as e.g. Hxk1p and Glk1p, which are known to be glucose repressed, were also up-regulated [202]. Taken together, this verified the hypothesis from Paper II that cells inside the capsule experienced a lack of glucose and hence starvation. The trehalose and glycogen pathway was significantly up-regulated, as expected from the findings of Talebnia and Taherzadeh [141], that the intracellular trehalose and glycogen levels increased upon sequential batches with encapsulated yeast (Figure 19). These two carbohydrates are important storage carbohydrates in yeast, accumulated mainly in starved or slow-growing cells [85]. However, trehalose is also important as a protector of proteins and membranes against stresses such as starvation, cold and heat [203].

Down-regulated proteins were often involved in protein synthesis and in transcription (see Figure 4, Paper III). This was not unexpected, as the biomass yield was decreased for encapsulated yeast compared to freely suspended, as observed also in Paper II. It has also been observed that the proportions of both RNA and protein decrease in encapsulated cells upon prolonged cultivation [141]. In contrast to these findings, levels of ribosomal proteins have been observed to be up-regulated in cells immobilised in alginate gel beads [204]. This shows that there are physiological differences between cells encapsulated in liquid core capsules and cells immobilised in porous gels.

The expression of stress response proteins was of special interest to study because of the seemingly starvation stressed cells. Genes under the control of the transcription factors Msn2p and Msn4p were enriched among those encoding the up-regulated proteins in the encapsulated yeast compared to the suspended yeast. These transcription factors are important parts of the ESR [197,205,206]. The occurrence of up-regulated proteins under control of Msn2/4 further strengthens the hypothesis from Paper II, which stated that the cells sensed a low stress level. Specifically, proteins involved in the heat shock response were up-regulated. Together with the elevated trehalose levels this explained the increased tolerance towards elevated temperatures observed by yeast encapsulated in alginate-chitosan capsules [46]. It has also been reported that the heat shock response and progression into stationary phase are similar on the gene transcription level [197].

Furthermore, it has been suggested that slow growth might be perceived as ‘stress’ by the cells, similar to starvation, and lead to heat-shock resistance [207]. This is further discussed in section 6.4.

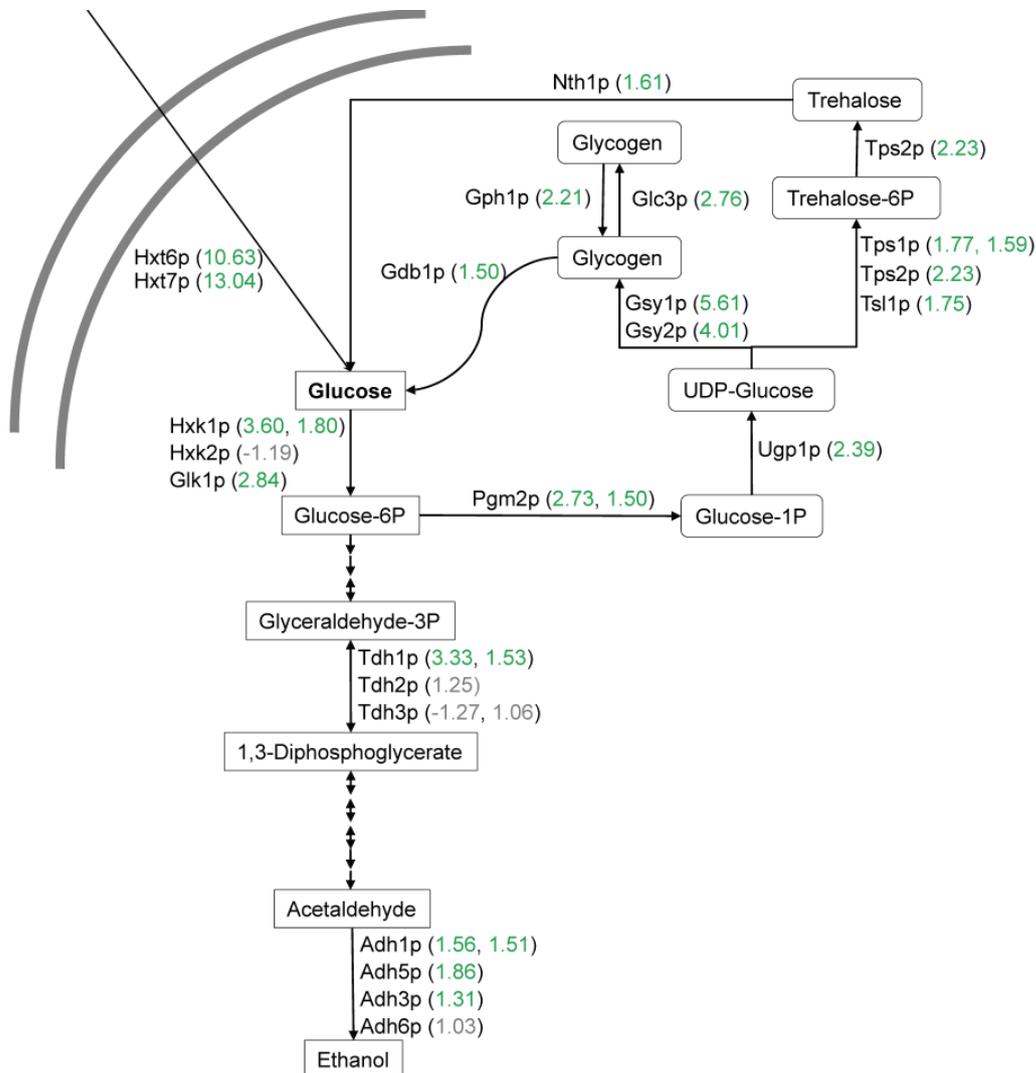


Figure 19 Proteomic changes upon encapsulation of *S. cerevisiae* revealed glucose starved cells. The high affinity hexose transporters Hxt6/7p were strongly up-regulated in encapsulated yeast. This indicated that the cells sensed significantly lower glucose levels than the more than 10 g/l present in the medium outside the capsules. The proteins involved in trehalose and glycogen synthesis were also strongly up-regulated, as a response to the carbon starvation. The fold change (encapsulated cells compared to suspended cells) is shown in parentheses next to the proteins, with significant up-regulation shown in green. The first numbers in the parentheses are the ratios obtained by n-LS-MS/MS and the second numbers are from 2D-DIGE, where applicable. [Figure adapted from Paper III.]

Several of the proteomic responses observed in the encapsulated yeast were similar to what has been observed in previous studies on yeast cells treated with furfural [103,104]. Based on the fact that these changes are evident in the encapsulated cells already before they are subjected to furfural, it is plausible that the cells are better prepared to handle the addition of furfural to the medium. The increased expression of alcohol dehydrogenases was of special importance for the observed increase in tolerance towards furfural by the encapsulated yeast (Figure 19). These enzymes are, as mentioned in Chapter 3, responsible for reduction of the furan aldehydes to less inhibitory alcohols under anaerobic conditions [99]. Up-regulation of chaperones was also observed in both furfural treated cells and encapsulated cells [103].

6.4 Stress cross-tolerance and stress resistance by slow growth

The existence of stress cross-tolerance in *S. cerevisiae* has been shown in numerous publications, e.g. [90,208-211]. Cross-tolerance means that after being subjected to one stress, the tolerance towards a second stress is increased. This often includes the expression of heat shock proteins, hsps, as well as intracellular accumulation of trehalose. The actual stress responses are specific for each stress, but the overlap between the responses are substantial, as observed with new technologies. With microarray technology, genome wide expression analysis of responses to various conditions led to a deeper understanding of the stress responses of *S. cerevisiae* [197,198,212]. It was found that more than half of the genome was involved in responses to various environmental changes and, more importantly, a subset of ~10% of the yeast genes was common to all responses, showing both up- and down-regulation. The studies emphasised the importance of the transcription factors Msn2p/Msn4p, which were also observed to be activated in the work in Paper III. These two homologous transcription factors were initially thought to be functionally redundant [206]. However, it has now been shown that they have specific roles in the response to different stresses. Often, both are required for full induction of gene transcription [205].

It has been shown that stress cross-tolerance is, at least some times, based on asymmetrical anticipatory regulation [213]. Thus, a certain stress might lead to protection against a second stress, but not vice versa. An example of asymmetrical anticipatory regulation is heat shock and ethanol stress, which both lead to protection against

oxidative stress. The stresses are unrelated with regard to their effect on the cells, but follow each other naturally during wine making. Hence, the one way cross-protection is likely an evolutionary evolved trait.

It has been observed that a large number of the genes classified as involved in stress responses are actually affected in similar directions by a decrease in growth rate, which is a natural effect of most stresses [124,214]. This implies that at least a part of the expression changes in the ESR might not be direct responses to the stresses, but rather effects arising from the change in growth rate [124,214]. It has indeed been shown that slow growth in itself leads to heat-shock resistance in *S. cerevisiae* [207], a link that was first observed in 1993 [215]. However, approximately as many genes displayed expression changes correlated to only heat, as those whose expression was correlated to both growth rate and temperature changes. The ESR growth rate relationship is thus not true for all expression changes [207]. Further investigations of slow growth have recently shown that it is a mediator of resistance also to various other stresses [216]. Chemostat cultures at low dilution rates were observed to be significantly more resistant to oxidative stress, heat stress, as well as acetic acid stress at pH 3, compared to cultures at higher dilution rates. However, in this case the survival rate was determined after the cells had been subjected to the stress for 10 minutes. This may not be relevant to the performance in fermentations with extended periods of stress, such as the batch cultures in this thesis.

With decreased growth rates due to gradients of nutrients in the capsules, it is understandable that the encapsulated yeast will have an increased stress resistance. Encapsulated cells subjected to a medium with convertible inhibitors will show an improved fermentation performance compared to free cells, partly thanks to the increased stress resistance. The initial stress resistance helps the cells to survive while they convert the inhibitors. However, when continuously subjected to non-convertible inhibitors, they may not exhibit an overall improved fermentation performance. In this case, the stress is not relieved by conversion of the inhibitors into less inhibitory compounds. An analogy can be the use of sunscreen on a sunny day. If you apply sunscreen in the morning, it helps to protect your skin while pigment is produced, which decreases the stress from the sun. This is analogous to conversion of inhibitors, although with the inhibitors, the stress is decreased instead of the protection increased. However, if you are unable to produce pigment, the sunscreen cannot help you very long if you do not continuously apply more.

In the specific case of the acids, anion accumulation inside the cells or ATP depletion could be reasons why the increased stress resistance cannot help against a long-term continuous stress. The cells are not able to adapt themselves enough against the high concentrations of the acids, regardless of the initial state of readiness of the cells. Against other stresses that cannot be 'detoxified' by the cells, the stress response from encapsulation might help even in the long-term. This would happen if the cells can be protected by the increased stress tolerance while additional, more specific, stress responses are developed. Further experiments are needed to elucidate whether this is the case. For temporary perturbations, e.g. in an industrial process, encapsulation of cells would likely increase the robustness of the system almost regardless of the source. Such perturbations could be e.g. an accidental temperature increase, or a pulse of a non-convertible toxic compound into a continuous process. The compound would be washed out before long, while the cells are retained. In all, it is likely that changes occurring in the cells due to encapsulation, or rather from growth inside the capsules, lead to a more robust system. At least some cells, dependent on concentration gradients along the radius of the cell pellet, will be better prepared to handle additional stresses.

6.5 Improved xylose and glucose co-consumption by encapsulated cells

The results and hypothesis from Papers II & III, gave rise to an additional hypothesis. It was hypothesised that the gradients through the cell pellet would increase not only the tolerance towards convertible inhibitors, but also the ability of the yeast population to simultaneously utilise different sugars.

As mentioned in Chapter 3, *S. cerevisiae* has a natural preference for glucose as a carbon source, with a strong repression of the utilisation of most other energy sources, as long as there is glucose present [60,217]. This is an issue when it comes to fermentation of lignocellulosic hydrolysates, which contain various different sugars (e.g. the hexoses glucose, mannose and galactose, and the pentoses xylose and arabinose). The total fermentation time becomes quite long if the sugars are utilised sequentially. Recombinant *S. cerevisiae* with the ability to utilise xylose, first consume the glucose. Not until most of the glucose is consumed will they start to utilise the xylose present [75,76,78,79,84,218,219]. This sequential utilisation is mainly an effect of both sugars being transported into the yeast cells by the same transporters, the hexose transporter

system. These transporters have a significantly higher affinity for glucose than xylose, which is why glucose transport is preferred as long as glucose is present near the cells [200,220-222]. However, what could plausibly be the case for encapsulated yeast is that there will be cells that experience a high, an intermediate, a low and zero concentrations of glucose, present in a cultivation at the same time. This gradient of glucose would be present despite a high ‘extracapsular’ concentration of glucose in the medium. As observed in Paper III, the expression of the high affinity hexose transporters, Hxt6p and Hxt7p, was increased in cells inside the capsules, despite high levels of ‘extracapsular’ glucose. Normally, these transporters are only expressed at low levels of glucose [199,201]. The cells that expressed Hxt6p and Hxt7p at high levels were most likely cells closer to the core of the capsules. Cells closer to the surface of the capsules plausibly expressed more of the low affinity transporters instead. The transporter Hxt7p (and Hxt6p by homology) has been shown to be a significantly better xylose transporter than e.g. Hxt1 [221], which is expressed at higher levels of glucose [199]. A low concentration of glucose has also been observed to be beneficial for maximal xylose utilisation [75,200]. It has been speculated that glucose metabolism leads to production of intermediary metabolites for the initial steps of xylose metabolism and the pentose phosphate pathway [78]. In traditional batch fermentations, the period with a low level of glucose will be very short. For encapsulated yeast, however, there will most likely be cells already in the beginning of a batch that experience a high xylose concentration and a low glucose concentration simultaneously. With this background information, it was hypothesised that encapsulation of a xylose utilising yeast strain would increase the simultaneous co-utilisation of glucose and xylose. Cells close to the surface of the capsule will consume more glucose, so that cells closer to the core can utilise more xylose. This was simulated by finite element modelling [223]. The results showed that encapsulation would indeed improve the simultaneous utilisation of glucose and xylose, especially in the case of a medium that contained convertible inhibitors. This would mainly be an effect of the concentration gradients of the sugars, which led to different uptake rates by cells in different parts of the capsules (see Figure 2, Paper IV).

To experimentally investigate the notion of increased simultaneous hexose and pentose co-consumption, the recombinant xylose fermenting *S. cerevisiae* CEN.PK XXX, developed at Chalmers University of Technology by Dr Nicklas Bonander, was encapsulated [Paper IV]. Following the results of the *in silico* simulations, the yeast was

examined for its xylose and glucose co-utilisation in the presence and absence of the inhibitor furfural. Media with single carbon sources and furfural were also used in anaerobic batch experiments for comparison. For the encapsulated yeast, in some fermentations there were cells that grew outside of the capsules. This happened since cells escaped from occasional broken capsules during the precultivations. These cells clung to the capsules used in the anaerobic batch fermentations despite thorough rinsing. However, the occurrence of free cells was not believed to have influenced the conclusions drawn from the study. Rather, the difference between the systems is believed to have decreased.

The freely suspended recombinant *S. cerevisiae* CEN.PK XXX was able to utilise xylose anaerobically as the sole carbon source. However, the rate was significantly slower compared to when only glucose was fermented. When a mixture of glucose and xylose was fermented, the glucose was consumed rapidly, with only a small fraction of the xylose consumed during the glucose consumption phase. After glucose depletion, a small part of the xylose that remained was consumed. This phase was followed by an almost ceased consumption at a residual xylose concentration of approximately 30 g/l, around 24 hours after consumption of the last glucose. From this point on, only a very slow consumption was observed until the fermentations were stopped at 96 h. During the glucose consumption phase, there was almost no accumulation of xylitol, but a high production of glycerol. The cells could thus balance the need for reoxidation of NADH by glycerol production (Figure 20). Upon depletion of the glucose, the balances shifted. Xylitol started to accumulate and the cells could likely not adapt their metabolism fast enough to the changed conditions (Figure 20). Since neither NAD(P)H, NAD(P)⁺, ATP, ADP, nor PPP metabolites were quantified, the reasons for the slow xylose consumption after glucose depletion can only be hypothesised. However, decreased xylose consumption under anaerobic conditions after exhaustion of the glucose has been reported previously [78,81]. The reason for the slowed or stopped consumption has been hypothesised to be caused by redox imbalance, or the fact that glucose consumption provides important intermediary metabolites for xylose consumption. For example, there might be a shortage of NADPH, which is generated through the oxidative PPP, or NAD⁺, generated from glycerol production. It is also possible that ATP was depleted by e.g. too high xylulokinase activity [224]. However, it was evident that the cells could not adapt their metabolism fast enough to maintain stable conditions while fermenting the

remaining xylose in the batch fermentation. In the medium with only xylose as the carbon source, the consumption of xylose continued throughout the batch. The reason for this could be that the cells in this case had a slower metabolism already from the start of the batch. The cells' metabolism was thus better adapted for xylose utilisation. Thanks to the adapted metabolism, the cells were able to balance the production of glycerol from xylose, with maintained cell growth and ethanol production. However, there was significant accumulation of xylitol, showing that there was also a redox problem in this case.

Encapsulation of the xylose fermenting yeast completely changed its performance. First, as described previously in this thesis for another yeast strain, the tolerance towards the high level of furfural was increased. Encapsulated, the yeast was able to ferment the medium with 2 g/l furfural at only a slightly lower rate than without furfural. This medium was too inhibitory for the freely suspended yeast at the same initial cell concentration in the batch. Secondly, it was clear that the encapsulated yeast was able to ferment a significant portion of the xylose simultaneously with the glucose (Figure 20). As the glucose was depleted from the medium, the xylose consumption rate decreased, similar to what was observed for the freely suspended cells. However, the rate never decreased as much as for the suspended cells. Most probably, and supported by the *in silico* simulations, the reason for the better xylose utilisation was that many cells, closer to the core of the cell pellet, fermented xylose almost exclusively from early on in the batch cultivations. These cells, hence, had a balanced metabolism and did not experience a severe change in metabolism upon glucose depletion. The decreased xylose consumption rate would thus occur mainly for the cells closer to the membrane of the capsules. These cells would have fermented mostly glucose during the glucose phase. Similar to the freely suspended cells, there was a significant production of glycerol and accumulation of xylitol. However, xylitol was accumulated from the beginning of the batches in the case of the encapsulated yeast.

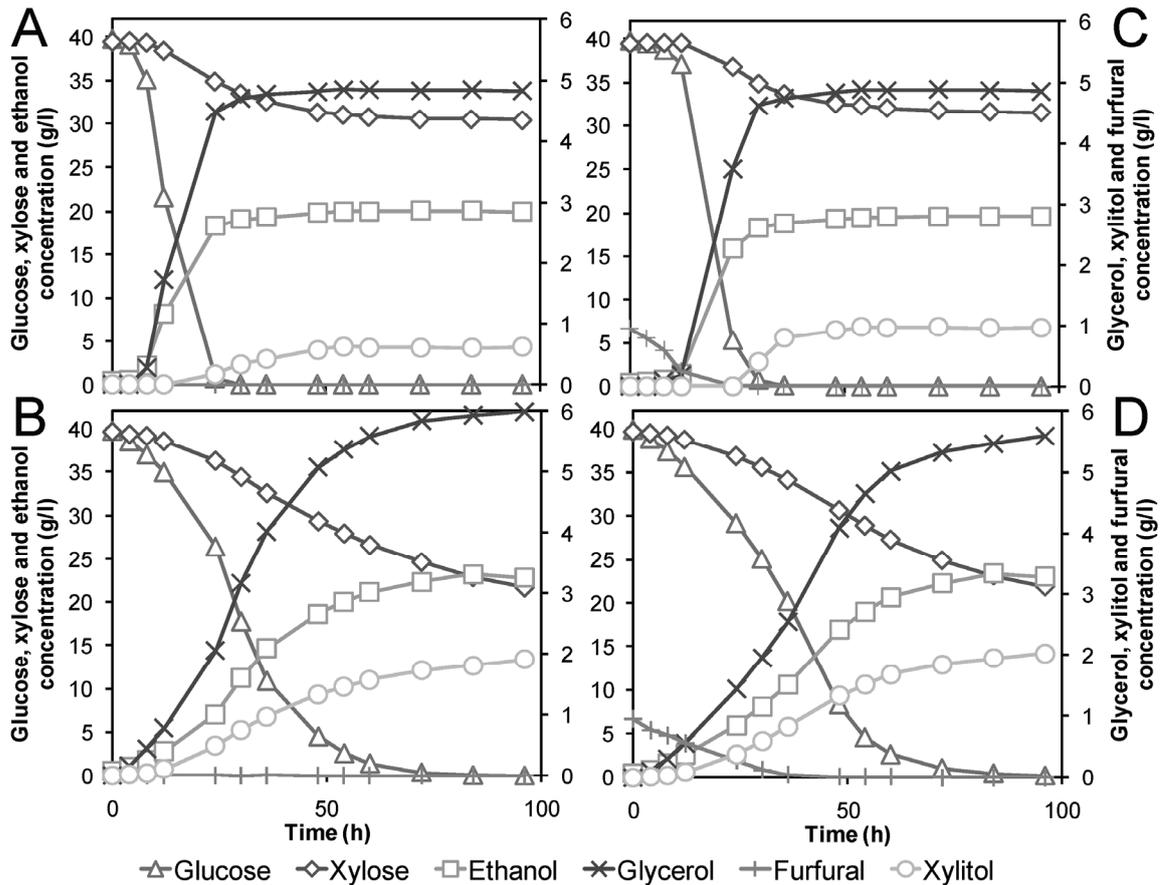


Figure 20 Improved simultaneous fermentation of glucose and xylose by yeast encapsulation. The results from the fermentations with 40 g/l glucose, 40 g/l xylose and 0 g/l (A and B) or 1 g/l (C and D) furfural in the medium showed that the freely suspended yeast (A and C) consumed the glucose present rapidly once enough of the furfural in the medium was converted. Xylose was consumed once the glucose concentration was at a low level. The encapsulated yeast (B and D) consumed glucose and xylose as well as converted furfural simultaneously, already from the start of the batch. Furthermore, significantly more xylose was consumed by the encapsulated yeast over the 96 hours that the batch lasted. Hence, higher ethanol titres were reached for the encapsulated yeast. Significant amounts of glycerol were produced to reoxidise NADH in all cases. However, xylitol accumulation occurred from the beginning of xylose consumption for the encapsulated yeast, but only once the glucose was depleted for the freely suspended yeast. [Data from Paper IV.]

Improvements in the simultaneous glucose and xylose consumption through encapsulation of the fermenting yeast were also evident from the ethanol yields per consumed glucose (Figure 21 A & B). Yields calculated from the consumption and production, between the sample points, showed that the encapsulated yeast in cultures with two sugars had a higher yield than in the reference case medium with only glucose throughout the batch. For the suspended yeast, on the other hand, substantial co-consumption was not observed until the concentration of glucose in the medium was low.

The ethanol yield for the suspended yeast was also than that theoretically possible on only glucose, 0.51 g/g, between many sample points. This showed clearly that co-fermentation of the sugars into ethanol occurred. The amount of xylose consumed per glucose consumed, also showed that the encapsulated yeast consumed more xylose simultaneously with glucose than the freely suspended yeast (Figure 21 C & D).

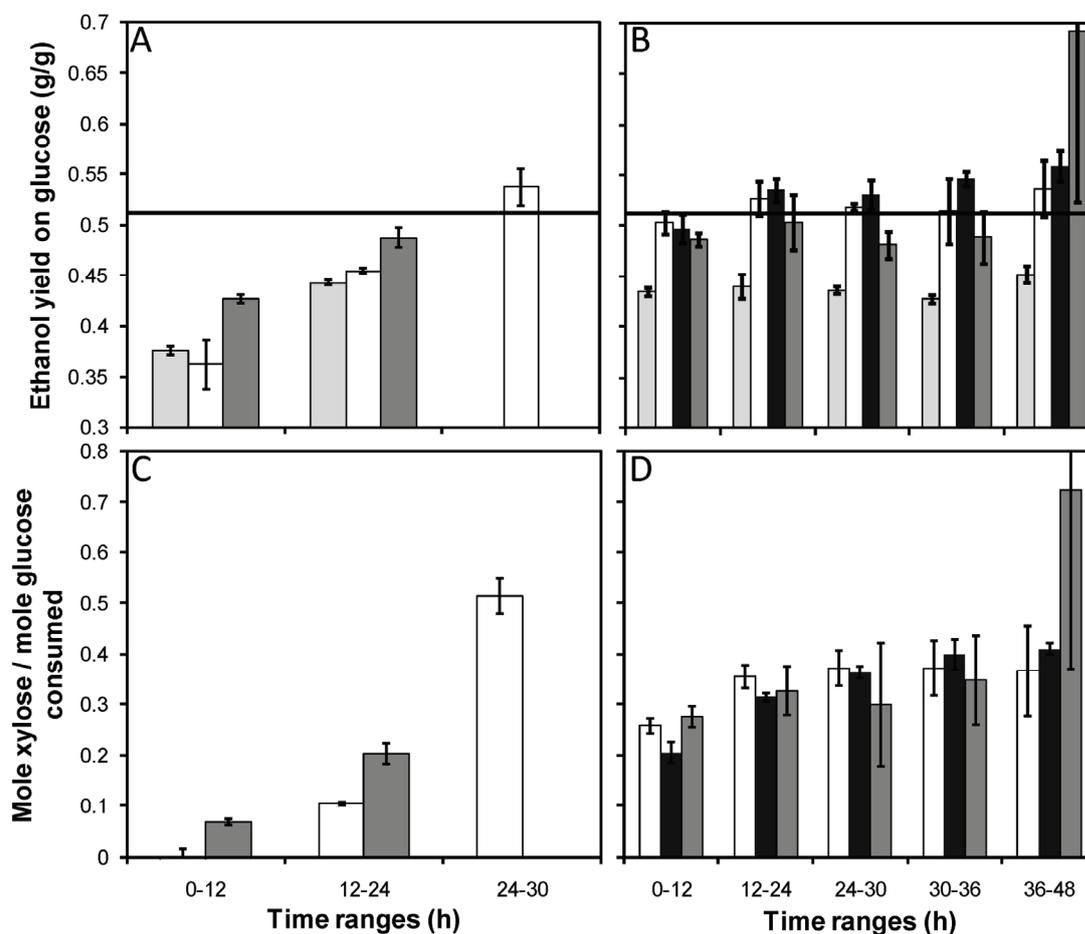


Figure 21 Improved co-consumption by encapsulation. The ethanol yields calculated per consumed glucose over time intervals (A, B) indicated a small degree of co-utilisation, which was more pronounced at low glucose concentrations for the suspended yeast (A). However, the encapsulated yeast (B) showed a higher ethanol yield already from the start of the fermentations for the mixed carbohydrate cultures compared to the culture with only glucose. It was also evident that the encapsulated yeast was able to reach yields that were higher than what is theoretically possible on glucose alone (as indicated by the black line at 0.51 g/g) over consecutive time ranges. This was not observed for the freely suspended yeast. Xylose consumed per glucose consumed over different time intervals (C, D) also showed that the simultaneous utilisation of glucose and xylose was improved significantly for the encapsulated yeast (D) compared to the freely suspended yeast (C). Medium with 40 g/l glucose and 1 g/l furfural (light grey bars); medium with 40 g/l glucose, 40 g/l xylose and 0 g/l furfural (dark grey bars); medium with 40 g/l glucose, 40 g/l xylose and 1 g/l furfural (white bars); and medium with 40 g/l glucose, 40 g/l xylose and 2 g/l furfural (black bars). [Data from Paper IV.]

As proof of the concept, a complete spruce hydrolysate was also fermented by the freely suspended and encapsulated *S. cerevisiae* CEN.PK XXX. The suspended yeast showed a long lag phase prior to sugar consumption, during which the furfural in the medium was converted (see Figure 5 B & C, Paper IV). Following furfural conversion, the cells rapidly consumed the glucose and mannose, followed by the xylose and galactose. The consumption of the latter two started at low concentrations of glucose. Also, the xylose consumption almost stopped shortly after full consumption of all hexoses, similar to what was observed in the defined media. The encapsulated CEN.PK XXX exhibited simultaneous utilisation of all sugars, except arabinose, as well as conversion of the furfural (Figure 22). Simultaneous utilisation of galactose and glucose is generally not possible in a system with suspended cells, since the expression of *GAL* genes is repressed by glucose [65]. However, with gradients of glucose in the cell pellet inside the capsules, cells that experience none or a low concentration of glucose can express the *GAL* genes. In this way, galactose and glucose can be consumed simultaneously by the system.

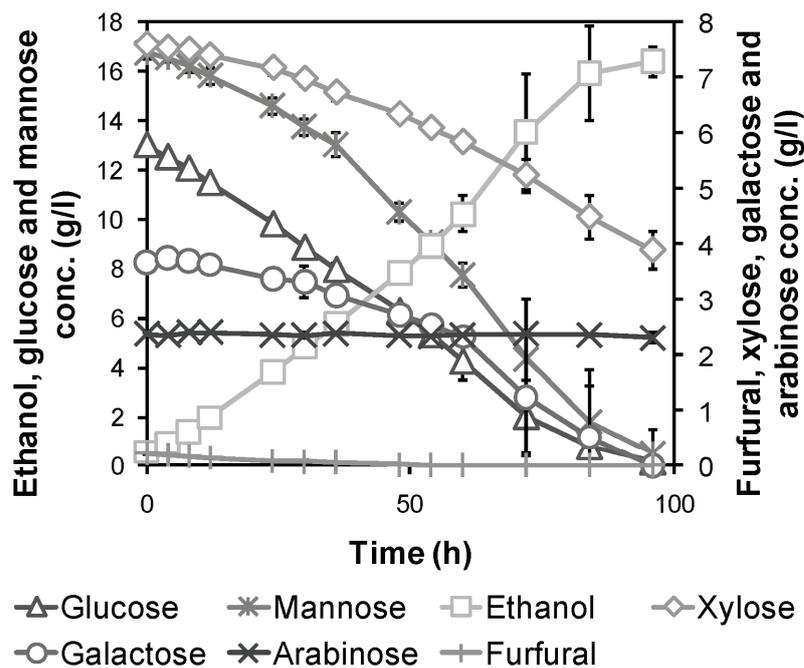


Figure 22 Simultaneous consumption of sugars in spruce hydrolysate by encapsulated xylose utilising yeast. The encapsulated *S. cerevisiae* CEN.PK XXX exhibited simultaneous co-consumption of all sugars in the spruce hydrolysate except arabinose, which it could not utilise. Concurrently the furfural was converted and ethanol produced. Although the xylose consumption was not complete, it was clear that xylose was utilised simultaneously with glucose already from the start of the fermentation. [Data from Paper IV.]

6.6 Improved inhibitor tolerance by flocculating yeast

Encapsulated yeast is at times difficult to work with in the laboratory. Due to capsules breaking from rapid CO₂ evolution as mentioned in Chapter 3, some cells can be released into the medium. This results in a mixture of suspended and encapsulated yeast. In an industrial setting, this would not necessarily have to be a major concern. Especially in a continuous process at a high dilution rate, the proportion of suspended cells would be rather small. However, it has been a major headache during this PhD project, as a lot of cultivations have had to be discarded. Furthermore, industrially, encapsulation of yeast would likely be very costly, at least in relation to the low price of fuel ethanol. It is, therefore, questionable if encapsulated yeast for 2nd generation bioethanol production could ever be made economically feasible. An alternative and simpler way to achieve high local cell density is flocculation.

Under certain conditions, yeast aggregates created during the cultivation of a flocculent yeast strain can become highly similar to the encapsulated yeast. Certain similarities were also observed on the gene expression level between encapsulated and strongly flocculating yeast (Figure 23). From Papers II & III, it was evident that the inhibitor tolerance of encapsulated yeast was an effect of high local cell density and not a direct effect of the membrane. This made a hypothesis that arose during the work on Paper I more plausible and interesting to investigate: Does flocculation in itself increase the inhibitor tolerance of *S. cerevisiae* during fermentations? In order to investigate this in a systematic manner, a strain of non-flocculating yeast had to be made flocculating, or vice versa. In this way the inhibitor tolerance of two otherwise identical strains could be compared. To this end, variants of *FLO1* were expressed in *S. cerevisiae* CEN.PK 113–7D as described in Chapter 5.

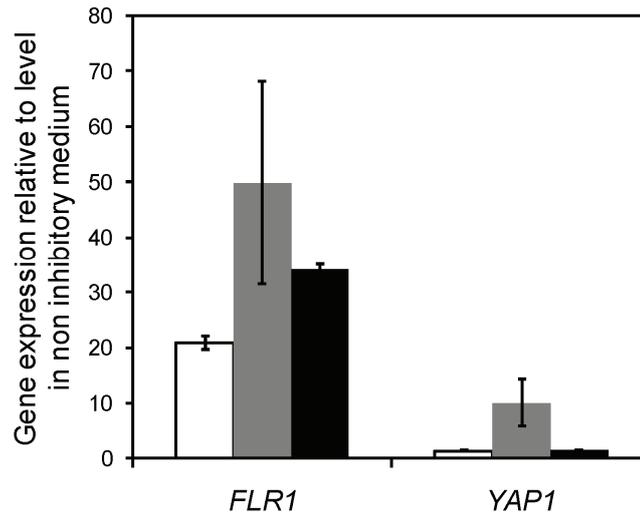


Figure 23 Gene expression changes in hydrolysate indicate a relationship between encapsulation and flocculation. Encapsulated CBS8066 (white bars), and the strongly flocculating CCUG53310 (black bars), showed similarities in the gene expression changes of *FLR1* and *YAP1*, involved in the resistance towards lignocellulosic inhibitors, when cultivated in a spruce hydrolysate compared to a non inhibitory medium. The freely suspended CBS8066 (grey bars), generally exhibited larger changes in the levels of the transcripts. [Data from Papers I & II.]

6.6.1 Batch fermentations reveal flocculation-induced inhibitor tolerance

The developed flocculating strains, as well as the non-transformed CEN.PK 113–7D, were investigated at similar initial cell concentrations in anaerobic batch fermentations in various media. The results of the anaerobic cultivations showed that the different strains reacted in different ways to different media (Figure 24). In non-inhibitory medium with 20 g/l glucose, the non-flocculating, non-transformed, strain performed best. For the other strains, stronger flocculation led to slower glucose consumption (Figure 24 A). The slower consumption rate was likely an effect of mass transfer limitations of nutrients to the cells in the flocs, as well as the increased burden of constitutive recombinant protein production by the cells. This would result in slower growth and thus slower glucose consumption in the batch. However, in a preliminary test where the non-flocculating and the strongly flocculating strains fermented a spruce hydrolysate, the difference between the strains was reversed and the flocculating strain consumed the glucose and mannose faster than the non-flocculating (see Figure 4, Paper V). The toxicity of the spruce hydrolysate, however, was not severe even for the non-flocculating strain under the experimental conditions. Therefore, for further experiments, 1.5 g/l furfural was added to the hydrolysate to increase its inhibitory effect. The results of the fermentations of the

furfural supplemented hydrolysate showed an even clearer increased inhibitor tolerance of the strongly, and to some extent also the intermediately, flocculating strains when compared to the non-flocculating wild type (Figure 24 B). The weakly flocculating strain, on the other hand, exhibited a fermentation profile highly similar to the non-flocculating strain. These results showed that flocculation *per se* does not result in increased tolerance, but only when it is strong enough to form dense cell flocs.

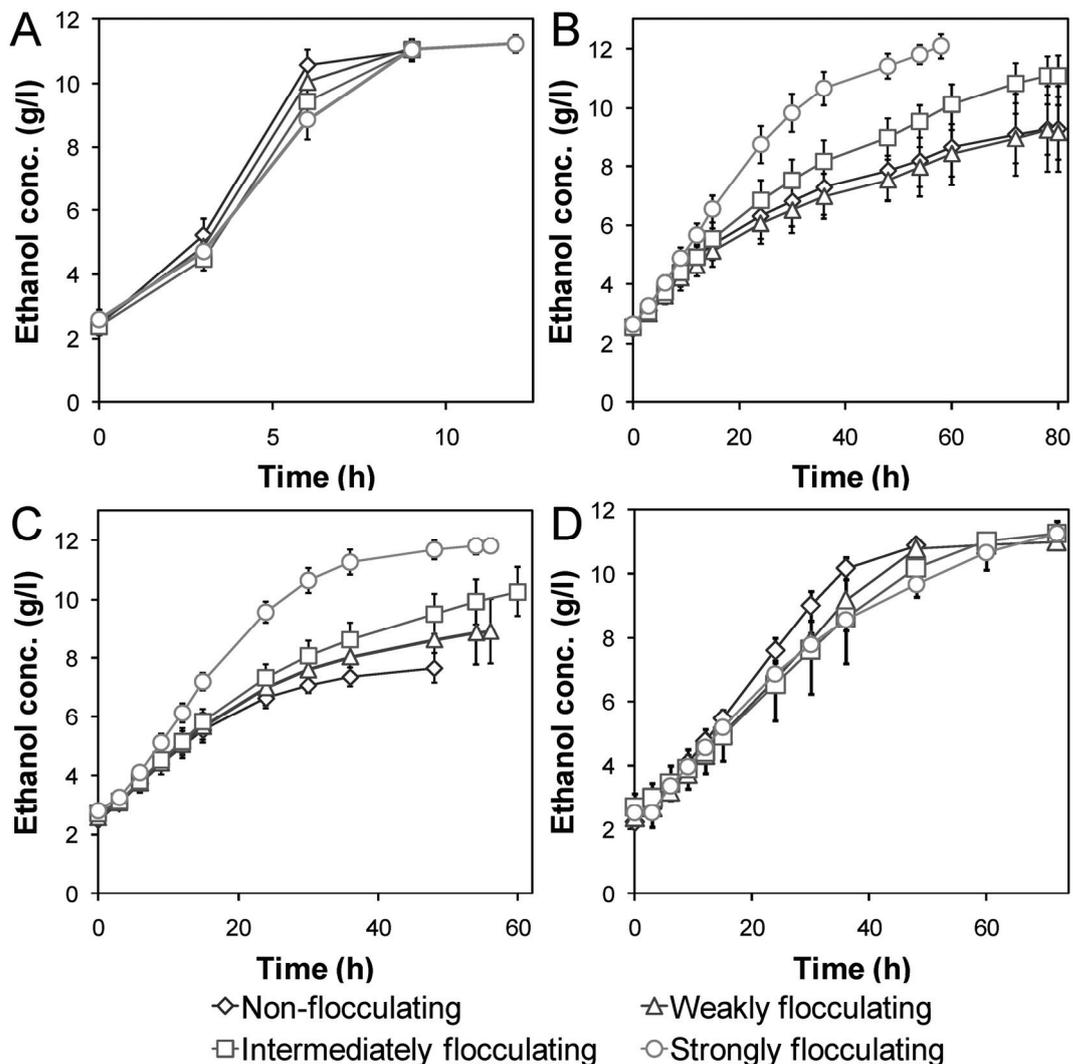


Figure 24 The degree of flocculation strongly influenced the ability of the strains to ferment the different media investigated. The different strains showed distinctly different ethanol production profiles in the different media tested. **A)** defined medium without added inhibitors, **B)** spruce hydrolysate with 1.5 g/l extra furfural, **C)** defined medium with 5 g/l furfural and **D)** defined medium with 200 mM each of formic, acetic and levulinic acid. With strong flocculation (dense cell flocs), the tolerance towards the readily convertible inhibitor furfural, as well as the spruce hydrolysate, was increased. For the not readily convertible acids, as well as the non-inhibitory medium, mass transfer limitations through the flocs decreased the fermentation rates. This led to longer fermentation times the stronger the flocculation. [Data from Paper V.]

6.6.2 Increased tolerance is specific to convertible inhibitors

To investigate the specificity of the increased inhibitor tolerance, the strains were subjected to fermentations of medium containing either 5 g/l furfural or 200 mM each of formic, acetic and levulinic acid in a medium otherwise identical to the non-inhibitory medium. As described in Paper II, these inhibitors can be classified into readily convertible *in situ* under anaerobic conditions, and not readily convertible, respectively. The results of the fermentations were very clear in that the strongly flocculating strain could easily ferment the medium that contained furfural, which inhibited the non-flocculating and weakly flocculating strains strongly (Figure 24 C).

Investigations of the furfural tolerance of high cell density yeast cultures in a membrane bioreactor (MBR) have been reported. There, it was found that cells at a cell density exceeding 150 g DW/l could tolerate 17 g/l furfural in the feed, after stepwise increases from lower concentrations [106]. However, when the concentration of furfural was further increased, the conversion capacity of the cells was evidently not enough and the furfural concentration in the outlet of the reactor started to increase while the ethanol concentration decreased. It was concluded that the cells could detoxify the furfural as long as the concentration was below a certain level in the inlet. This level must be strongly correlated to the amount of cells in the reactor and the state that they are in. It was also found that cells in an MBR could tolerate pulses of furfural of 21.8 g/l. The furfural per biomass ratio was in this case approximately 0.17 g furfural per g DW of cells. In all, it is obvious that the total detoxification capacity of the system is of great importance for its performance. It is not likely that the high cell density in itself had an impact on each cells' ability to tolerate furfural.

In the experiments with the yeast strains in Paper V, the furfural to cell ratio was approximately 6 g furfural per g DW of cells, which was detoxified by all strains. For the non-flocculating and weakly flocculating strains, it was clear that the cells were severely affected after complete detoxification. This effect is similar to what appeared to be the case for the cells in the membrane bioreactor at high concentrations of furfural. However, cells at a high *local* cell density had a clear benefit when compared to freely suspended cells at the same cell concentration. The strongly flocculating cells can 'sacrifice' the outer lying cells, letting them convert furfural at full capacity. The furfural that they

cannot convert will be left for the next 'layer' of cells, which will then experience a lower concentration. In this way a high fermentation capacity can be maintained for a longer time. The fermenting cells inside the flocs can likely also still grow. Accordingly, new cells that can convert furfural are created. This is not the case for freely suspended cells, where the presence of an equally high furfural concentration for all cells inhibits cell growth.

All strains were negatively affected by the medium with carboxylic acids. Instead, the medium with acids was fermented faster by the non-flocculating strain. Most likely, this was an effect of the glucose diffusion limitations hypothesised for the flocculating strains in the non-inhibitory medium, only pronounced by the longer fermentation time in the medium with acids.

In all, the results showed clearly that strong flocculation increased the tolerance significantly only towards convertible inhibitors. This is similar to what was observed for the encapsulated yeast in Paper II and likely for the same reasons. The existence of diffusion limitations inside yeast flocs has been shown previously. The size of the yeast flocs was shown to affect the rates of growth and ethanol formation for flocs of *S. cerevisiae* SPSC01 larger than 100 μm [225]. This is significantly smaller than the flocs of the strongly flocculating mutant in the current study. These were usually approximately 3–4 mm in diameter. Furthermore, the effective diffusivity of glucose in yeast flocs of *S. cerevisiae* NRRL Y265 has been measured to be between 7–17% of the diffusivity in pure water [226].

The difference in hydrolysate fermentation between the non-flocculating and strongly flocculating strains lay mainly in the consumption of mannose, rather than that of glucose (see Figure 4 C & D, Paper V). This can be compared with the encapsulated yeast, where simultaneous consumption of different sugars was observed [Paper IV]. *S. cerevisiae* has a higher affinity for glucose uptake than for mannose uptake [62]. Hence, concentration gradients in the yeast flocs could explain the observed difference. Interestingly, the furfural conversion in the defined medium with furfural did not differ between the strains, despite the significant difference in glucose consumption even after full conversion (see Figure 5, Paper V). However, it was evident that the glucose consumption rate also decreased for the strongly flocculating cells over time with furfural in the medium. The decreased consumption rate can be explained by the fact that glucose

consumption became more limited by diffusion into the flocs over time. After complete conversion of the furfural, the cells in the outer part were strongly negatively affected, similar to *all* of the non-flocculating cells. As long as there is a high concentration of furfural in the medium, cells further and further into the flocs will be severely affected, until there is a balance between the conversion and diffusion of the furfural. Thus, the affected cells might not consume glucose, and the glucose must diffuse further into the flocs to be fermented. In the more complex spruce hydrolysate, on the other hand, there was also a large difference in the furan aldehydes consumption (see Figure 5, Paper V). This is probably due to the additional adversary effects on the outer lying cells of the flocs, by other inhibitory compounds in the hydrolysate. Cells further inside the flocs then have to convert the furan aldehydes. For the non-flocculating strain, all cells face the same concentration of all inhibitors. The system as a whole was thus more affected, and converted the furan aldehydes, as well as consumed the sugars, more slowly.

Like the encapsulated cells, it is very likely that the flocculating cells also have an improved general stress resistance. In a comparison between a non-flocculating strain and the same strain expressing *FLO1*, it was found that multidrug transporter genes as well as other genes involved in stress resistance were up-regulated [227]. The similarities to the results obtained in Papers II & III for encapsulated yeast are striking. Thus, it can be said that flocculation is nature's way of encapsulating yeast.

When the results obtained for the constructed flocculating strains were compared with the performance of the naturally flocculating *S. cerevisiae* CCUG53310, it was obvious that the high tolerance towards carboxylic acids of CCUG53310 cannot have been an effect of its strong flocculation. However, the tolerance towards furan aldehydes may very well, at least in part, be an effect of the flocculation. The sensitivity of flocculating strains to phenolic compounds remains to be investigated. It is likely to be related to the rate of conversion to less inhibitory compounds. However, the tolerance might also be affected by the hydrophobicity of the compounds. If the compounds are attracted to the cells due to the cells' increased hydrophobicity, it is possible that the strongly flocculating strain might experience higher local concentrations and thus stronger inhibition.

6.7 Possibilities and strategies for industrial applications

There are obvious advantages with utilising yeast flocculation rather than encapsulation in industrial settings. Encapsulation of yeast in 2nd generation bioethanol production would likely increase the cost of the final product too much to make the process economically feasible. Flocculation, on the other hand, does not require expensive processing of the yeast prior to the fermentation step, and still it enables easy recovery and reuse of the biocatalyst. However, constitutive flocculation is most of the time unfavourable for the yeast, since mass transfer limitations decrease the specific fermentation rate of the yeast. One can imagine how expression of *FLO1* under the control of a non-constitutive promoter could lead to expression, for example, only in the event of furan aldehydes in the medium. This could possibly be done using the promoter of *FLR1*. *FLR1* was found to be highly expressed in the furan aldehydes medium [Papers I & II]. A similar approach has been taken to obtain flocculation at high ethanol concentration [228]. A combination of these two systems could result in a yeast strain, which flocculated under inhibitory conditions, as well as in the end of the batch, but not under non-inhibitory conditions. An alternative to the expression at high ethanol concentrations could be constitutive expression of a NewFlo phenotype *FLO* gene. This would result in flocculation only at low sugar concentrations. With both options, one could make use of two of the benefits of flocculation – increased inhibitor tolerance and easier cell recovery.

CHAPTER 7

CONCLUDING REMARKS

The main goal of the thesis was to investigate the physiology of *S. cerevisiae* at high local cell density for 2nd generation bioethanol production. In particular, a driving force at the start-up of the project was the elucidation of the reason for the observed increase in inhibitor tolerance of encapsulated yeast. From the encapsulated yeast, parallels could be drawn to flocculating yeast, which is nature's way of making the yeast grow at a high local cell density. Investigations conducted in this thesis demonstrated that the tolerance towards certain inhibitors could be directly linked to the fact that the cells were living tightly packed together.

The first hypothesis described in the introduction, that is, diffusion limitations would decrease the concentrations of inhibitors locally, showed to be correct in part. Some cells, 'acting as body guards', are exposed to the inhibitors and more or less forced to convert them. Other cells, further into the cell pellet of the floc or capsule, are thus virtually unaffected by the inhibitors. A prerequisite for such beneficial concentration gradients is that the cell population is able to convert the inhibitors into less inhibitory compounds.

The second hypothesis, that is, encapsulation triggers stress responses, was shown to be correct. The slow growth and nutrient limitations inside the capsules, and likely also inside dense yeast flocs, indeed led to a starvation stress response. Through stress cross-protection, the tolerance to additional stress of various kinds is thus believed to be increased. However, no additional specific effects of the encapsulation that would lead to increased tolerance to toxic hydrolysates could be elucidated through the investigation of the proteome.

The outcomes of the different subprojects are concluded as:

- The constitutively flocculating yeast *S. cerevisiae* CCUG53310 was tolerant towards furan aldehydes and carboxylic acids, but sensitive towards phenolic compounds.

- Encapsulation-induced stress helped *S. cerevisiae* to tolerate convertible lignocellulose derived inhibitors, but not non-convertible.
- The increased stress tolerance of *S. cerevisiae* encapsulated in liquid core alginate-chitosan capsules came from nutrient limitation and slow growth. This led to a stress response, which is believed to increase the tolerance towards further stress.
- Encapsulation of a xylose fermenting yeast strain increased the cell community's ability to simultaneously utilise all hexoses and xylose present in a spruce hydrolysate medium.
- Strong flocculation with dense cell flocs increased the cell community's resistance towards lignocellulosic hydrolysates, and specifically towards the convertible inhibitor furfural. However, it did not result in visible benefits towards the not readily convertible carboxylic acids.

CHAPTER 8

FUTURE DIRECTIONS

To enable industrial implementations of the results obtained during my PhD project, and to obtain further proof of the hypotheses, a number of further projects are conceivable:

- ❖ The next logical step is the creation of a xylose fermenting flocculating yeast strain. If all hypotheses are correct, this would merge the knowledge obtained during my PhD project into a superior yeast strain.
- ❖ The cell surface hydrophobicity was significantly increased by the expression of flocculins. Furthermore, the high cell surface hydrophobicity was hypothesised to be a possible reason for the sensitivity to phenolic compounds of CCUG53310. Thus, in order to elucidate if the tolerance to the compounds is related to their hydrophobicity, investigations of the tolerance to different phenolic compounds need to be performed for the strongly flocculating yeast.
- ❖ Visualisation of the proposed gradients in the cellular physiology inside the capsules and flocs would give direct evidence in favour of the hypotheses laid forward in this thesis. During the work of the thesis, attempts were made to do this. Cells that expressed a fusion protein of GFP-Yap1p were encapsulated and grown in different media: non-inhibitory defined medium and spruce hydrolysate, until the capsules were full with cells. The cells in the capsules were thereafter fixed using various techniques, e.g. glutaraldehyde or formaldehyde, followed by embedding in agar or agarose and rapid freezing in N₂(l). The embedded capsules were subsequently cut using a microtome, resulting in slices of capsules with only a few micrometres thickness. However, the attempts to visualise the gradients proved to be unsuccessful due to difficulties in quenching the autofluorescence caused by the fixation step. Less severe fixation led to leakage of the cells out of the cut capsules.

Using a microtome/cryostat to cut thin slices of yeast flocs expressing fluorescent proteins (preferably able to mature in anaerobic conditions), under the expression

of different promoters, could enable visualisation of the proposed gradients inside the flocs. Flocculating cells naturally attach to each other. Hence, the treatment needed to quench the autofluorescence could plausibly be performed without disruption of the cells in the cross-sections. This was not possible for the encapsulated cells of a non-flocculating yeast strain. However, encapsulation of the flocculating strains could possibly even simplify the visualisation of the gradients, as the cells would be better kept in place by the capsule membrane.

- ❖ In conjunction with the visualisation of different cell physiology, it would be of interest to investigate different sizes of capsules. Results from the visualisation could indicate an optimal size, where the inhibitor tolerance is still maintained and the specific productivity is as high as possible. This can also most likely be done using a strongly flocculating yeast strain where the size of the flocs can be adjusted by changing the reactor design and operational parameters.
- ❖ Finally, a necessary step on the way to industrial implementation is to scale up the fermentation of hydrolysates with flocculating xylose fermenting *S. cerevisiae* of an optimised floc size. Keeping the cell flocs at the optimal size without breaking, by e.g. the impellers or the high pressure in a larger reactor, is a challenge that might be encountered.

NOMENCLATURE

2D-DIGE	Two Dimensional Difference Gel Electrophoresis
ADP/ATP	Adenosine Di/Tri-Phosphate
CBP	Consolidated Bio Processing
cDNA	Complementary DNA
DGM	Defined Glucose Medium
DW	Cell Dry Weight
ESR	Environmental Stress Response
FAD/FADH ₂	Flavine Adenine Dinucleotide
HMF	5-HydroxyMethyl-2-Furaldehyde
GRAS	Generally Recognised As Safe
MBR	Membrane BioReactor
mRNA	Messenger RNA
nLC-MS/MS	Nano-Liquid Chromatography Mass Spectrometry/Mass Spectrometry
NAD/NADH	Nicotinamide Adenine Dinucleotide
NADP/NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PTMs	Post Translational Modifications
SSF	Simultaneous Saccharification and Fermentation
SHF	Separate Hydrolysis and Fermentation
TCA cycle	TriCarboxylic Acid cycle
qPCR	Quantitative Polymerase Chain Reaction

ACKNOWLEDGEMENTS

The road to the PhD degree is surely longer than most people dare imagining. Reaching the goal has been my own responsibility, but fortunately there have been a lot of people cheering and helping along the way.

Calle and Mohammad, my supervisors, thank you for the never ending support and all the time you have set aside for discussions with me.

Lisbeth, my examiner, thank you for keeping an eye on my progress over the years and for letting me be a part of the Industrial Biotechnology group.

I would like to thank everyone working at the School of Engineering (University of Borås) for my almost five years there. I especially want to thank Jonas for all his help in the lab, Tatiana for discussions on anything and everything, Peter for his understanding of a PhD student's problems and needs and Elisabeth for microbiology discussions. I would also like to thank the local Saco-s union board for giving me a break from research and teaching.

My gratitude also goes to the staff at Chalmers that I have come to know over the years.

I would also like to thank the (former and current) PhD students and post docs that I have come to know over the years at both the University of Borås and at Chalmers. Especially Päivi for all our discussions during both good and less good times of our studies!

The master's students whose thesis works I have supervised over the years as a PhD student also deserve a thank you for all their hard work in the lab.

Finally, I would like to thank my family, especially Supansa, 'Dr Aom', without whom this journey would have seemed a lot longer.

Johan Westman
January 2014

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