Biotechnology Journal
www.biotechnology-journal.com

Biotechnol. J. 2015, 10, 1185–1195

DOI 10.1002/biot.201400581

Review

Current progress in high cell density yeast bioprocesses for bioethanol production

Johan O. Westman and Carl Johan Franzén

Department of Biology and Biological Engineering, Division of Industrial Biotechnology, Chalmers University of Technology, Gothenburg, Sweden

High capital costs and low reaction rates are major challenges for establishment of fermentation-based production systems in the bioeconomy. Using high cell density cultures is an efficient way to increase the volumetric productivity of fermentation processes, thereby enabling faster and more robust processes and use of smaller reactors. In this review, we summarize recent progress in the application of high cell density yeast bioprocesses for first and second generation bioethanol production. High biomass concentrations obtained by retention of yeast cells in the reactor enables easier cell reuse, simplified product recovery and higher dilution rates in continuous processes. High local cell density cultures, in the form of encapsulated or strongly flocculating yeast, furthermore obtain increased tolerance to convertible fermentation inhibitors and utilize glucose and other sugars simultaneously, thereby overcoming two additional hurdles for second generation bioethanol production. These effects are caused by local concentration gradients due to diffusion limitations and conversion of inhibitors and sugars by the cells, which lead to low local concentrations of inhibitors and glucose. Quorum sensing may also contribute to the increased stress tolerance. Recent developments indicate that high cell density methodology, with emphasis on high local cell density, offers significant advantages for sustainable second generation bioethanol production.

Received 15 DEC 2014
Revised 16 JUN 2015
Accepted 06 JUL 2015

Keywords: Bioencapsulation · Biofuels · Biorefinery · Flocculation · Industrial biotechnology

1 Introduction

Fermentation of sugars to ethanol by microorganisms is a natural process that has been utilized by humans for thousands of years. However, the production of ethanol for use as fuel has only relatively recently gained a greater interest [1]. The production of fuel ethanol differs from that of beverages in that a slow process is not necessary for the quality of the final product. Rather, a rapid and complete fermentation process is required for maximizing the profitability of the process. Fuel ethanol is a low-priced bulk chemical and, hence, the productivity and product yield based on total sugars fed to the system are the most important factors for the overall economy of the process [2, 3]. Furthermore, the final ethanol concentration is of utmost significance, as it decides the energy consumption needed for the distillation as well as for drying of the stillage, which is used as animal feed.

The volumetric productivity determines the required reactor volume and the process time, which are directly linked to the process economy. Factors affecting the productivity in bioprocesses are, e.g., the medium composition, product inhibition, pH, temperature and choice of microbial strain. Increased volumetric productivity can be achieved by running the process in continuous mode, or by increasing the specific rates of conversion by metabolic or evolutionary engineering. However, the easiest way to improve the volumetric productivity is to increase the amount of biocatalyst, i.e. the active microorganism, in the reactor.

Correspondence: Dr. Carl Johan Franzén, Department of Biology and Biological Engineering, Division of Industrial Biotechnology, Chalmers University of Technology, Kemivägen 10, 412 96 Gothenburg, Sweden
E-mail: franzen@chalmers.se

Abbreviations: DW, dry weight; **SHF**, separate hydrolysis and fermentation; **SSF**, simultaneous saccharification and fermentation

There are several ways of achieving such high cell concentration, also called high cell density. The simplest method is to add more cells at the start of a batch or fed-batch fermentation. To keep the cost of cell propagation down, cells must be reused, recycled or retained inside the reactor. This can be achieved by reuse of the cells in sequential batches after separation of the product using e.g. centrifugation or filtration, or by cell sedimentation and withdrawal of spent medium, followed by addition of new substrate (Fig. 1A).

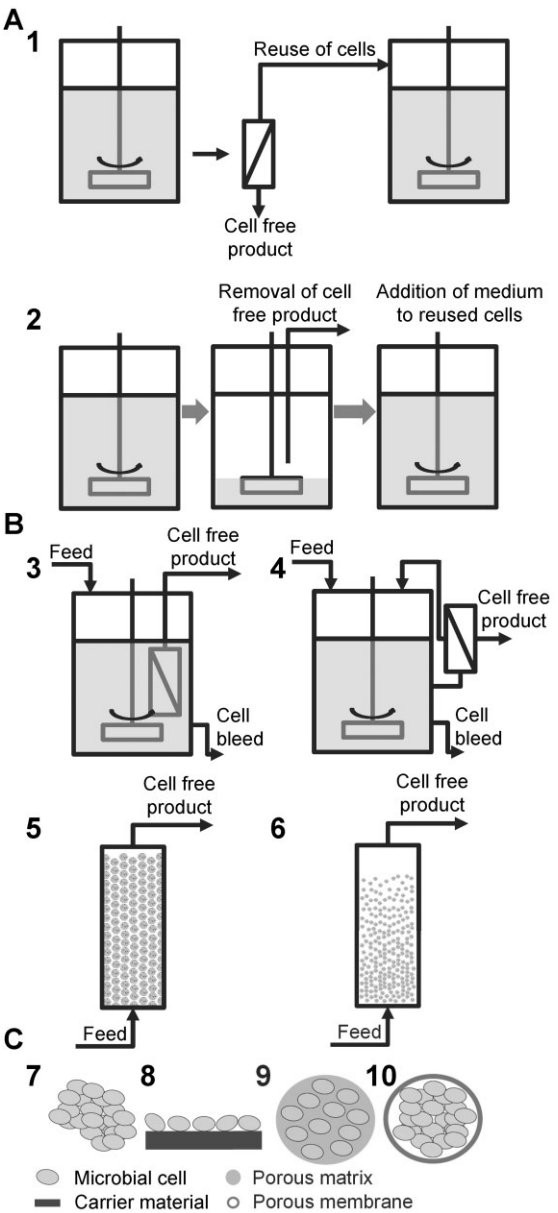

Figure 1. High cell density process options. (**A**) Batch or fed-batch operations at high cell density. 1) Centrifugation or filtration of medium after harvest, for cell recovery and reuse. 2) Sedimentation of cells (possibly immobilized to/in a carrier) at end of process, followed by product removal and addition of new medium in the same reactor. (**B**) Continuous operations at high cell density. 3) Membrane bioreactor with submerged filter for removal of cell free product and retention of cells. 4) Reactor with external separation of a cell free product, using e.g. membrane filtration or sedimentation in a settler. 5) Packed bed reactor with immobilized cells. 6) Fluidized bed reactor with immobilized or flocculating cells. (**C**) Various immobilization methods: 7) Flocculating cells, 8) cells adsorbed to a surface, 9) cells immobilized in a matrix and 10) cells encapsulated in a semi-permeable membrane (adapted from [69]).

In continuous processes on the other hand, retention of the cells inside the reactor is necessary in order to achieve accumulation of cells. Cell retention can be achieved by e.g. filtration of the product through a membrane, immobilization on a carrier material or by exploiting cellular flocculation (Fig. 1B–C). Furthermore, these cell retention methods can be used in combination with different reactor configurations (Fig. 1B) [4].

All of these strategies enable a high global cell density inside the bioreactor, and some of them also enable extremely high local cell densities. For example, encapsulation inside a semi-permeable membrane can give local cell concentrations of more than 300 g DW/L [5], and strong flocculation can give a very high local cell concentration within dense cell flocs, several mm in diameter [6].

High cell density brings about several benefits to a fermentation process. The most obvious is that it enables significantly faster fermentations than at a lower biocatalyst concentration in the reactor. Furthermore, when cells are reused at a high cell density in consecutive batches or during continuous cultivation, the unproductive lag and cell growth phases are eliminated [7]. This means that smaller reactors can be used to meet production goals, or that higher production goals can be met. Both of these improvements decrease the capital cost of the production plant as part of the unit production cost. In a continuous process the substrate uptake is more complete at higher cell density, leading to higher product concentration in the product stream [8]. It has also been observed that the inhibitory effect of ethanol is reduced at a higher cell density in the reactor [9]. Furthermore, most modes of high cell density allow for easier product separation, since reuse of the cells requires that cells are removed from the product stream.

The drawbacks of high cell density and prolonged cell reuse include contamination issues [10], low specific productivities and extra costs of membranes, immobilization materials and additional equipment. High cell density may increase the complexity of process unit operations. For example, stirring becomes more difficult due to the higher viscosity of the fermentation broth compared to broths at low cell density. High local cell density may lead to reduced productivity due to diffusion limitations. In contrast, we have shown that the diffusion limitations in flocs and encapsulated cells may cause increased conversion rates in cases of highly inhibitory media [6, 11, 12]. It is worth to point out that utilization of high cell density fermentations is only feasible when the benefits of higher volumetric productivity and prolonged cell usage

outweigh the decreased specific productivity and the extra costs incurred for cell separation processes and immobilization.

In this review, we discuss the applications of high cell density cultures to bioethanol production using sugar cane, starch, and lignocellulosic raw materials. We address the benefits and drawbacks of different types of cultivations in relation to both first and second generation bioethanol production. Furthermore, we discuss high local cell density as a special case of high cell density giving additional benefits especially valuable for applications in second generation bioethanol production from lignocellulosic raw materials.

2 High cell density production of first generation bioethanol

The production of fuel ethanol is categorized into first and second generation, depending on the feedstock used as raw material. First generation bioethanol is produced from materials rich in sugar or starch, such as sugar cane and various cereals [13]. The production methods differ depending on the raw material used, since starch materials require hydrolysis prior to fermentation. Ethanol from sugar cane is made from sugar cane juice, containing 15% sucrose, and/or molasses, a rest product from the sugar production industry that can contain up to 50% sucrose [1]. These high sugar content feedstocks contain low concentrations of inhibitory compounds and can be easily fermented by yeast, resulting in high concentrations of ethanol within short periods of time. Starch based ethanol production is made from enzymatically hydrolysed cereals. Similar to sugar cane juice, starch hydrolysates contain low amounts of inhibitors, and enough sugar to reach ethanol concentrations of 10–12% [14]. However, the high sugar concentrations and the high ethanol concentrations produced may be stressful for the cells.

With the high sugar concentrations reached from first-generation raw materials, the cell concentration during the fermentation will determine the volumetric production rate of ethanol. Hence, in Brazilian ethanol production plants using sugar cane as raw material, 90–95% of the yeast cells are recycled after each fed-batch, resulting in cell densities of 8–17% (wet weight/volume) in the reactors [15, 16]. After each fermentation, the yeast is separated by centrifugation and prior to reuse treated with H_2SO_4 to reduce the amount of contaminating bacteria [15, 16]. An alternative to the centrifugation is to let the cells sediment, followed by withdrawal of most of the fermentation broth and subsequent addition of new medium. This is most easily achieved with flocculating cells.

Flocculation can be said to be a natural way of yeast immobilization, where the yeast cells are clustered in large flocs. The importance of flocculation for biotechnological processes has been the subject of recent reviews [17, 18]. Flocculating yeast cells create large cell complexes of up to millimeter size, that rapidly sediment if undisturbed. Repeated batch fermentations with flocculating cells are commonly used in beer production and in e.g. cachaça distilleries [19, 20]. However, utilization of both flocculating and immobilized cells will always result in mass transfer limitations due to the diffusion-limited mass transport to cells that are not in immediate contact with the well mixed medium. Flocculation is thus not always desired [21]. On a case to case basis, the benefits of easier cell reuse at high cell density must be weighed against the drawbacks of lower specific productivity because of limited mass transfer rates. Ma et al. showed that with a short sedimentation period, partial medium removal and addition of fresh medium, sequential batches could be performed with increasing volumetric productivities [22].

Rather than reusing the cells in consecutive batches, cell recycling into a reactor can be achieved with an external settler during continuous cultivation [23]. The cells are left unagitated in the settler, usually entering at mid height. The effluent is removed from the top, and cells are transferred back to the reactor from the bottom. These systems include a bleed of cells with the effluent stream, which can be adjusted with changes in the recirculation and dilution rates, and in the settler design. Due to the cell bleed a steady state is reached at a maximum cell concentration supported by the reactor configuration. The method benefits strongly from utilization of rapidly sedimenting yeast strains, such as flocculating strains. Already in 1977, Cysewski and Wilke used a cell recycle reactor with a settler and vacuum ethanol separation, reaching an ethanol productivity of 82 g/L/h on a 33.4% glucose medium [23]. Tang et al. showed successful fermentation of a molasses medium using a flocculating yeast strain in serial bioreactors with settlers, yielding an ethanol concentration of 80 g/L and a productivity of 6.6 g/L/h for more than one month [24]. In a similar setup, Wang et al. reached productivities of 6.9–7.5 g/L/h from sucrose, with less than 2% unconverted sugar in the product stream [25]. Viegas et al. used flocculating yeast in two tower fluidized bed reactors in series, with no external settler, reaching cell densities of approximately 45 g/L and an ethanol productivity of up to 15.4 g/L/h from sucrose [26].

Significantly higher cell concentrations can be reached by using membrane filtration instead of sedimentation. For example, Chaabane et al. used a two stage bioreactor with cell recycling and an ultrafiltration module connected to the outlet of the second reactor to reach cell concentrations of up to 157 g dry cell weight/L [27]. With this setup they reached an ethanol productivity of 41 g/L/h and a concentration of 65 g/L, with complete conversion of the glucose [27].

3 High cell density production of second generation bioethanol

Second generation bioethanol is made from lignocellulosic raw materials, in which carbohydrates are strongly bound in a matrix of cellulose, hemicellulose and lignin [28]. Due to the high recalcitrance of these materials, they have to be pretreated prior to enzymatic hydrolysis into fermentable sugars. There are different methods to perform the hydrolysis, the simplest being separately from the fermentation step, so called separate hydrolysis and fermentation, SHF. The hydrolysis and fermentation can also be performed in a single, simultaneous saccharification and fermentation operation, SSF. If the fermenting microorganisms in an SSF also produce the hydrolysing enzymes, the process is termed consolidated bioprocessing. Regardless of the approach, cellulose is hydrolysed to glucose monomers that can be easily fermented by yeast into ethanol. Hemicellulose, on the other hand, has a divergent composition which is highly dependent on the source plant. Hydrolysis of hemicellulose releases pentoses such as xylose and arabinose, in addition to hexoses such as glucose, mannose and galactose. To reach as high ethanol concentration as possible, all these sugars must be fermented to ethanol. Wild-type *Saccharomyces cerevisiae* cannot ferment pentoses into ethanol, but recombinant yeast strains harboring pentose-converting pathways have been developed, as reviewed in [29].

Lignocellulosic raw materials also contain up to 35% lignin, a non-fermentable complex and heterogenous aromatic polymer [30]. In SSF processes, the lignin is not separated from the cellulose prior to the fermentation. At high cellulose loading there will also be lignin present at high concentration, and cells are not easily separated from the lignin residues. Therefore, cell reuse is difficult, since the lignin would also accumulate in sequential batches and increase the viscosity of the fermentation broth [3]. Moreover, the potentially high content of solids and high viscosity make the packed and fluidized bed bioreactors unsuitable for SSF processes. In second generation bioprocesses, the enzymatic hydrolysis of cellulose is generally the rate limiting step, rather than the fermentation of the released sugars [31]. Hence, high cell density cultures do not seem to provide the same benefit as in the first-generation case. However, consolidated bioprocessing would benefit from increased cell density. Matano et al. attempted repeated batch SSF with a cellulase-expressing yeast at a cell concentration of approximately 100 g wet cells/L [32]. By using two sequential centrifugation steps after each batch, they managed to first remove larger particles, and in the second step separate the cells from the medium. Cells were reused in sequential batches. These experiments were performed in 10 ml scale, and scale up of the process might prove difficult.

In SHF processes, the lignin residue can instead be removed prior to fermentation. The difficulty in SHF processes is to reach high sugar concentrations in the hydrolysis step, due to the viscosity of the material at high concentrations and end-product inhibition of the enzymes [33]. However, in recent years, progress has been made in the field of high gravity lignocellulose pretreatment and hydrolysis, and end-product inhibition is becoming less of a problem [33]. Sarks et al. used the SHF approach with high cell density and cell reuse by centrifugation, reaching ethanol concentrations of approximately 40 g/L over five consecutive batches [34].

Cell recycling with an external settler or membrane filtration during continuous cultivation has been performed also with lignocellulose-derived media. Brandberg et al. fermented dilute acid spruce hydrolysates with cell retention of a flocculent yeast to significantly increase the sugar consumption [35, 36]. Purwadi et al. used a flocculating yeast strain and cell recycling in two continuous stirred tank bioreactors and an external settler in series, to reach cell densities of 35 g DW/L from a spruce hydrolysate at a dilution rate of 0.52 h^{-1} and a sugar conversion of 94% [37].

4 Case specific benefits and characteristics of high cell density cultures

As illustrated above, high cell density technology may not be generally applicable in bioethanol production. Nevertheless, high cell density may lead to special benefits and desirable characteristics depending on the reactor configuration or type of cell retention used.

4.1 Contamination reduction

In continuous fermentations with cell retention, a dilution rate faster than the maximum specific growth rate of the cells can be used. If the yeast is retained by other means than membrane filtration, contaminating microorganisms will be washed out of the reactor. This may happen even if the maximum growth rate of the contaminant is higher than the dilution rate, since the high cell concentration of the retained microorganism depletes the medium of nutrients. This has been shown for a recombinant flocculating *S. cerevisiae* in an air-lift reactor, deliberately contaminated with *Escherichia coli* when operating at steady state [38]. The beneficial effect has also been observed when fermenting a lignocellulose hydrolysate in continuous mode with a flocculent yeast strain [39]. Processes utilizing cell recycling in consecutive batches are instead rather susceptible to contamination, as no cells are removed from the fermentations [10].

4.2 Alleviation of the toxic effect of inhibitors

The harsh treatment needed to enable hydrolysis of the cellulose in the lignocellulosic material also releases and creates various compounds that are inhibitory to yeast

fermentation, and the more concentrated the hydrolysate, the more inhibitory it becomes [33, 40]. Some of the inhibitors can be detoxified in situ by the yeast, by conversion into less toxic compounds. The furan aldehydes furfural and 5-hydroxymethyl furfural are examples of this. Under anaerobic conditions, these aldehydes are reduced to their corresponding alcohols [41, 42]. In batch fermentations of a medium containing furfural, a lag phase is often observed while the inhibitor is being converted. However, if the concentrations of the inhibitors are too high, the detoxification capacity of the cells will be insufficient, resulting in stuck fermentations [43].

Using high cell density is a way to alleviate this problem. More cells increase the detoxification capacity of the system, albeit not necessarily of the individual cells. This has been shown to be an effective strategy when using high inoculum levels [43, 44], an external settler in continuous culture [35] and a membrane bioreactor to increase the cell density [45]. For example, a medium containing 17.0 g/L furfural could be continuously fermented by using a membrane bioreactor to obtain cell densities of up to 180 g cell dry weight/L [45]. Furthermore, Ylitervo et al. showed that continuous fermentation of spruce hydrolysate was possible at dilution rates of 0.8 h^{-1} in a submerged membrane bioreactor at cell densities of 60 g cell dry weight/L [46].

Although the system's detoxification capacity is increased by a high cell density, the long term viability and fermentation capacity of cells in lignocellulose-derived media is lower than for cells in first generation media [47]. The time that the cells can be reused or kept in a reactor thus strongly depends on the toxicity of the lignocellulosic hydrolysate and the robustness of the cells.

4.3 Physiological effects

High cell density induces changes in the physiology of the cells, mainly due to the more scarce nutrient availability per cell, which leads to low specific growth rates. At low growth rates, a larger proportion of the utilized energy source is used for meeting maintenance and other non-growth related energy requirements, which leads to lower biomass yields and higher yields of energy-related products than at higher growth rates [48]. In anaerobic yeast cultures, this means higher yield of ethanol on consumed sugars [49]. This effect is emphasized in inhibitory media, where more energy is required for counteracting the effect of the inhibitors. For example, weak acids cause metabolic uncoupling, presumably by activation of ATP-driven ion pumps to maintain homeostasis [50, 51]. Low nutrient availability may also cause cell death, which counterintuitively may decrease the need of nutrient addition. Thomas et al. suggested that lysed yeast cells release nutrients that help to maintain the viability of the yeast population, as no nutrient supplementation was necessary at high pitching rate [52].

Immobilization also affects the physiology of the cells. Cells immobilized in calcium alginate beads have been reported to have significantly higher activities and levels of glycolytic enzymes compared to cells grown in suspension, leading to faster ethanol production rates [53–55].

For flocculating cells it has been observed that the membrane lipid composition is affected by the floc size, and is likely correlated to the ethanol tolerance [56]. The highest ergosterol content was observed in cells in the flocs that displayed the highest ethanol tolerance. Differences in the membrane composition have also been observed for cells immobilized in calcium alginate gel beads, with lower content of unsaturated fatty acids in the immobilized, more ethanol tolerant, cells [57]. In contrast, strongly flocculating cells display a gene expression profile that indicates sterol deprivation for cells embedded in the flocs [58]. These somewhat contradictory observations might depend on the size distribution of the investigated flocs, as Lei et al. observed a decrease in the ergosterol content and ethanol tolerance for flocs larger than 300 μm [56].

Smukalla et al. also observed upregulation of many genes involved in stress resistance and multidrug transporters in yeast flocs [58]. We have made similar observations by comparing the proteomic profile of encapsulated and suspended yeast cells [5], and we have observed upregulation of multidrug transporter genes in encapsulated compared to freely suspended yeast [12]. The intracellular trehalose level, which is important for the cellular stress tolerance, also increases in encapsulated yeast [59, 60]. Sun et al. observed an increase in the osmotolerance of encapsulated cells, possibly an effect of the increased accumulation of intracellular trehalose and glycerol [60].

Activation of the Pdr1p transcription factor and upregulation of the multidrug transporter gene *PDR5* have also been observed in response to the aromatic alcohols tryptophol and tyrosol, which are known quorum sensing molecules in *S. cerevisiae* [61]. Quorum sensing, i.e. cell density dependent cell-to-cell communication via extracellular signalling molecules, has been shown to induce various stress responses in bacteria at high cell density [62, 63]. This partially explains the resistance of microbial biofilms to both therapeutic agents and host immunoresponses [64, 65]. In fungi, quorum sensing is involved in the transition between single cell and filamentous growth [66]. In *S. cerevisiae*, the aromatic alcohols phenylethanol and tryptophol induce filamentous growth by induction of *FLO11* via the Ras-cAMPK protein kinase Tpk2p [67], and tryptophol induces flocculation by induction of *FLO1* [58]. Moreover, the expression of several other transcription factors, like Mig1p and Cat8p, respond to treatment with aromatic alcohols [68]. Since these transcription factors are involved in various stress responses, quorum sensing may play a role in the development of stress tolerance in high cell density yeast cultures.

5 Advantages of high local cell density

High local cell density enables all the general benefits of cell retention, but also changes other characteristics of the yeast cell system, that are relevant for, especially, second generation bioethanol production. High local cell density can be combined with both high and low total cell concentration. Although high local cell density may lead to mass transfer limitations, and hence often lower specific productivity [12], it may give extra benefits in addition to the ones common for all types of high cell density.

High local cell density can be obtained if cells, suspended in a liquid droplet, are entrapped inside a thin membrane, rather than in a bead of a porous matrix [69]. Encapsulation by this method enables extremely high local cell densities in the capsules, reaching above 300 g DW/L [5]. In a gel matrix, on the other hand, cells are present at a high concentration only in the outer layer of the bead. For example, Purwadi and Taherzadeh reported a cell density of approximately 130 g DW/L in the outer layer with rapidly dropping cell concentration towards the center of the bead [70].

Flocculation may also lead to high local cell density, if the yeast flocs form dense cell pellets. This does not seem to be possible for all flocculating yeast strains [6]. Andrietta et al. assessed the performance of 12 flocculating yeast strains in two serial reactors without cell recycling and found that only three of them formed pellets during the fermentation [71]. Flocculation is a highly variable and rapidly evolving trait in *S. cerevisiae* strains [72, 73]. The strength of the flocculation can be varied by expression of different flocculation genes or gene variants [6, 74]. A number of recombinant flocculating yeast strains have been developed, e.g. with constitutive flocculation [6, 75] or with inducible flocculation by expression under control of the *TPS1* promoter [76], the *TPI1* promoter [77] or the *ADH2* promoter [78].

5.1 Inhibitor tolerance

The inhibitor tolerance of yeast can be improved also at a low global cell density when *S. cerevisiae* cells are encapsulated in semi-permeable alginate-chitosan capsules to obtain a high local cell density [8, 47]. Specifically, we have shown that encapsulation increases the tolerance towards furan aldehydes, but not towards carboxylic acids [12]. The difference can be explained by the convertible and non-convertible nature of the compounds. Since the furan aldehydes can be converted to alcohols anaerobically, their concentration decreases along the radius inside the capsules, leaving sub-inhibitory levels for a large fraction of the encapsulated yeast cells (Fig. 2). These interior cells are thus able to ferment the sugars. Carboxylic acids, on the other hand, are not converted anaerobically by the yeast. Hence, after sufficient time, acids will be present throughout the capsules at the same concentration as in the surrounding medium.

By the same reasoning, nutrient limitation will occur because of gradients in the substrate concentrations. Nutrient limitation triggers stress responses in yeast,

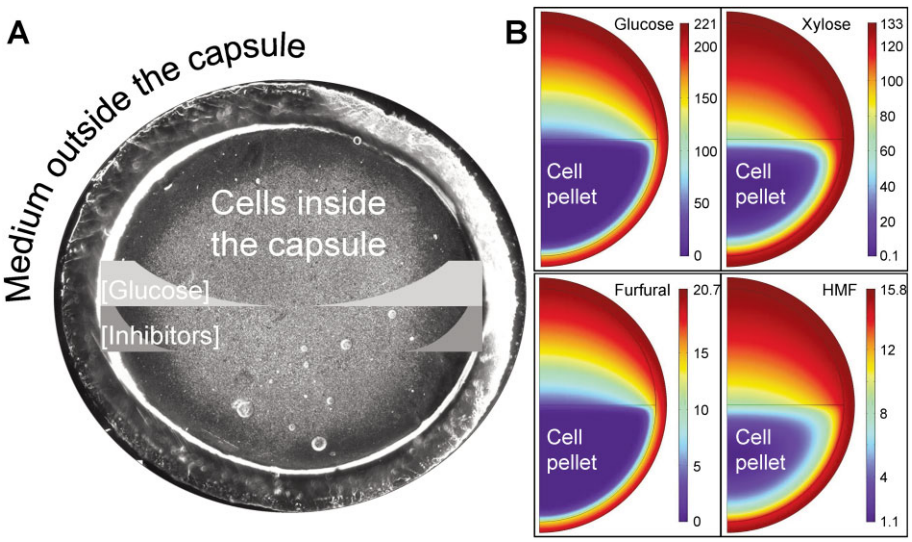

Figure 2. High local cell density leads to concentration gradients. (**A**) Micrograph of a cross-section of an alginate-chitosan capsule filled with *S. cerevisiae*, with a schematic interpretation of concentration profiles (Westman, unpublished). (**B**) Simulation of concentration profiles in capsules half full with yeast cells [11]. In high local cell density systems, concentration gradients are formed that lead to different carbohydrate availabilities for cells at different depth inside the system, as shown here in capsules half filled with cells at steady state. As an effect, the system can "bypass" the strong preference for glucose utilization and simultaneously ferment different carbohydrates that would be sequentially fermented by cells evenly distributed in the medium. Furthermore, cells deep in the cell pellet are protected from convertible inhibitors such as the furan aldehydes, as cells closer to the membrane convert them to less inhibitory compounds. Numbers are in mmol/L. See [11] for details on simulation procedures.

which we have observed on mRNA and protein level [5, 12]. The stress response increases the robustness of the yeast and leads to improved tolerance towards additional stress, such as heat [79]. In addition to the protective effect given by the high local cell density, the capsule membrane may provide a more direct protective effect by excluding certain compounds, such as the hydrophobic compound limonene [80].

Furthermore, we have shown that strong flocculation, causing dense cell flocs, leads to similar improvements in the inhibitor tolerance as observed with encapsulation. We created a set of three constitutively flocculating yeast strains showing different strength of flocculation [6]. We evaluated these strains in batch fermentations and observed that the most strongly flocculating strain had a clear advantage in both furfural-containing defined medium and in a complete spruce hydrolysate. However, no improvements in acid tolerance could be observed, similar to what was shown for encapsulated yeast. Furthermore, Ge and Bai have shown that there are internal diffusion limitations in flocs larger than 100 µm of the yeast strain SPSC01 [81], and naturally flocculating yeast strains have often been reported to be inhibitor tolerant [34, 82–84].

It has been shown that the tolerance to ethanol is increased by immobilization in gel beads [85, 86] and by flocculation [56]. Qun et al. showed that yeast tolerance to organic solvents was increased by immobilization in calcium alginate beads [87]. In immobilized cell systems, the immobilization support can act as a protective agent against, e.g. heavy metals as well as non-optimal pH and temperature [7].

Quorum sensing may, at least partially, increase the stress tolerance of flocculating cells [88]. However, the stress responsive pathways linked to quorum sensing

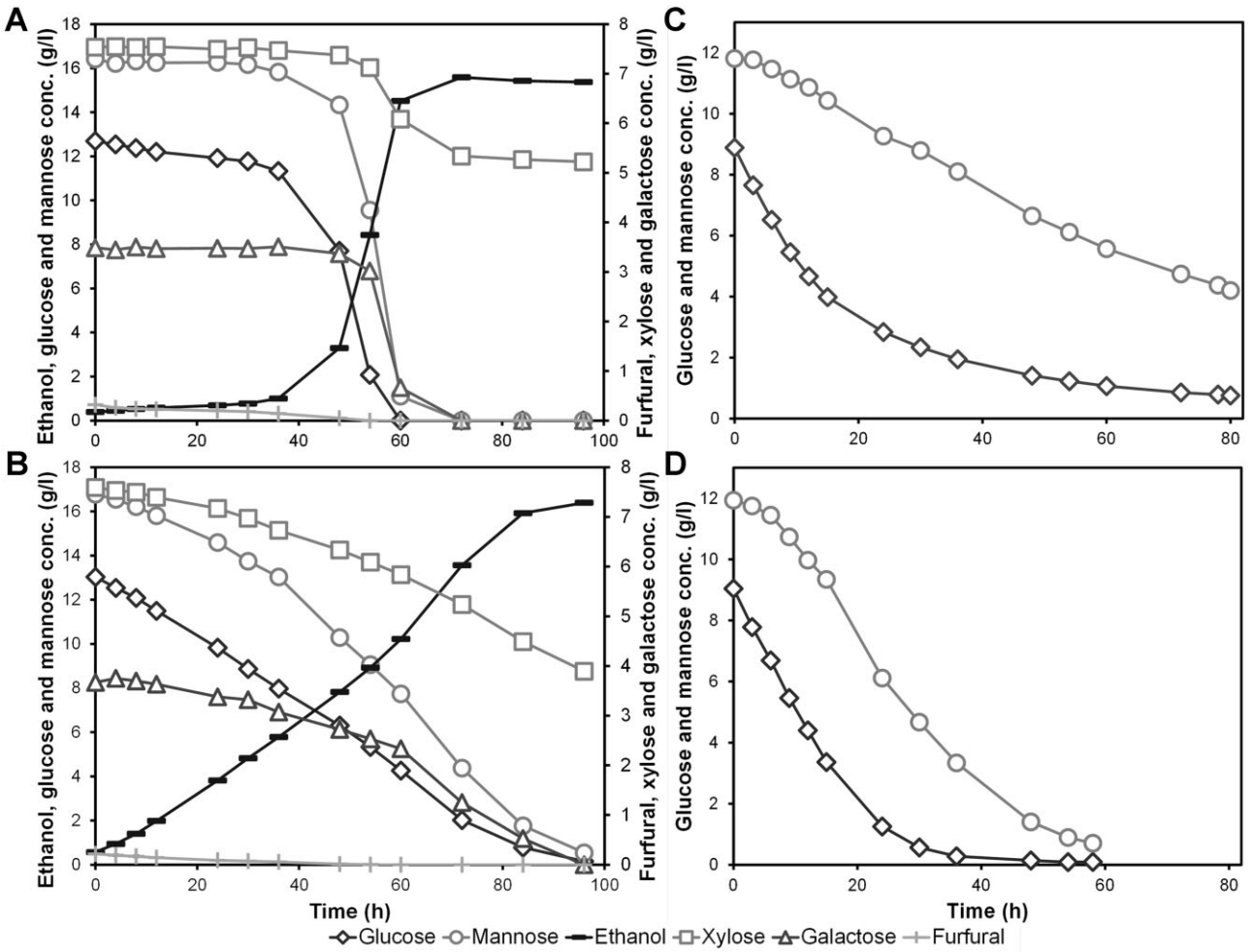

Figure 3. High local cell density leads to simultaneous utilization of different sugars. While yeast suspended in a spruce hydrolysate (**A**) displayed a long lag phase prior to fermentation of glucose and subsequently of the other sugars present in the medium, encapsulated yeast (**B**) displayed simultaneous utilization of all fermentable sugars from the beginning of the batch. Similarly, a strongly flocculating yeast mutant (**D**) fermented the glucose and the mannose in a spruce hydrolysate simultaneously and more rapidly than the non-flocculating parental strain (**C**) at the same total cell concentration. Adapted from [6, 11].

(e.g. Ras-cAMPK), are involved in nutrient sensing and stress responses even at low cell density [89, 90]. Therefore, improved stress tolerance in direct response to nutrient limitation, caused by concentration gradients due to diffusion-limited mass transfer, offers a more direct explanation. We have indeed observed that the improvement in inhibitor tolerance was linked to the size and compactness of the cell flocs, which directly affect the mass transfer rates through the flocs [6].

5.2 Simultaneous sugar utilization

S. cerevisiae has a natural preference for glucose as a carbon source. The utilization of other carbon and energy sources is strongly repressed as long as there is glucose present [89, 91]. This becomes a problem in second generation bioethanol production, since lignocellulosic hydrolysates typically contain several sugars. Other sugars are not utilized until most of the glucose is consumed, leading to long fermentation times [92, 93]. The sequential utilization is in part an effect of most sugars being transported into the yeast cells by the same transporters. These hexose transporters have a significantly higher affinity for glucose, their natural substrate, than for e.g. xylose [94, 95]. However, in cell aggregates at a high enough local cell density, the cell population is exposed to an entire range of glucose concentrations at the same time (Fig. 2). As a result, all fermentable sugars, including glucose, mannose, galactose and xylose, could be fermented simultaneously in a batch fermentation by a recombinant xylose fermenting *S. cerevisiae* encapsulated in alginate-chitosan capsules [11]. The medium was concomitantly detoxified by in situ conversion of furan aldehydes. When suspended in the medium at the same average cell concentration, the yeast fermented the sugars mainly sequentially and only after detoxification of the medium during an extended lag phase (Fig. 3A and 3B). Furthermore, we have observed that strongly flocculating yeast has an improved simultaneous fermentation of glucose and mannose in spruce hydrolysate (Fig. 3C and 3D) [6]. In all, even at a low total cell density, cells at a high local cell density can aid against the two major issues for second generation bioethanol production: sequential utilization of several sugars and inhibitory media.

6 Concluding remarks

High cell density contributes to reaching high reaction rates in fermentation processes. In first generation bioethanol production it is commonly used, resulting in short fermentation times because of high volumetric sugar consumption rates. In second generation bioethanol production additional benefits that are necessary for obtaining a feasible process are realized at high cell density. Above all, high cell density increases the tolerance to inhibitory compounds by forming a system with a higher total in situ detoxification capacity. Encapsulation or flocculation of cells leads to high local cell density, which causes both improved tolerance to convertible inhibitors, enables simultaneous utilization of the different sugars present in lignocellulose-derived hydrolysates, and simplifies separation and recycling of cells. High cell density processes can be expected to be an integral part of any successful biochemical production from sugar- and starch-containing agricultural feedstocks as well as lignocellulosic raw materials.

Dr. Johan Westman is a postdoctoral researcher at the division of Industrial Biotechnology, Chalmers University of Technology, Sweden. Dr. Westman holds a PhD in Biosciences and an MSc in Chemical Biology. His current research interests are second generation bioethanol production and yeast physiology, with a focus on simultaneous saccharification and fermentation of pretreated lignocelluloses. Previously, Dr. Westman worked as a PhD candidate at the University of Borås, Sweden, with research focus on flocculating and encapsulated *Saccharomyces cerevisiae*.

Associate Professor Carl Johan Franzén obtained his PhD degree in Chemical Reaction Engineering at Chalmers University of Technology in 1997, with a thesis on feed-back RQ control of microaerobic, continuous cultures of *Saccharomyces cerevisiae*. He has extensive experience with high cell density yeast biotechnology for robust second generation bioethanol production, using cell retention, recirculation, encapsulation and flocculation approaches. In 2005 he was appointed Associate Professor in Bioreaction Engineering, and in 2008 he joined the Industrial Biotechnology division. He is an active teacher and Head of the five-year MScEng Bioengineering program and the international MSc program in Biotechnology.

Financial support under grants P13872-2 and P37353-1 from the Swedish Energy Agency, grant 2009-4514 from the Swedish Research Council, and from the Chalmers Energy Initiative (http://www.chalmers.se/en/areas-of-advance/energy/cei/) is gratefully acknowledged.

The authors declare no financial or commercial conflict of interest.

7 References

[1] Walker, G. M., 125th Anniversary review: Fuel alcohol: Current production and future challenges. *J. Inst. Brew.* 2011, *117*, 3–22.
[2] Galbe, M., Sassner, P., Wingren, A., Zacchi, G., Process engineering economics of bioethanol production, in: Olsson, L. (Ed.), *Biofuels*, Springer, Berlin Heidelberg 2007, pp. 303–327.
[3] Galbe, M., Zacchi, G., A review of the production of ethanol from softwood. *Appl. Microbiol. Biotechnol.* 2002, *59*, 618–628.
[4] Obradovic, B., Nedovic, V., Bugarski, B., Willaert, R., Vunjak-Novakovic, G., Immobilised Cell Bioreactors, in: Nedovic, V., Willaert, R. (Eds.), *Fundamentals of Cell Immobilisation Biotechnology*, Springer, Netherlands 2004, pp. 411–436.
[5] Westman, J. O., Taherzadeh, M. J., Franzén, C. J., Proteomic analysis of the increased stress tolerance of *Saccharomyces cerevisiae* encapsulated in liquid core alginate-chitosan capsules. *PLoS One* 2012, *7*, e49335.
[6] Westman, J. O., Mapelli, V., Taherzadeh, M. J., Franzén, C. J., Flocculation causes inhibitor tolerance in *Saccharomyces cerevisiae* for second-generation bioethanol production. *Appl. Environ. Microbiol.* 2014, *80*, 6908–6918.
[7] Kourkoutas, Y., Bekatorou, A., Banat, I. M., Marchant, R., Koutinas, A. A., Immobilization technologies and support materials suitable in alcohol beverages production: A review. *Food Microbiol.* 2004, *21*, 377–397.
[8] Talebnia, F., Taherzadeh, M. J., In situ detoxification and continuous cultivation of dilute-acid hydrolyzate to ethanol by encapsulated *S. cerevisiae*. *J. Biotechnol.* 2006, *125*, 377–384.
[9] Vega, J. L., Navarro, A. R., Clausen, E. C., Gaddy, J. L., Effects of inoculum size on ethanol inhibition modeling and other fermentation parameters. *Biotechnol. Bioeng.* 1987, *29*, 633–638.
[10] Basso, L. C., De Amorim, H. V., De Oliveira, A. J., Lopes, M. L., Yeast selection for fuel ethanol production in Brazil. *FEMS Yeast Res.* 2008, *8*, 1155–1163.
[11] Westman, J. O., Bonander, N., Taherzadeh, M. J., Franzén, C. J., Improved sugar co-utilisation by encapsulation of a recombinant *Saccharomyces cerevisiae* strain in alginate-chitosan capsules. *Biotechnol. Biofuels* 2014, *7*, 102.
[12] Westman, J. O., Manikondu, R. B., Franzén, C. J., Taherzadeh, M. J., Encapsulation-induced stress helps *Saccharomyces cerevisiae* resist convertible lignocellulose derived inhibitors. *Int. J. Mol. Sci.* 2012, *13*, 11881–11894.
[13] Naik, S. N., Goud, V. V., Rout, P. K., Dalai, A. K., Production of first and second generation biofuels: A comprehensive review. *Renewable Sustainable Energy Rev.* 2010, *14*, 578–597.
[14] Bothast, R. J., Schlicher, M. A., Biotechnological processes for conversion of corn into ethanol. *Appl. Microbiol. Biotechnol.* 2005, *67*, 19–25.
[15] Basso, L. C., Basso, T. O., Rocha, S. N., Ethanol production in Brazil: The industrial process and its impact on yeast fermentation, in: Bernardes, M. A. D. S. (Ed.), *Biofuel Production-Recent Developments and Prospects*, InTech, Rijeka 2011, pp. 85–100.
[16] Wheals, A. E., Basso, L. C., Alves, D. M. G., Amorim, H. V., Fuel ethanol after 25 years. *Trends Biotechnol.* 1999, *17*, 482–487.
[17] Zhao, X. Q., Bai, F. W., Yeast flocculation: New story in fuel ethanol production. *Biotechnol. Adv.* 2009, *27*, 859–865.
[18] Bauer, F., Govender, P., Bester, M., Yeast flocculation and its biotechnological relevance. *Appl. Microbiol. Biotechnol.* 2010, *88*, 31–39.
[19] Silva, C. L. C., Rosa, C. A., Oliveira, E. S., Studies on the kinetic parameters for alcoholic fermentation by flocculent *Saccharomyces cerevisiae* strains and non-hydrogen sulfide-producing strains. *World J. Microbiol. Biotechnol.* 2006, *22*, 857–863.
[20] Verstrepen, K. J., Derdelinckx, G., Verachtert, H., Delvaux, F. R., Yeast flocculation: What brewers should know. *Appl. Microbiol. Biotechnol.* 2003, *61*, 197–205.
[21] Abreu-Cavalheiro, A., Monteiro, G., Solving ethanol production problems with genetically modified yeast strains. *Braz. J. Microbiol.* 2013, *44*, 665–671.
[22] Ma, K., Wakisaka, M., Sakai, K., Shirai, Y., Flocculation characteristics of an isolated mutant flocculent *Saccharomyces cerevisiae* strain and its application for fuel ethanol production from kitchen refuse. *Bioresour. Technol.* 2009, *100*, 2289–2292.
[23] Cysewski, G. R., Wilke, C. R., Rapid ethanol fermentations using vacuum and cell recycle. *Biotechnol. Bioeng.* 1977, *19*, 1125–1143.
[24] Tang, Y.-Q., An, M.-Z., Zhong, Y.-L., Shigeru, M. et al., Continuous ethanol fermentation from non-sulfuric acid-washed molasses using traditional stirred tank reactors and the flocculating yeast strain KF-7. *J. Biosci. Bioeng.* 2010, *109*, 41–46.
[25] Wang, F.-S., Li, C.-C., Lin, Y.-S., Lee, W.-C., Enhanced ethanol production by continuous fermentation in a two-tank system with cell recycling. *Process Biochem.* 2013, *48*, 1425–1428.
[26] Viegas, M. C., Andrietta, S. R., Andrietta, M. G. S., Use of tower reactors for continuous ethanol production. *Braz. J. Chem. Eng.* 2002, *19*, 167–173.
[27] Chaabane, F. B., Aldiguier, A., Alfenore, S., Cameleyre, X. et al., Very high ethanol productivity in an innovative continuous two-stage bioreactor with cell recycle. *Bioprocess. Biosyst. Eng.* 2006, *29*, 49–57.
[28] Gray, K. A., Zhao, L., Emptage, M., Bioethanol. *Curr. Opin. Chem. Biol.* 2006, *10*, 141–146.
[29] Kim, S. R., Park, Y.-C., Jin, Y.-S., Seo, J.-H., Strain engineering of *Saccharomyces cerevisiae* for enhanced xylose metabolism. *Biotechnol. Adv.* 2013, *31*, 851–861.
[30] Zhao, X., Zhang, L., Liu, D., Biomass recalcitrance. Part I: The chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuel. Bioprod. Bior.*2012, *6*, 465–482.
[31] Olofsson, K., Bertilsson, M., Lidén, G., A short review on SSF-an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol. Biofuels* 2008, *1*, 1–14.
[32] Matano, Y., Hasunuma, T., Kondo, A., Cell recycle batch fermentation of high-solid lignocellulose using a recombinant cellulase-displaying yeast strain for high yield ethanol production in consolidated bioprocessing. *Bioresour. Technol.* 2013, *135*, 403–409.
[33] Koppram, R., Tomás-Pejó, E., Xiros, C., Olsson, L., Lignocellulosic ethanol production at high-gravity: Challenges and perspectives. *Trends Biotechnol.* 2014, *32*, 46–53.
[34] Sarks, C., Jin, M., Sato, T., Balan, V., Dale, B., Studying the rapid bioconversion of lignocellulosic sugars into ethanol using high cell density fermentations with cell recycle. *Biotechnol. Biofuels* 2014, *7*, 73.
[35] Brandberg, T., Karimi, K., Taherzadeh, M. J., Franzén, C. J., Gustafsson, L., Continuous fermentation of wheat-supplemented lignocellulose hydrolysate with different types of cell retention. *Biotechnol. Bioeng.* 2007, *98*, 80–90.
[36] Brandberg, T., Sanandaji, N., Gustafsson, L., Franzén, C. J., Continuous fermentation of undetoxified dilute acid lignocellulose hydrolysate by *Saccharomyces cerevisiae* ATCC 96581 using cell recirculation. *Biotechnol. Prog.* 2005, *21*, 1093–1101.
[37] Purwadi, R., Brandberg, T., Taherzadeh, M. J., A possible industrial solution to ferment lignocellulosic hydrolyzate to ethanol: Continuous cultivation with flocculating yeast. *Int. J. Mol. Sci.* 2007, *8*, 920–932.
[38] Domingues, L., Lima, N., Teixeira, J. A., Contamination of a high-cell-density continuous bioreactor. *Biotechnol. Bioeng.* 2000, *68*, 584–587.
[39] Tang, Y., An, M., Liu, K., Nagai, S. et al., Ethanol production from acid hydrolysate of wood biomass using the flocculating yeast *Sac-*

charomyces cerevisiae strain KF-7. *Process Biochem.* 2006, *41*, 909–914.

[40] Almeida, J. R. M., Modig, T., Petersson, A., Hähn-Hägerdal, B. et al., Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J. Chem. Technol. Biotechnol.* 2007, *82*, 340–349.

[41] Taherzadeh, M. J., Gustafsson, L., Niklasson, C., Lidén, G., Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* 1999, *87*, 169–174.

[42] Taherzadeh, M. J., Gustafsson, L., Niklasson, C., Lidén, G., Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 2000, *53*, 701–708.

[43] Kapu, N. S., Piddocke, M., Saddler, J., High gravity and high cell density mitigate some of the fermentation inhibitory effects of softwood hydrolysates. *AMB Express* 2013, *3*, 15.

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

[45] Ylitervo, P., Franzén, C. J., Taherzadeh, M., Impact of furfural on rapid ethanol production using a membrane bioreactor. *Energies* 2013, *6*, 1604–1617.

[46] Ylitervo, P., Doyen, W., Taherzadeh, M. J., Fermentation of lignocellulosic hydrolyzate using a submerged membrane bioreactor at high dilution rates. *Bioresour. Technol.* 2014, *164*, 64–69.

[47] Talebnia, F., Niklasson, C., Taherzadeh, M. J., Ethanol production from glucose and dilute-acid hydrolyzates by encapsulated *S. cerevisiae*. *Biotechnol. Bioeng.* 2005, *90*, 345–353.

[48] Pirt, S. J., The maintenance energy of bacteria in growing cultures. *Proc. R. Soc. Ser. B* 1965, *163*, 224–231.

[49] Boender, L. G. M., de Hulster, E. A. F., van Maris, A. J. A., Daran-Lapujade, P. A. S., Pronk, J. T., Quantitative physiology of *Saccharomyces cerevisiae* at near-zero specific growth rates. *Appl. Environ. Microbiol.* 2009, *75*, 5607–5614.

[50] Taherzadeh, M. J., Niklasson, C., Lidén, G., Acetic acid-friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae*? *Chem. Eng. Sci.* 1997, *52*, 2653–2659.

[51] Verduyn, C., Postma, E., Scheffers, W. A., van Dijken, J. P., Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *J. Gen. Microbiol.* 1990, *136*, 395–403.

[52] Thomas, K. C., Ingledew, W. M., Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes. *J. Ind. Microbiol.* 1992, *10*, 61–68.

[53] Galazzo, J. L., Bailey, J. E., Growing *Saccharomyces cerevisiae* in calcium-alginate beads induces cell alterations which accelerate glucose conversion to ethanol. *Biotechnol. Bioeng.* 1990, *36*, 417–426.

[54] Hilge-Rotmann, B., Rehm, H.-J., Comparison of fermentation properties and specific enzyme activities of free and calcium-alginate-entrapped *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 1990, *33*, 54–58.

[55] Pham, T. K., Wright, P. C., Proteomic analysis of calcium alginate-immobilized *Saccharomyces cerevisiae* under high-gravity fermentation conditions. *J. Proteome Res.* 2008, *7*, 515–525.

[56] Lei, J., Zhao, X., Ge, X., Bai, F., Ethanol tolerance and the variation of plasma membrane composition of yeast floc populations with different size distribution. *J. Biotechnol.* 2007, *131*, 270–275.

[57] Hilge-Rotmann, B., Rehm, H.-J., Relationship between fermentation capability and fatty acid composition of free and immobilized *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 1991, *34*, 502–508.

[58] Smukalla, S., Caldara, M., Pochet, N., Beauvais, A. et al., *FLO1* Is a variable green beard gene that drives biofilm-like cooperation in budding yeast. *Cell* 2008, *135*, 726–737.

[59] Talebnia, F., Taherzadeh, M. J., Physiological and morphological study of encapsulated *Saccharomyces cerevisiae*. *Enzyme Microb. Tech.* 2007, *41*, 683–688.

[60] Sun, Z.-J., Lv, G.-j., Li, S.-y., Yu, W.-t. et al., Differential role of microenvironment in microencapsulation for improved cell tolerance to stress. *Appl. Microbiol. Biotechnol.* 2007, *75*, 1419–1427.

[61] Hlavácek, O., Kucerová, H., Harant, K., Palková, Z., Váchová, L., Putative role for ABC multidrug exporters in yeast quorum sensing. *FEBS Lett.* 2009, *583*, 1107–1113.

[62] Fux, C. A., Costerton, J. W., Stewart, P. S., Stoodley, P., Survival strategies of infectious biofilms. *Trends Microbiol.* 2005, *13*, 34–40.

[63] Albuquerque, P., Casadevall, A., Quorum sensing in fungi – a review. *Med. Mycol.* 2012, *50*, 337–345.

[64] Bjarnsholt, T., Jensen, P. Ø., Burmølle, M., Hentzer, M. et al., *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. *Microbiology* 2005, *151*, 373–383.

[65] Garcia-Contreras, R., Nunez-Lopez, L., Jasso-Chavez, R., Kwan, B. W. et al., Quorum sensing enhancement of the stress response promotes resistance to quorum quenching and prevents social cheating. *ISME J.* 2015, *9*, 115–125.

[66] Sprague, G. F., Winans, S. C., Eukaryotes learn how to count: Quorum sensing by yeast. *Gene. Dev.* 2006, *20*, 1045–1049.

[67] Chen, H., Fink, G. R., Feedback control of morphogenesis in fungi by aromatic alcohols. *Gene. Dev.* 2006, *20*, 1150–1161.

[68] Wuster, A., Babu, M. M., Transcriptional control of the quorum sensing response in yeast. *Mol. Biosyst.* 2009, *6*, 134–141.

[69] Westman, J. O., Ylitervo, P., Franzén, C., Taherzadeh, M. J., Effects of encapsulation of microorganisms on product formation during microbial fermentations. *Appl. Microbiol. Biotechnol.* 2012, *96*, 1441–1454.

[70] Purwadi, R., Taherzadeh, M. J., The performance of serial bioreactors in rapid continuous production of ethanol from dilute-acid hydrolyzates using immobilized cells. *Bioresour. Technol.* 2008, *99*, 2226–2233.

[71] Andrietta, S. R., Steckelberg, C., da Graca Andrietta Stupiello, M., Study of flocculent yeast performance in tower reactors for bioethanol production in a continuous fermentation process with no cell recycling. *Bioresour. Technol.* 2008, *99*, 3002–3008,.

[72] Van Mulders, S. E., Ghequire, M., Daenen, L., Verbelen, P. J. et al., Flocculation gene variability in industrial brewer's yeast strains. *Appl. Microbiol. Biotechnol.* 2010, *88*, 1321–1331.

[73] Zhao, X.-Q., Li, Q., He, L.-Y., Li, F. et al., Exploration of a natural reservoir of flocculating genes from various *Saccharomyces cerevisiae* strains and improved ethanol fermentation using stable genetically engineered flocculating yeast strains. *Process Biochem.* 2011, *47*, 1612–1619.

[74] van Mulders, S. E., Christianen, E., Saerens, S. M. G., Daenen, L. et al., Phenotypic diversity of Flo protein family-mediated adhesion in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 2009, *9*, 178–190.

[75] He, L.-Y., Zhao, X.-Q., Bai, F.-W., Engineering industrial *Saccharomyces cerevisiae* strain with the *FLO1*-derivative gene isolated from the flocculating yeast SPSC01 for constitutive flocculation and fuel ethanol production. *Appl. Energ.* 2012, *100*, 33–40.

[76] Li, Q., Zhao, X.-Q., Chang, A. K., Zhang, Q.-M., Bai, F.-W., Ethanol-induced yeast flocculation directed by the promoter of *TPS1* encoding trehalose-6-phosphate synthase 1 for efficient ethanol production. *Metab. Eng.* 2012, *14*, 1–8.

[77] Wang, F.-Z., Shen, W., Rao, Z.-M., Fang, H.-Y. et al., Construction of a flocculating yeast for fuel ethanol production. *Biotechnol. Lett.* 2008, *30*, 97–102.

[78] Cunha, A. F., Missawa, S. K., Gomes, L. H., Reis, S. F., Pereira, G. A. G., Control by sugar of *Saccharomyces cerevisiae* flocculation for industrial ethanol production. *FEMS Yeast Res.* 2006, *6*, 280–287.

[79] Ylitervo, P., Franzén, C. J., Taherzadeh, M. J., Ethanol production at elevated temperatures using encapsulation of yeast. *J. Biotechnol.* 2011, *156*, 22–29.
[80] Pourbafrani, M., Talebnia, F., Niklasson, C., Taherzadeh, M. J., Protective effect of encapsulation in fermentation of limonene-contained media and orange peel hydrolyzate. *Int. J. Mol. Sci.* 2007, *8*, 777–787.
[81] Ge, X. M., Bai, F. W., Intrinsic kinetics of continuous growth and ethanol production of a flocculating fusant yeast strain SPSC01. *J. Biotechnol.* 2006, *124*, 363–372.
[82] Sanchez i Nogue, V., Bettiga, M., Gorwa-Grauslund, M., Isolation and characterization of a resident tolerant *Saccharomyces cerevisiae* strain from a spent sulfite liquor fermentation plant. *AMB Express* 2012, *2*, 68.
[83] Matsushika, A., Inoue, H., Murakami, K., Takimura, O., Sawayama, S., Bioethanol production performance of five recombinant strains of laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. *Bioresour. Technol.* 2009, *100*, 2392–2398.
[84] Westman, J. O., Taherzadeh, M. J., Franzén, C. J., Inhibitor tolerance and flocculation of a yeast strain suitable for 2nd generation bioethanol production. *Electron. J. Biotechnol.* 2012, *15*, 1–14.
[85] Norton, S., Watson, K., D'Amore, T., Ethanol tolerance of immobilized brewers' yeast cells. *Appl. Microbiol. Biotechnol.* 1995, *43*, 18–24.
[86] Dror, Y., Cohen, O., Freeman, A., Stabilization effects on immobilized yeast: Effect of gel composition on tolerance to water miscible solvents. *Enzyme Microb. Technol.* 1988, *10*, 273–279.
[87] Qun, J., Shanjing, Y., Lehe, M., Tolerance of immobilized baker's yeast in organic solvents. *Enzyme Microb. Tech.* 2002, *30*, 721–725.
[88] Zi, L.-H., Liu, C.-G., Xin, C.-B., Bai, F.-W., Stillage backset and its impact on ethanol fermentation by the flocculating yeast. *Process Biochem.* 2013, *48*, 753–758.
[89] Conrad, M., Schothorst, J., Kankipati, H. N., Van Zeebroeck, G. et al., Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 2014, *38*, 254–299.
[90] Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O. et al., Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* 2000, *11*, 4241–4257.
[91] Carlson, M., Glucose repression in yeast. *Curr. Opin. Microbiol.* 1999, *2*, 202–207.
[92] Ho, N. W., Chen, Z., Brainard, A. P., Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl. Environ. Microbiol.* 1998, *64*, 1852–1859.
[93] Krahulec, S., Petschacher, B., Wallner, M., Longus, K. et al., Fermentation of mixed glucose-xylose substrates by engineered strains of *Saccharomyces cerevisiae*: Role of the coenzyme specificity of xylose reductase, and effect of glucose on xylose utilization. *Microb. Cell Fact.* 2010, *9*, 16.
[94] Sedlak, M., Ho, N. W. Y., Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast. *Yeast* 2004, *21*, 671–684.
[95] Saloheimo, A., Rauta, J., Stasyk, O. V., Sibirny, A. A. et al., Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases. *Appl. Microbiol. Biotechnol.* 2007, *74*, 1041–1052.

Cover illustration

Special Issue: ESBES. This issue of BTJ highlights a selection of articles presented at the 10th European Symposium on Biochemical Engineering Sciences (ESBES), which was held in Lille, France, September 7–10, 2014. The issue is edited by Guilherme Ferreira and Philippe Jacques and includes articles on metabolic engineering, protein expression and bioprocess development. The cover shows the Grande Place in Lille. © bbsferrari – Fotolia.com

Biotechnology Journal – list of articles published in the August 2015 issue.

Research Article
Fermentation broth components influence droplet coalescence and hinder advanced biofuel recovery during fermentation
Arjan S. Heeres, Karin Schroën, Joseph J. Heijnen, Luuk A. M. van der Wielen and Maria C. Cuellar
http://dx.doi.org/10.1002/biot.201400570

Research Article
Modeling leucine's metabolic pathway and knockout prediction improving the production of surfactin, a biosurfactant from *Bacillus subtilis*
François Coutte, Joachim Niehren, Debarun Dhali, Mathias John, Cristian Versari and Philippe Jacques
http://dx.doi.org/10.1002/biot.201400541

Research Article
A xeno-free microcarrier-based stirred culture system for the scalable expansion of human mesenchymal stem/stromal cells isolated from bone marrow and adipose tissue
Joana G. Carmelo, Ana Fernandes-Platzgummer, Maria Margarida Diogo, Cláudia Lobato da Silva and Joaquim M. S. Cabral
http://dx.doi.org/10.1002/biot.201400586

Research Article
Dynamic flux balancing elucidates NAD(P)H production as limiting response to furfural inhibition in *Saccharomyces cerevisiae*
Pornkamol Unrean and Carl J. Franzen
http://dx.doi.org/10.1002/biot.201400833

Research Article
Newly designed and validated impedance spectroscopy setup in microtiter plates successfully monitors viable biomass online
Bettina Luchterhand, Jannis Nolten, Sadik Hafizovic, Tino Schlepütz, Sandra Janine Wewetzer, Elke Pach, Kristina Meier, Georg Wandrey and Jochen Büchs
http://dx.doi.org/10.1002/biot.201400534

Research Article
A versatile, non genetically modified organism (GMO)-based strategy for controlling low-producer mutants in *Bordetella pertussis* cultures using antigenic modulation
Philippe Goffin, Thomas Slock, Vincent Smessaert, Philippe De Rop and Philippe Dehottay
http://dx.doi.org/10.1002/biot.201400539

Research Article
Maximizing the utilization of *Laminaria japonica* as biomass via improvement of alginate lyase activity in a two-phase fermentation system
Yuri Oh, Xu Xu, Ji Young Kim and Jong Moon Park
http://dx.doi.org/10.1002/biot.201400860

Research Article
Improving recombinant protein production in the *Chlamydomonas reinhardtii* chloroplast using vivid Verde Fluorescent Protein as a reporter
Stephanie Braun-Galleani, Frank Baganz and Saul Purton
http://dx.doi.org/10.1002/biot.201400566

Research Article
Regulation of the NADH pool and NADH/NADPH ratio redistributes acetoin and 2,3-butanediol proportion in *Bacillus subtilis*
Teng Bao, Xian Zhang, Xiaojing Zhao, Zhiming Rao, Taowei Yang and Shangtian Yang
http://dx.doi.org/10.1002/biot.201400577

Research Article
Engineering a branch of the UDP-precursor biosynthesis pathway enhances the production of capsular polysaccharide in *Escherichia coli* O5:K4:H4
Donatella Cimini, Elisabetta Carlino, Alfonso Giovane, Ottavia Argenzio, Ileana Dello Iacono, Mario De Rosa and Chiara Schiraldi
http://dx.doi.org/10.1002/biot.201400602

Research Article
Phenotypic variability in bioprocessing conditions can be tracked on the basis of on-line flow cytometry and fits to a scaling law
Jonathan Baert, Romain Kinet, Alison Brognaux, Anissa Delepierre, Samuel Telek, Søren J. Sørensen, Leise Riber, Patrick Fickers and Frank Delvigne
http://dx.doi.org/10.1002/biot.201400537