Available online at www.sciencedirect.com
ScienceDirect
Biotechnology Advances 26 (2008) 89–105
BIOTECHNOLOGY ADVANCES
www.elsevier.com/locate/biotechadv

Research review paper

Ethanol fermentation technologies from sugar and starch feedstocks

F.W. Bai [a,b,*], W.A. Anderson [a], M. Moo-Young [a]

[a] *Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1*
[b] *Department of Bioscience and Bioengineering, Dalian University of Technology, Dalian 116023, China*

Received 14 November 2006; received in revised form 26 August 2007; accepted 4 September 2007
Available online 8 September 2007

Abstract

This article critically reviews some ethanol fermentation technologies from sugar and starch feedstocks, particularly those key aspects that have been neglected or misunderstood. Compared with *Saccharomyces cerevisiae*, the ethanol yield and productivity of *Zymomonas mobilis* are higher, because less biomass is produced and a higher metabolic rate of glucose is maintained through its special Entner–Doudoroff pathway. However, due to its specific substrate spectrum as well as the undesirability of its biomass to be used as animal feed, this species cannot readily replace *S. cerevisiae* in ethanol production. The steady state kinetic models developed for continuous ethanol fermentations show some discrepancies, making them unsuitable for predicting and optimizing the industrial processes. The dynamic behavior of the continuous ethanol fermentation under high gravity or very high gravity conditions has been neglected, which needs to be addressed in order to further increase the final ethanol concentration and save the energy consumption. Ethanol is a typical primary metabolite whose production is tightly coupled with the growth of yeast cells, indicating yeast must be produced as a co-product. Technically, the immobilization of yeast cells by supporting materials, particularly by gel entrapments, is not desirable for ethanol production, because not only is the growth of the yeast cells restrained, but also the slowly growing yeast cells are difficult to be removed from the systems. Moreover, the additional cost from the consumption of the supporting materials, the potential contamination of some supporting materials to the quality of the co-product animal feed, and the difficulty in the microbial contamination control all make the immobilized yeast cells economically unacceptable. In contrast, the self-immobilization of yeast cells through their flocculation can effectively overcome these drawbacks.

Keywords: Ethanol fermentation; *Saccharomyces cerevisiae*; *Zymomonas mobilis*; Kinetics; Immobilization/self-immobilization; Very high gravity

Contents

* Corresponding author. Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1.
E-mail address: fwbai@dlut.edu.cn (F.W. Bai).

0734-9750/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.biotechadv.2007.09.002

1. Introduction

In recent years, a new round of enthusiasm in biomass and bioenergy has been initiated with the recognition that the global crude oil reserve is finite, and its depletion is occurring much faster than previously predicted. In addition, the environmental deterioration resulting from the over-consumption of petroleum-derived products, especially the transportation fuels, is threatening the sustainability of human society. Ethanol, both renewable and environmentally friendly, is believed to be one of the best alternatives, leading to a dramatic increase in its production capacity. Fig. 1 illustrates the increasing trend of ethanol production in the United States since 1980. As can be seen, a total production capacity of 4.89 billion gallons was achieved in 2006, representing a treble increase compared with that of 1.63 billion gallons in 2000. Other countries, such as China and India, are following this trend. The National Development and Reform Commission of the People's Republic of China initiated Chinese National Fuel Ethanol Program in 2002. Three large fuel ethanol plants with a total annual production capacity of 1.2 million tons were approved thereafter, and put into operation in 2005.

For such a bulk product, any improvements, especially in its major fermentation technologies, will be economically very attractive. In practice, the R&D in ethanol fermentation, especially using lignocellulosic biomass to replace current sugar and starch feedstocks, has never been interrupted since the shock of the crude oil crisis in the 1970s, except for a slowdown in the 1980s and 1990s when the price of crude oil was lower, and the economic prospect of fuel ethanol seemed to be less promising.

Currently, the global ethanol supply is produced mainly from sugar and starch feedstocks. Although Iogen, a Canadian company located in Ottawa, established a pilot plant in 2002, which can process 25 ton wheat straw per week and correspondingly, produce 320,000 liter ethanol per year (www.iogen.ca), the prospect of lignocellulosic ethanol, as Bungay (2004) later predicted, is still economically problematic. This review article focuses on the ethanol fermentation technologies from sugar and starch feedstocks, critically reviewing some key technical and economic challenges.

2. Microorganisms for ethanol production

Among many microorganisms that have been exploited for ethanol production, *Saccharomyces cerevisiae* still remains as the prime species. *Zymomonas*

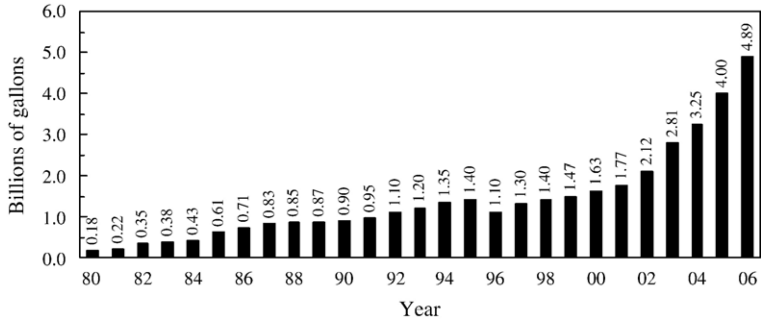

Fig. 1. Annual ethanol production volumes in the United States. (Data from the website of American Coalition for ethanol, www.ethanol.org/production.html).

mobilis has also been intensively studied over the past three decades and repeatedly claimed by some researchers to replace *S. cerevisiae* in ethanol production, because this species possesses some "superior characteristics" compared to its counterpart *S. cerevisiae*.

2.1. Saccharomyces cerevisiae

Although many researchers studied the ethanol fermentation with *S. cerevisiae*, in some cases a lack of recognition of its metabolic pathway led to approaches that are unlikely to yield significant improvements. The main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden–Meyerhof–Parnas or EMP pathway), through which one molecule of glucose is metabolized, and two molecules of pyruvate are produced (Madigan et al., 2000), as illustrated in Fig. 2.

Under anaerobic conditions, the pyruvate is further reduced to ethanol with the release of CO_2. Theoretically, the yield is 0.511 for ethanol and 0.489 for CO_2 on a mass basis of glucose metabolized. Two ATPs produced in the glycolysis are used to drive the biosynthesis of yeast cells which involves a variety of energy-requiring bioreactions. Therefore, ethanol production is tightly coupled with yeast cell growth, which means yeast must be produced as a co-product. Without the continuous consumption of ATPs by the growth of yeast cells, the glycolytic metabolism of glucose will be interrupted immediately, because of the intracellular accumulation of ATP, which inhibits phosphofructokinase (PFK), one of the most important regulation enzymes in the glycolysis. This very basic principle contradicts the ethanol fermentation with the yeast cells immobilized by supporting materials, particularly by gel entrapments, which physically restrict the yeast cells and significantly retard their growth.

In addition to ethanol and CO_2, various by-products are also produced during ethanol fermentation. Glycerol, producing at a level of about 1.0% (w/v) for most ethanol fermentations, is the main one. Higher mash pH,

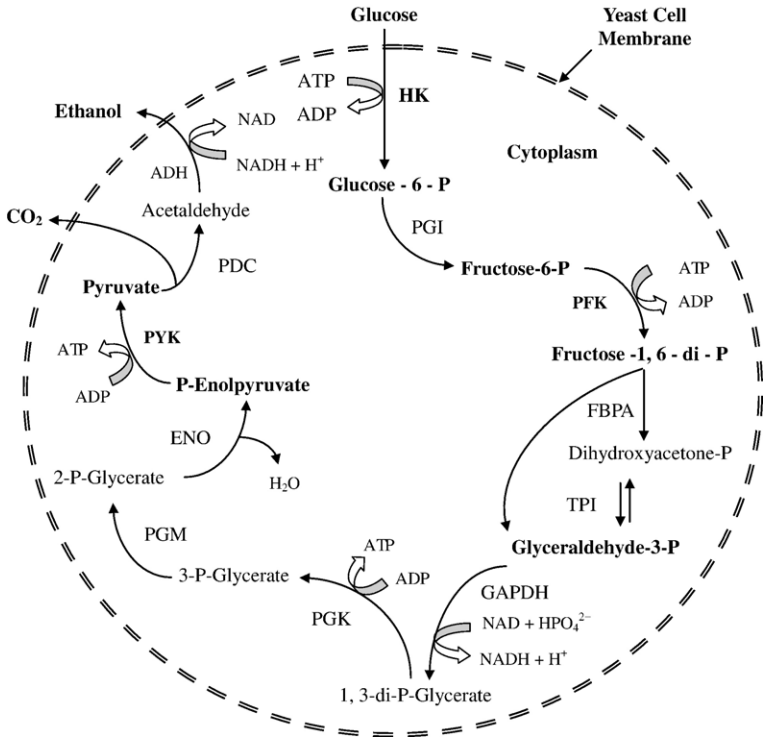

Fig. 2. Metabolic pathway of ethanol fermentation in *S. cerevisiae*. Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase.

increased osmotic pressure, lower flux of pyruvate due to the utilization of glycolytic intermediates subsequent to the step in the pathway producing reduced NAD for biosynthesis all can stimulate the conversion of dihydroxyacetone phosphate to glycerol (Ingledew, 1999). Other by-products such as organic acids and higher alcohols are produced at much lower levels. The production of these by-products as well as the growth of yeast cells inevitably direct some glycolytic intermediates to the corresponding metabolic pathways, decreasing the ethanol yield to some extent. In the industry, the ethanol yield that is calculated based on the total sugar feeding into the fermentation system without deduction of the residual sugar can be as high as 90–93% of its theoretical value of ethanol to glucose (Ingledew, 1999). Therefore, the residual sugar must be controlled at a very low level. For example, no more than 2 g l^{-1} and 5 g l^{-1} are controlled for the residual reducing sugar and total sugar, respectively, in the ethanol production from starch materials. Any ethanol fermentation research which is expected to be practical needs to bear these criteria.

During ethanol fermentations, yeast cells suffer from various stresses. Some are environmental such as nutrient deficiency, high temperature and contamination, while the others are from the yeast cell metabolism such as ethanol accumulation and its corresponding inhibition on yeast cell growth and ethanol production, especially under very high gravity (VHG) conditions that will be discussed later. Fig. 3 summarizes some of these stresses. Many of them are synergistic, affecting yeast cells more severely than any single one, leading to reduced yeast viability and vigor as well as lower ethanol yield.

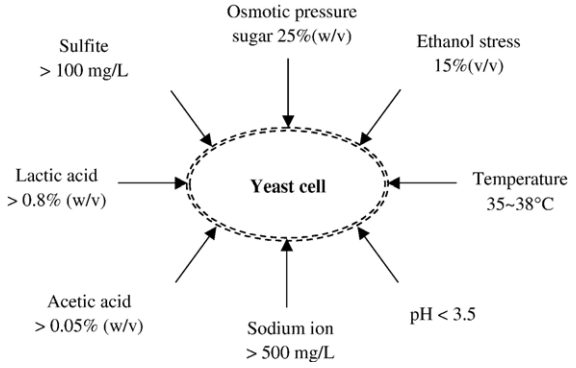

Fig. 3. Potential environmental stresses on *S. cerevisiae* during ethanol fermentation (Ingledew, 1999).

2.2. Zymomonas mobilis

Z. mobilis is an anaerobic, gram-negative bacterium which produces ethanol from glucose *via* the Entner–Doudoroff (ED) pathway in conjunction with the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Conway, 1992), as illustrated in Fig. 4. This microorganism was originally discovered in fermenting sugar-rich plant saps, *e.g.* in the traditional pulque drink of Mexico, palm wines of tropical African, or ripening honey (Swings and Deley, 1977).

Compared with the EMP pathway of *S. cerevisiae*, which involves the cleavage of fructose-1, 6-bisphosphate by fructose bisphosphate aldolase to yield one molecule each of glyceraldehydes-3-phosphate and dihydroxyacetone phosphate, the ED pathway forms glyceraldehyde-3-phosphate and pyruvate by the cleavage of 2-keto-3-deoxy-6-phosphogluconate by 2-keto-3-deoxy-gluconate aldolase, yielding only one molecule ATP per glucose molecule. As a consequence, *Z. mobilis* produces less biomass than *S. cerevisiae*, and more carbon is funneled to the ethanol fermentation. It was reported that the ethanol yield of *Z. mobilis* could be as high as 97% of the theoretical yield of ethanol to glucose (Sprenger, 1996), while only 90–93% can be achieved for *S. cerevisiae*. Also, as a consequence of the low ATP yield, *Z. mobilis* maintains a higher glucose metabolic flux, and correspondingly, guarantees its higher ethanol productivity, normally 3–5 folds higher than that of *S. cerevisiae* (Sprenger, 1996).

Despite these advantages, *Z. mobilis* is not suitable for the industrial ethanol production. Firstly, this species has a very specific substrate spectrum including only three sugars: D-glucose, D-fructose, and sucrose. Its growth on sucrose is accompanied by the extracellular formation of fructose oligomers (levan) and sorbitol, with a significant decrease in its ethanol yield (Sprenger, 1996), making it unsuitable for the ethanol production from molasses. Since it can effectively ferment only glucose in the hydrolysate of starch materials, not other sugars such as sucrose, fructose and maltose formed in the cooking and saccharifying, it is also unsuitable for the ethanol production from starch materials. The ethanol fermentation industry cannot use pure glucose as its raw material like many researchers did in their laboratory studies. Secondly, although *Z. mobilis* is generally regarded as safe (GRAS) (Lin and Tanaka, 2006), its biomass is not commonly acceptable to be used as animal feed, which inevitably generates the problem for its biomass disposal if it replaces *S. cerevisiae* in

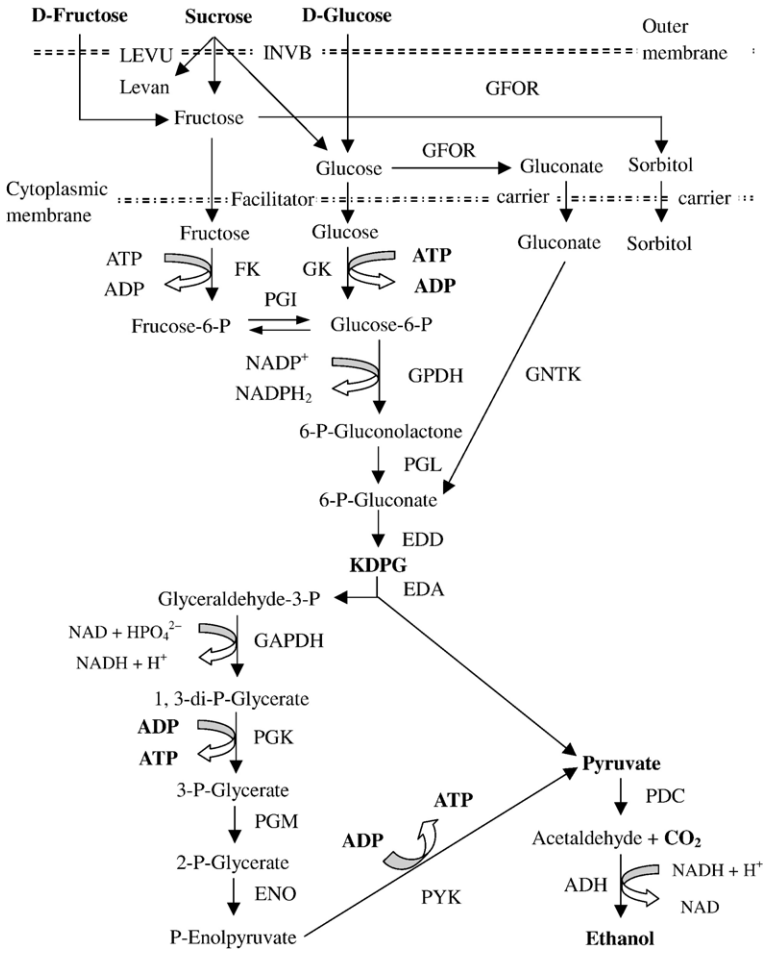

Fig. 4. Carbohydrate metabolic pathways in *Z. mobilis* (Sprenger, 1996). Abbreviations: LEVU: levansucrase, INVB: invertase, GFOR: glucose–fructose oxidoreductase, FK: fructokinase, GK: glucokinase, GPDH: glucose-6-phosphate dehydrogenase, PGL: phosphogluconolactonase, EDD: 6-phosphogluconate dehydratase, KDPG: 2-keto-3-deoxy-6-phosphogluconate, EDA: 2-keto-3-deoxy-gluconate aldolase, GNTK: gluconate kinase. See Fig. 2 for PGI, GAPDH, PGK, PGM, ENO, PYK, PDC and ADH.

the industrial ethanol production. And finally, the continuous ethanol fermentation with *Z. mobilis* tends to be oscillatory (Daugulis et al., 1997; McLellan et al., 1999). Although these oscillations can ameliorate the stresses exerted on the species such as the inhibitions by ethanol and sugar, and improve the ethanol productivity of the fermentation system, they also risk increasing the average residual sugar, and correspondingly, decreasing the ethanol yield.

Taking into account these drawbacks, some investigations involving the ethanol fermentation with *Z. mobilis* seem misdirected, although certainly of scientific interest. Some researchers who concluded this species would replace *S. cerevisiae* are likely too optimistic in their assessments.

3. Kinetics and process design

Kinetic theories and models are the very basis for process design, optimization and plant operation. However, the applications of these techniques in ethanol fermentations are still largely qualitative rather than quantitative. Some reasons are discussed in the following sections.

3.1. Steady or instantaneous kinetics

It is well known that ethanol is inhibitory to both yeast cell growth and ethanol production. Also, ethanol is a primary metabolite whose production is tightly coupled with the growth of yeast cells. In some of the

earliest kinetic research, Aiba et al. (1968) reported their results in the 1960s, in which a chemostat fermentation system was established and the media with initial glucose concentrations of 10 and 20 g l^{-1} were fed into the fermentation system at different dilution rates. Ethanol was added into the fermenter to reach the designated ethanol concentrations in order to investigate their inhibitions on yeast cell growth and ethanol production, since ethanol produced by yeast cells at these glucose concentrations was too low in concentrations to be inhibitory. The following kinetic models were proposed and correlated by their experimental data.

$$\mu = \mu_0 e^{-k_1 p} \frac{S}{K_S + S} \tag{1}$$

$$v = v_0 e^{-k_2 p} \frac{S}{K_S^* + S} \tag{2}$$

where μ and v are the specific rates for yeast cell growth and ethanol production, the subscript "$_0$" donates no ethanol inhibition, K_S and K_S^* are the Monod constants for yeast cell growth and ethanol fermentation, S is the limiting sugar concentration, p is the ethanol concentration, and k_1 and k_2 are constants.

Although ethanol inhibition effect is reflected in the Aiba's models, these models predict that ethanol concentration can approach infinity before yeast cell growth and ethanol production are completely inhibited, which appears unreasonable. On the other hand, when continuous ethanol fermentation is operated at a low dilution rate, especially when a medium containing low sugar concentration is used, the limiting sugar concentration S is likely to be undetectable. Under these conditions, the specific rates for yeast cell growth and ethanol production predicted by Eqs. (1) and (2) are zero, which are also incorrect since the broth containing yeast cells and ethanol is continuously produced in practice. These models have been heavily cited since they were proposed, with little discussion for their structural inadequacies.

A few other kinetic expressions have also been suggested, such as the linear models (Holzberg et al., 1967) and the parabolic models (Bazua and Wilke, 1977). However, these two groups of kinetic models were strictly limited to the experimental conditions in which they were correlated. Based on these work, Levenspiel (1980) further proposed a generalized non-linear equation to account for the influence of ethanol.

In the middle of the 1980s, Luong (1985) summarized the research progress in the kinetics of ethanol inhibition, and pointed out that the inhibition of yeast cell growth by ethanol is non-competitive, similar to non-competitive enzymatic reactions. The kinetic models below were proposed and the model parameters α and β were obtained through the batch fermentation experiments in which ethanol was externally added into the fermentation system, similar to the approach used by Aiba et al. (1968).

$$\frac{\mu}{\mu_0} = 1 - \left(\frac{P}{P_m}\right)^{\alpha} \tag{3}$$

$$\frac{v}{v_0} = 1 - \left(\frac{P}{P'_m}\right)^{\beta} \tag{4}$$

where P_m and P'_m are the ethanol concentrations at which yeast cell growth and ethanol production are completely inhibited, and α and β are the model parameters which can be evaluated by experimental data.

Some researchers reported that externally added ethanol was less toxic than ethanol produced by yeast cells during fermentations (Nagodawithana and Steinkraus, 1976; Ferreira, 1983), while others believed that the inhibition effect of ethanol, whether added or produced, was the same because of the excellent permeability of yeast cell membranes to ethanol (Guijarro and Lagunas, 1984). However it is preferable that reliable kinetic models are developed under the real fermentation conditions in which a high gravity (HG) medium is used, and high ethanol concentration is achieved by fermentation rather than by external addition.

In addition to ethanol inhibition, yeast cell concentration was found to be another inhibitor when extremely high biomass concentration was achieved through cell immobilization or the recycling of yeast slurry concentrated by membrane filtration or centrifugation. Lee and Chang (1987) established a continuous ethanol fermentation system coupled with a membrane unit to retain yeast cells, and the kinetic models for yeast cell growth and ethanol production were correlated with yeast cell concentration. They extrapolated and obtained the maximum yeast concentrations of 255 g (DCW) l^{-1} and 640 g (DCW) l^{-1}, respectively, at which yeast cell growth and ethanol production were completely inhibited. Porto et al. (1987) also investigated the impact of yeast cell concentration on ethanol production in a fermentation system similar to Chang's, and an exponential correlation between the specific ethanol production rate and yeast cell concentration was established. Unfortunately, such a high yeast cell concentration cannot be achieved in the industry, making the research with little practical use.

Although some researchers observed that the maximum ethanol concentration at which yeast cell growth was completely inhibited was different from that at

which ethanol production was completely inhibited, there were no reasonable explanations until Groot et al. (1992a,b) introduced the concept of maintenance into ethanol fermentation. They proposed that ethanol is continuously produced until the maximum concentration P'_{max} is approached, at which ethanol production is completely inhibited. This occurs at a higher ethanol concentration than P_{max} at which yeast cell growth is completely inhibited ($P'_{max} > P_{max}$). They further obtained a quantitative relationship between substrate consumption, ethanol production and maintenance through the continuous fermentation experiments run at different dilution rates using the media with initial glucose concentrations ranging from 120 g l^{-1} to 280 g l^{-1}. Thus, different ethanol concentrations were achieved through the fermentations rather than by adding ethanol into the fermentation system. This treatment is more reasonable, but has not been widely acknowledged to date.

Substrate inhibition was not taken into account in the Aiba's and Luong's models, nor in the Groot's work. Thus, these models may significantly deviate from the industrial situations where substrate inhibition potentially occurs, especially when the High gravity (HG) or VHG media are used to achieve high ethanol concentrations.

Substrate inhibition is a common phenomenon in other fermentations, and occurs when substrate concentration exceeds a strain-dependent level. Andrews (1968) generalized the work of Boon and Laudelout (1962) in the nitrite oxidation with *Nitrobacter winogradskyi*, studied the impact of substrate inhibition on batch and continuous cultures, and established the kinetic model in Eq. (5):

$$\mu = \mu_{max} \frac{S}{K_S + S + S^2/K_I} \quad (5)$$

where K_I is the substrate inhibition constant. Numerically, K_S and K_I are equivalent to the lowest and highest substrate concentrations, respectively, at which the specific growth rate μ is equal to one-half the maximum specific growth rate μ_{max}.

The kinetic investigations involving substrate inhibition in ethanol fermentations, especially in the genus of *S. cerevisiae*, are very limited, possibly because the simultaneous saccharification and fermentation is widely used in the ethanol production from starch materials, in which substrate inhibition is not significant since sugar is consumed by yeast cells immediately after it is released and a low sugar concentration is maintained within the fermentation system. However, for the ethanol production from molasses, substrate inhibition is more likely, due to the high initial sugar concentration in the medium.

In comparison with *S. cerevisiae*, more investigations have been done on the kinetics of *Z. mobilis*. On the one hand, this species, as an ethanol production alternative, was discovered relatively late in the early 1960s. On the other hand, since then it has been highly anticipated as a potential industrial species for ethanol production, replacing *S. cerevisiae*, because of its much faster fermentation rate and a higher ethanol yield from its ED pathway opposed to the EMP pathway in *S. cerevisiae*, as illustrated in Fig. 4. However, this species is not economically competitive with *S. cerevisiae* because of its narrow substrate spectrum and the problem for its biomass treatment, as discussed in Section 2.2.

In the studies of the ethanol fermentation with *Z. mobilis*, glucose was the only carbon source. In order to achieve a higher ethanol concentration, the HG medium was used, which inevitably generated substrate inhibition. Huang and Chen (1988) investigated the kinetic behavior of *Z. mobilis* in the batch fermentation using the HG medium containing 200 g l^{-1} glucose, and established the kinetic models characterized by both substrate and product inhibitions. In addition, because *Z. mobilis* can tolerate higher temperature than *S. cerevisiae*, the impact of temperature on its kinetic performance was also investigated (Stevsborg et al., 1986; Fieschko and Humphrey, 1983).

3.2. Process dynamics

All kinetic models currently available for the ethanol fermentations with *S. cerevisiae* are steady state for continuous fermentations or instantaneous for batch processes. Although unsteady states and oscillations are common phenomena in biological systems, and many studies in the oscillation of the glycolytic pathway in *S. cerevisiae* have been reported, there are only a few reports on the oscillations of sugar, ethanol and biomass in the continuous ethanol fermentations with *S. cerevisiae* (Borzani, 2001; Bai et al., 2004a). For the cascade fermentation system composed of 4–6 fermentors in the industry, the residual sugar, ethanol and yeast concentrations in the front main fermentors do oscillate up and down around their average levels, but these oscillations are gradually dampened as the fermented broth goes through the rear fermentors, and completely attenuated within the last fermentor. A much longer average fermentation time is needed to achieve the oscillation attenuation. For example, 50–70 h is commonly required to achieve the final ethanol

concentration of only 10–12% (v/v), but maintains the residual reducing and total sugars at no more than 2 g l^{-1} and 5 g l^{-1}, respectively, for the ethanol fermentation from starch materials, if no centrifuges are used to separate and recycle the yeast cells.

In case of oscillation, dynamic rather than steady state kinetics are required to explain, predict and optimize the corresponding fermentation process. A mechanistic analysis is the prerequisite to developing such kind of dynamic kinetic models. Although there are many investigations involving the oscillation in the continuous cultures of *S. cerevisiae* (Patnaik, 2003), the oscillation patterns observed in the continuous ethanol fermentations with *S. cerevisiae* and *Z. mobilis* are significantly different from those observed in the continuous aerobic cultures of *S. cerevisiae*, and the mechanisms triggering these oscillations are also different (Bai et al., 2004a).

Jöbses et al. (1986) analyzed the sustained oscillations in biomass, ethanol and glucose concentrations in the continuous ethanol fermentation with *Z. mobilis*, and pointed out that the theoretical possibility of such an oscillation system is a delayed response of *Z. mobilis* to its inhibitory product ethanol. A delayed inhibition can be imagined if the inhibitor does not act directly on the fermentation and thereby on its own production, but indirectly on what is coupled with its production, like ethanol which first inhibits the growth of bacteria or yeast cells that is tightly coupled with its production.

For a dynamic ethanol fermentation system, the inhibition of ethanol involves two aspects: the history of ethanol concentration and the rate of ethanol concentration change. Li et al. (1995) designed their ethanol fermentation experiments, and revealed that the rate of ethanol concentration change, especially the upward change rate, exerted much stronger inhibition than the history of ethanol concentration, *i.e.* the time experienced at a certain ethanol concentration. Furthermore, they developed the dynamic models incorporating this time delay and predicted the oscillatory behavior of *Z. mobilis* (Daugulis et al., 1997; McLellan et al., 1999). No similar work is available to date for the continuous ethanol fermentation with *S. cerevisiae*.

3.3. Intracellular metabolic dynamics

Metabolism is dynamic, and several mechanisms involving the glycolytic dynamics and oscillations of some intermediate metabolites have been elucidated (Madsen et al., 2005; Richard, 2003). PFK with its allosteric regulation, in particular its substrate inhibition by ATP, was pointed out as the source of the glycolytic dynamic behavior in yeast cells, and was termed "the oscillophore" in the early research (Ghosh and Chance, 1964). The basis for this conclusion was a special application of the crossover theorem (Chance et al., 1958), in which the enzymatic control points of the glycolytic pathway were identified as being those enzymes with the biggest phase-shifts between their substrates and products. The experimental work of Hess (1979) further supported this conclusion. From a contemporary point of view, this crossover theorem in the analysis of the glycolytic oscillation seems too simple and weak. However, the allosteric regulation of PFK does provide a key control in the dynamic regulation of the whole glycolytic pathway in *S. cerevisiae* (Boiteux et al., 1975; Yuan et al., 1990).

Betz and Chance (1965) also reported the phase relationships of the glycolytic intermediates in yeast cells. In addition to the components indicated by Ghosh and Chance (1964), dihydroxyacetone phosphate and pyruvate were also labeled at the branch points in the glycolytic network, where the fluxes through the branches depend on the availability of NADH (for the glycerol 3-phosphate dehydrogenase reaction in the case of dihydroxyacetone phosphate, and for the ADH reaction in the case of pyruvate), indicating the complexity of the dynamics of the glycolysis and the existence of more control sites than only PFK.

Distributed control is another mechanism of interest. One could expect that the oscillations, flux and concentration changes in the glycolysis in yeast cells are systemic properties, which are determined by the interplay between the intracellular constituents. Hence, PFK is surely not the only part of the network exerting control on glycolytic dynamic properties. Based on the phase angles of the glycolytic intermediates, Hynne et al. (2001) pointed out the importance of pyruvate kinase, the enzyme pair: phosphoglycerate kinase and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), hexose transport kinetics, and the glycolytic ATP stoichiometry. The redox feedback loop consisting of the conserved sum of NAD^+ and NADH has received attention, as it plays a key role in the acetaldehyde-based mechanism, which is believed to be responsible for the active synchronization of the oscillations among individual yeast cells. Acetaldehyde acts as the substrate for ADH, producing ethanol and oxidizing NADH to NAD^+. The altered NAD^+/NADH ratio then modulates the phases of the oscillations *via* the GAPDH reaction (Richard et al., 1996; Wolf et al., 2000).

Although there has been so much research progress in elucidating the mechanisms of the glycolytic

dynamics in yeast cells, the bridges between these scientific insights and the engineering aspects of ethanol fermentation are scarcely available, for example, the intrinsic connection between the intracellular metabolites and extracellular substrates and products. This makes the ethanol fermentation process design and plant operation, to a large extent, dependent on know-how rather than fundamental scientific understanding.

3.4. Process design

As discussed above, the kinetics of ethanol fermentation are characterized by strong product inhibition, especially under HG and VHG fermentation conditions. *In situ* removal of ethanol seems to be the best way to minimize this effect, increasing the fermentation rate and productivity (Roffler et al., 1984). Among the many technologies available for removing ethanol from the corresponding fermentation systems, the combination of pervaporation and fermentation has been widely investigated (Ikegami et al., 2002; Groot et al., 1992a,b).

O'Brien and Craig (1996) and O'Brien et al. (2000) analyzed the economic feasibility of coupling pervaporation with ethanol fermentation by comparing it with a typical dry-milling ethanol plant, and pointed out that the cost for the fermentation–pervaporation process is much higher. In addition, the problem of membrane fouling, when handling the fermented mash with a high concentration of raw material solid residue, is also a big problem (Vane, 2005), making this technology impractical for use in the dry-milling fuel ethanol production. Possibly, it could be an option for a wet-milling process in which most raw material residues is separated prior to the fermentation, and a liquid rather than a slurry mash is fed into the fermentors. However, the flux of the pervaporation membrane currently available is still not high enough to realistically meet the large ethanol production capacities of the wet-milling processes. In addition, the membrane fouling by yeast cells could be another problem that needs to be addressed.

The vacuum fermentation also can decrease the ethanol concentrations inside the fermentors, and this technology was developed for the ethanol fermentations with *S. cerevisiae* as well as *Z. mobilis* in the 1970s and 1980s (Cysewski and Wilke, 1977; Ghose et al., 1984; Lee et al., 1981). However, it is also impractical for ethanol fermentors, with their working volumes of hundreds to thousands of cubic meters, to be operated at a significant vacuum condition. The capital cost for tank manufacture and the energy consumption for maintaining the vacuum condition are likely economically unacceptable.

In another approach to *in situ* ethanol removal, Taylor et al. (2000) evaluated a small pilot plant operation in which the ethanol fermentation was coupled with a stripping column and condenser. Ethanol was stripped by the recycled CO_2 in the stripping column, and the CO_2 enriched with ethanol was passed through the condenser where the ethanol was absorbed by the circulated dilute ethanol condensate. The dry-milling corn mash containing 309 g l^{-1} dry solid was continuously fed into the fermentor at a flow rate of 3.36 kg h^{-1}, and the ethanol concentration inside the fermentor was decreased to 48 g l^{-1}, resulting in a significantly improved fermentation rate. The condensate containing 257 g l^{-1} ethanol was pumped out of the system at a flow rate of 0.879 kg h^{-1}. Meanwhile, the fermented mash containing 48 g l^{-1} ethanol was also pumped out of the system at a flow rate of 2.8 kg h^{-1}, mixing with the condensate containing 270 g l^{-1} ethanol, to maintain the mass balance of the fermentation system. The mixture with a lower ethanol concentration was distilled, making the distillation process still very energy-intensive. Another disadvantage of this system is the complexity of its process design and operation, although further research may lead to some improvements.

In the event that economically feasible technologies that can remove ethanol *in situ* to alleviate ethanol inhibition are not available, the bioreaction engineering strategies that can alleviate ethanol inhibition through decreasing the backmixing in the fermentation system could be an alternative. Theoretically, batch bioreactors (BBR) and plug flow bioreactors (PFBR) are the best options because of their lack of backmixing, which effectively reduces their time-averaged product inhibition (Levenspiel, 1999). Indeed, BBRs have been widely used in many conventional ethanol fermentations, especially in the plants with small production capacities. Currently, due to the dramatically increasing market demand for fuel ethanol, much larger scale ethanol fermentation plants with their annual production capacities of several hundred thousand tons are being planned, established and operated, in which the fermentors with working volumes in the order of thousands of cubic meters are required. For example, a total of 20 fermentors, each with a working volume of 3000 m^3, were constructed for an ethanol plant with a production capacity of half a million tons in Jining, Shandong Province, East China, in 2003. The disadvantage of a BBRs longer operational downtime required by mash adding, broth harvesting, and facility cleaning makes it unattractive for use in the large scale ethanol fermentation plants. As an average fermentation time of 48–72 h is normally required to achieve the final ethanol

concentration of 10–12% (Bothast and Schlicher, 2005), PFBRs with high superficial flow rates are also difficult to design and operate effectively.

The bioreactor engineering theory indicates that, compared with a single continuously stirred tank bioreactor (CSTR), a tanks-in-series system can effectively alleviate product inhibition by reducing the overall backmixing (Levenspiel, 1999). In fact, this strategy has been practiced in the ethanol fermentation industry for many years (Madson and Monceaux, 1999). Generally, 4–6 tanks in series are preferred because such a design tends to achieve the best balance between the ethanol fermentation kinetics and the capital investment for the tank manufacture. For the VHG ethanol fermentation, product inhibition is much worse than the regular fermentation, and substrate inhibition may also occur. Special considerations are needed.

4. Mechanisms of ethanol inhibition

Microbial cells exposed to ethanol correspondingly adjust their intracellular metabolisms, and this is manifested as ethanol inhibition. Not only are yeast cell growth and ethanol production inhibited, but also unsteady state and oscillation are triggered by the delayed response of yeast cells to the ethanol inhibition exerted on them. Understanding the mechanisms through which toxic ethanol inhibits yeast cells is the prerequisite to better exploitation of the potential of the strains as well as for the optimization of the fermentation process to effectively alleviate ethanol inhibition.

Ethanol inhibition has multiple effects and is very complicated. Fig. 5 shows some possible sites in yeast cells at which ethanol could exert a significant influence (D'Amore and Stewart, 1987).

Some key enzymes in the glycolytic pathway of yeast cells, such as hexokinase and ADH, may be affected by ethanol, and ethanol may also affect the nutrient uptake and membrane potential by decreasing the activity of the plasma membrane ATPase (Casey and Ingledew, 1986; Larue et al., 1984). Although many discrepancies exist among the studies of the mechanisms of ethanol inhibition, it has been commonly accepted that the membranes of some organelles and cells are the main targets of ethanol attack (D'Amore and Stewart, 1987). In many cases, the inhibition of ethanol is exacerbated by the presence of other fermentation by-products such as acetaldehyde and acetate, and other stresses such as high temperature (Jones, 1989).

Fatty acids, especially unsaturated fatty acids such as palmitoleic acid (C16:1) and oleic acid (C18:1), are key membrane components, which counteract ethanol inhibition by increasing the fluidity of the plasma membranes, in compensation for fluidity decreases resulting from ethanol effects. The levels of these two unsaturated fatty acids were measured to be higher for ethanol tolerant strains, or significantly increased after ethanol stress was exerted (You et al., 2003). They are synthesized in *S. cerevisiae* by the catalysis of the oxygen- and NADH-dependent desaturase of palmitic acid (C16:0) and stearic acid (C18:0). Therefore, a small amount of oxygen is required for yeast cells to

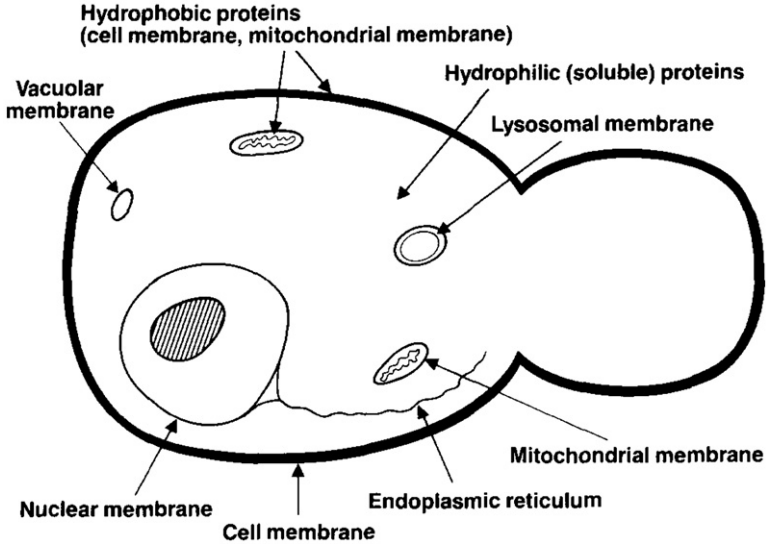

Fig. 5. Possible target sites for ethanol inhibition in yeast cells (D'Amore and Stewart, 1987).

synthesize these unsaturated fatty acids under anaerobic fermentation conditions. In fact, the role of a small amount of oxygen supply in improving the ethanol tolerance of yeast cells was investigated as early as in the 1980s (Ryu et al., 1984) and has been practiced in the ethanol fermentation industry for a long time. It is predicted that, under a VHG fermentation condition, the role of such "micro-aeration" in improving ethanol tolerance will be more significant since much stronger ethanol inhibition can develop.

The trans-membrane proton flow that drives the secondary transport of many nutrients has been found to be sensitive to ethanol inhibition. The dissipation of the proton gradient induced by ethanol was proposed to be involved in both the increase of the plasma membrane permeability (Pascual et al., 1988) and the inhibition of the proton-pumping plasma membrane ATPase activity (Salguerio et al., 1988). It was therefore pointed out that the plasma membrane ATPase is a key membrane protein, whose activity could be directly related to the ethanol tolerance of strains (Cartwright et al., 1987). Rosa and Sá-Correia (1992) reported that ethanol activated the plasma membrane ATPase of *S. cerevisiae* and *K. marxianus* at its lower levels, while it exerted inhibition at higher levels. Obviously, an adequate activity of the plasma membrane ATPase is a basis for yeast cells to maintain their intracellular physiological pH, because the H^+ produced during the ethanol fermentation needs to be continuously pumped out of the yeast cells by the proton motive force driven by the ATPase. Therefore, the ethanol inhibition in the VHG fermentation is expected to be alleviated by properly neutralizing its environmental H^+ and decreasing the H^+ gradient across the membranes when the plasma membrane ATPase is inhibited by the high ethanol concentration and cannot provide enough driving force to drive the H^+ out.

The pH values of the rear fermentors in a cascade fermentation system are observed to be higher than those of the front main fermentors. The main reasons may be the increase of the plasma membrane permeability as well as the decrease of the ATPase activity caused by the stronger ethanol inhibition, rather than by the by-product metabolism such as the uptake of the organic acids produced within the main fermentors, which is commonly assumed under the so-called condition of sugar depletion within the rear fermentors. In these fermentors, the ethanol concentration approaches to as high as 12% (v/v), causing very strong inhibition on the yeast cells, while the residual sugar in the range of 0.1–0.2% (w/v) is not yet entirely depleted.

Indeed, the mechanisms of ethanol inhibition are still not clear in many aspects, especially at the genetic level, and the investigations in this field are ongoing (Alper et al., 2006). Many factors, such as temperature and nutrients, directly or indirectly, affect the ethanol inhibition effect on the same strains, either exacerbating or alleviating it. Many strategies have been and will continue to be developed.

5. Yeast cell immobilization

5.1. Yeast cell immobilization using supporting materials

The concept of whole cell immobilization was proposed in the 1970s (Kierstan and Bucke, 1977), and was initially developed from the concept of enzyme immobilization, aiming at simplifying the complicated bioreactions that are catalyzed by multiple intracellular enzymes involving cofactors or coenzymes. It was assumed that the immobilized cells were more economically competitive than separate enzymes. However, because cells, whether living or dormant, are far more complicated, the immobilized cells in many cases do not work as efficiently as predicted. Recently, the research progress related to the ethanol fermentation using the immobilized yeast cells was reviewed (Kourkoutas et al., 2004; Brányik et al., 2005; Verbelen et al., 2006).

Theoretically, immobilized cells are more reasonable for the production of secondary metabolites than for the production of primary metabolites which is tightly coupled with the growth of cells. When cells are immobilized, their growth is affected by the factors such as the physical constraints, depletion of nutrients and accumulation of toxic metabolites because of the potential mass transfer limitation. On the other hand, the slowly growing cells within an immobilized cell system are difficult to be removed out of the system, especially when cells are immobilized by gel entrapments, one of the most commonly used cell immobilization strategies.

Ethanol is a typical primary metabolite whose production is tightly coupled with the growth of yeast cells. The ethanol fermentation rate of non-growing yeast cells is at least 30 folds slower than that of the growing ones (Ingledew, 1999), because the accumulation of ATP strongly inhibits the activity of PFK, one of the key regulation enzymes in the glycolysis pathway. Many studies involving the ethanol fermentation using the immobilized yeast cells supported by inert materials, especially entrapped by gels, have been performed within the past three decades. However, no successful cases have been reported since Nagashima et al. (1984) reported their pilot plant operation, in which yeast cells were immobilized by calcium alginate gel entrapment. From a metabolic basis, the ethanol fermentation using

the immobilized yeast cells through gel entrapments is not a productive direction, but this line of research has continued (Jamai et al., 2001; Saraydin et al., 2002; Öztop et al., 2003; Najafpour et al., 2004; Decamps et al., 2004).

Some yeast cell immobilization methods, especially by the surface adsorption, seem to be more reasonable than those by gel entrapment, membrane retention, or microencapsulation through which yeast cells are tightly constrained. When yeast cells are immobilized by the surface adsorption, their growth is not significantly affected, and some yeast cells can be washed out of the fermentation system, because the adsorption of yeast cells onto the surfaces is generally weak. When one of the authors (F. Bai) visited a molasses ethanol fermentation plant in Guangdong Province, South China in the middle of the 1990s, and found the workers there were using fibers to trap yeast cells to improve the fermentation, it was clear that this kind of yeast cell immobilization technology is more practical than many others. In fact, the yeast cells are temporarily immobilized onto the fiber surfaces, resulting in higher cell densities, but they are able to grow and be continuously renewed. In addition, such supporting materials are readily cleaned and microbial contamination can be effectively prevented.

The goal of using immobilized yeast cells in ethanol fermentation is to increase the ethanol productivity of the fermentation system, and correspondingly, decrease the requirement for the fermentor volume to save the capital investment for the fermentor manufacture. As the fermentors used for ethanol production are generally made of lower-grade inexpensive carbon steel, without powerful mixers, this projected saving of the capital investment is actually insignificant, especially when other process parameters are compromised. Therefore, it can be concluded that this goal is not readily achievable, both technically and economically.

However, when yeast cells aggregate together by their close contact when they are immobilized, the yeast cells can obtain much better protection from harsh and inhibiting ethanol fermentation conditions. Jirku (1999) studied *S. cerevisiae* immobilized by the gel entrapment, and found that the leakage of UV-absorbing intracellular substances was significantly decreased, compared with that of the free yeast cells. Further analysis of the membrane composition of the immobilized yeast cells indicated that fatty acids, phospholipids, and sterols increased, and therefore better protection was provided for the yeast cells under ethanol duress. Desimone et al. (2002) also reported that the specific death rate for the gel immobilized yeast cells decreased to one tenth of that of the free yeast cells, when the two groups were exposed to 50% (v/v) ethanol shock for 15 min. Recently, the research in the ethanol fermentation using the self-flocculating yeast cells showed similar results (Hu et al., 2005). The ethanol tolerance was significantly improved when the yeast cells self-flocculated and formed the flocs at a millimeter scale, compared with the free yeast cells at a micrometer scale. Not only was a membrane composition change generated from the self-flocculation, but the potential synergistic roles of the yeast cells also contributed to the ethanol tolerance improvement. These new research findings indicate the potential of the immobilized yeast cells in achieving a higher ethanol concentration in the VHG fermentation, which will be addressed later.

5.2. Yeast cell immobilization by self-flocculation

The flocculation of yeast cells, usually occurring spontaneously, has been investigated and used for separating yeast cells from beer in the brewing industry (Verstrepen et al., 2003). Technically, when yeast cells flocculate and form the flocs with an appropriate size range, they can be effectively retained and immobilized within the fermentors. In nature, it is a kind of yeast cell self-immobilization technology, which seems to be superior to the yeast cell immobilization technologies by supporting materials.

Firstly, no supporting material is consumed, which not only makes the process more simple and economically competitive compared with the yeast cell immobilization by supporting materials, but also completely eliminates the potential contamination to the quality of the co-product animal feed by the supporting materials. Secondly, the growth of yeast cells is not significantly affected, guaranteeing the ethanol fermentation to be carried out effectively. Thirdly, the yeast flocs can be purged from the fermentors under controlled conditions, maintaining the biomass concentrations inside the fermentors at designated levels. And finally, the yeast flocs purged from the fermentors can be recovered by sedimentation rather than by centrifugation which is widely used in the recovery of free yeast cells, saving the capital investment for centrifuges as well as the energy consumption for centrifuge operation.

The ethanol fermentations using the self-flocculating yeast strains were reported in the 1980s and 1990s. Different fermentor configurations were developed, including air-lift fermentors (Bu'lock et al., 1984), single packed column fermentors (Gong and Chen, 1984; Jones et al., 1984; Admassu and Korus, 1985) and two-stage packed

column fermentors coupled with settlers (Kuriyama et al., 1993) and without settlers (Kida et al., 1990), a CO_2 suspended-bed fermentor with baffle plates inside and separation tanks outside (Limtong et al., 1984) or only with a separation tank for CO_2 to be separated and recycled to the fermentor to suspend the yeast flocs (Kida et al., 1989). However, for unclear reasons, the research in this field has been relatively sparse in the past decade except for one of the authors' groups in China (F. Bai), reporting some fundamental research progress, such as the on-line monitoring and characterization of the self-flocculating yeast flocs, the intrinsic and observed kinetics, as well as some considerations for the corresponding fermentation process design (Xu et al., 2005; Ge et al., 2005; Ge and Bai, 2006; Ge et al., 2006a,b).

In 2005, the ethanol fermentation with the self-flocculating yeast has been commercialized, and a fuel ethanol plant with an annual production capacity of 200,000 tons was established at BBCA, one of the three fuel ethanol producers in China. Fig. 6 illustrates its fermentation process design. Six fermentors, each with a working volume of 1000 m^3, were designed and arranged in a cascade mode. Corn meal hydrolysate, with a sugar concentration of 200–220 g l^{-1}, is fed into the fermentation system at a dilution rate of 0.05 h^{-1}. The fermented broth with an ethanol concentration of 11–12% (v/v) is discharged from the fermentation system. The yeast flocs are retained within the fermentors by the baffles and effectively immobilized during the continuous fermentation, and the yeast-free broth overflows into the next fermentor or the storage tank for the downstream distillation treatment. The yeast slurry is circulated by a pump, passing through an external plate heat exchanger to control the fermentation temperature at 32–34 °C, and a small yeast stream bleeding to the next fermentor can balance the growth of the yeast cells within the front fermentor, maintaining the biomass concentrations within the fermentors at the levels of 40–60 g (DCW) l^{-1}. The yeast slurry bleeding from the last fermentor is transferred to the sedimentation tank where the yeast flocs are separated, and the yeast paste is collected for its post-processing.

6. VHG fermentation

High ethanol concentration has been continuously pursued in the industry, because significant energy savings can be achieved for the downstream distillation and waste distillage treatment. HG ethanol fermentation was proposed in the 1980s, and successfully commercialized thereafter, making the final ethanol concentration increase dramatically from the previous level of 7–8% (v/v) to the current value of 10–12% (v/v). Research in yeast physiology has revealed that many strains of *S. cerevisiae* can potentially tolerate far higher ethanol concentration than previously believed (Casey and Ingledew, 1986; Thomas and Ingledew, 1992), usually without any conditioning or genetic modifications. Thus, VHG ethanol fermentation using the medium containing sugar in excess of 250 g l^{-1} to achieve over 15% (v) ethanol was proposed in the 1990s (Thomas et al., 1996; Wang et al., 1999; Bai et al., 2004a,b). Among many ethanol fermentation technologies, the VHG fermentation is very promising for its industrial application. On the one hand, the energy cost is the second largest part in ethanol production only after the cost of the raw material consumption. On the other hand, the availability of the VHG mash in mass

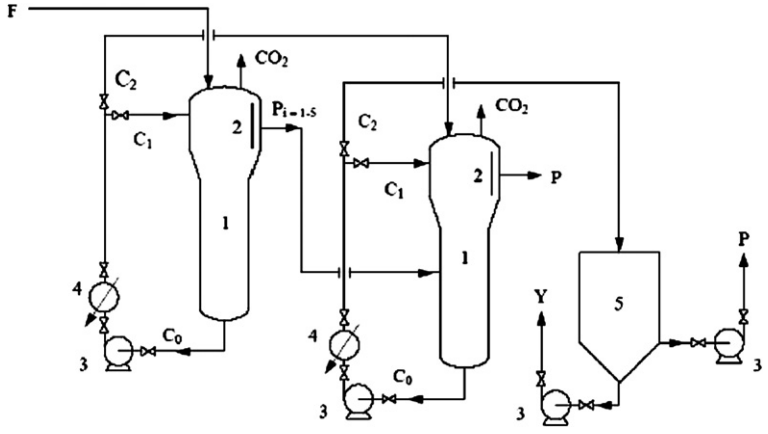

Fig. 6. Process diagram for the continuous ethanol fermentation with the self-flocculating yeast. 1. fermentors, 2. baffles, 3. pumps, 4. heat exchangers, 5. sedimentation tank. F: substrate stream, $P_{i=1-5}$: fermented broth, P: final product stream, Y: yeast paste for post-processing, C_1: circulating stream, C_2: yeast bleeding stream. *i*: number of fermentors in the cascade fermentation system ($i=5$, last fermentor not included).

quantities is now economically feasible, because the low cost and highly efficient enzymes such as α-amylases, glucoamylases, and proteases are now available. Meanwhile, the concept of the bio-refinery requires the separation of most raw material solid residues in the pretreatment, especially for those ethanol fermentation plants with large processing capacities, which further guarantees the reliable supply of the VHG mash. However, both the scientific research and applied technology development in the VHG ethanol fermentation have not been given enough attention, and only a few groups seem to be working in this field (Bayrock and Ingledew, 2001; Lin et al., 2002; Bai et al., 2004a,b; Devantier et al., 2005).

Research has revealed that the ability of yeast strains to achieve a high level of ethanol strongly depends on the nutritional conditions and protective functions that some nutrients can provide. Assimilable nitrogen is the most important component in the fermentation medium and has been reported to be a limiting nutrient in the VHG ethanol fermentation using wheat mash. Thomas and Ingledew (1990) fermented wheat mash with 350 g l^{-1} dissolved solids and produced 17.1% (v/v) ethanol in 8 days at 20 °C. When supplemented with 0.9% yeast extract, the fermentation time achieving the same ethanol concentration was reduced to 3 days. Considering that yeast extract was too costly for the industrial use, Jones and Ingledew (1994a) further studied the possibility of replacing it with typical industrial nutrient supplements, and found that urea was a good alternative. When proteases were used, the proteins in the mash were hydrolyzed into free amino acids and small peptides, providing nutrients to the yeast cells, and the VHG fermentation was significantly improved (Jones and Ingledew, 1994b).

Under the VHG fermentation conditions, substrate inhibition affects yeast cells by exerting a high osmotic pressure (Devantier et al., 2005). Glycine was found to be one of the most effective osmoprotectants, which helped in maintaining high viability of yeast cells (Thomas et al., 1994). Reddy and Reddy (2005, 2006) reported that horse gram (*Dolichos biflorus*) and finger millet (*Eleusine coracana*) improved the activity of *S. cerevisiae* under the VHG conditions because of their roles as osmoprotectants and nutrients.

In addition to product and substrate inhibition, other stresses may also exist. Although high temperature is desirable in the ethanol fermentation industry because of the potential savings in the cooling costs, the negative impact of high temperature on the ethanol fermentation performance is much worse under the VHG conditions than the regular fermentation. Jones and Ingledew (1994c) investigated the impact of temperature on the VHG fermentation and found that the fermentation time was dramatically extended when the temperature was increased from 17 °C to 33 °C.

Among all the aspects being investigated, the most important point is that the improvement of the VHG fermentation must be economically feasible and acceptable by the industry. Many medium supplements used in laboratory research, such as amino acids, vitamins, sterols and unsaturated fatty acids, are too expensive to be used in the industry, although they may provide insights into the fermentation fundamentals. More likely, the improvements in the process engineering design and operation, which can help optimize the physiological environment for the yeast cells under a variety of stresses, will be more economically feasible and a focus for continuing development. For example, increasing the number of the tanks-in-series systems for new plants or adding baffles inside the existing tanks can significantly decrease the overall backmixing and alleviate the ethanol inhibition effect. Unfortunately, the published research in this field is very limited. Exceptions include the work by Bayrock and Ingledew (2001), and Lin et al. (2002), who reported on the continuous VHG ethanol fermentation in a multistage fermentation system. Also, Bai et al. (2004a) investigated a bioreactor system composed of a CSTR and three tubular bioreactors in series.

7. Conclusions

As a well established industry, the ethanol fermentation has developed its own technical and economic criteria to evaluate emerging technologies. A significant gap between academic research and industry exists, making many proposed technical developments impractical although of scientific interest. The ethanol fermentation with *Z. mobilis* is one of them, and the yeast cell immobilization by supporting materials, particularly by gel entrapments, is another. On the other hand, the VHG fermentation, with its potential for significantly saving the energy consumption, has been largely neglected. Since the production cost of ethanol is primarily derived from the consumptions of raw materials and energy, the following conventions should be followed to advance ethanol fermentation technologies with immediate practical applications:

1) The ethanol fermentation industry is using heterogeneous raw materials rather than pure glucose, and the residual sugar at the end of the fermentations is strictly controlled at a very low level, such that the ethanol yield that is calculated based on the total

sugar feeding into the fermentation systems without deduction of the residual can be as high as 90–93% of its theoretical value of ethanol to glucose.

2) The HG substrate containing 180–220 g l^{-1} total sugars is already used to achieve a corresponding ethanol concentration of 10–12% (v/v). Low gravity fermentation significantly increases the energy consumption in the downstream processes such as the distillation and waste distillage treatment. The VHG substrate containing over 250 g l^{-1} total sugars is encouraged as a focus for further research into ethanol fermentations.

Acknowledgement

The support of the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

References

Admassu W, Korus RA. Ethanol fermentation with a flocculating yeast. Chem Eng J 1985;31:B1–8.

Aiba S, Shoda M, Nagatani M. Kinetics of product inhibition in alcohol fermentation. Biotechnol Bioeng 1968;10:845–64.

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 2006;314:1565–8.

Andrews JF. A mathematical model for the continuous culture of micro-organisms utilizing inhibitory substrates. Biotechnol Bioeng 1968;10:707–23.

Bai FW, Chen LJ, Anderson WA, Moo-Young M. Parameter oscillations in very high gravity medium continuous ethanol fermentation and their attenuation on multi-stage packed column bioreactor system. Biotechnol Bioeng 2004a;88:558–66.

Bai FW, Chen LJ, Zhang Z, Anderson WA, Moo-Young M. Continuous ethanol production and evaluation of yeast cell lysis and viability loss under very high gravity medium conditions. J Biotechnol 2004b;110:287–93.

Bayrock DP, Ingledew WM. Application of multistage continuous fermentation for production of fuel alcohol by very-high-gravity fermentation technology. J Ind Microbiol Biotechnol 2001;27:87–93.

Bazua CD, Wilke CR. Ethanol effects on kinetics of a continuous fermentation with *Saccharomyces-cerevisiae*. Biotechnol Bioeng Symp 1977;7:105–18.

Betz A, Chance B. Phase relationship of glycolytic intermediates in yeast cells with oscillatory metabolic control. Arch Biochem Biophys 1965;109:585–94.

Boiteux A, Goldbeter A, Hess B. Control of oscillating glycolysis of yeast by stochastic, periodic, and steady source of substrate — model and experimental study. Proc Natl Acad Sci U S A 1975;72:3829–33.

Boon B, Laudelout H. Kinetics of nitrite oxidation by *Nitrobacter winorradskyi*. Biochem J 1962;85:440–7.

Borzani W. Variation of the ethanol yield during oscillatory concentrations changes in undisturbed continuous ethanol fermentation of sugar-cane blackstrap molasses. World J Microbiol Biotechnol 2001;17:253–8.

Bothast RJ, Schlicher MA. Biotechnological process for conversion of corn into ethanol. Appl Microbiol Biotechnol 2005;67:19–25.

Brányik T, Vicente AA, Dostálek P, Teixeira JA. Continuous beer fermentation using immobilized yeast cell bioreactor systems. Biotechnol Prog 2005;21:653–63.

Bu'lock JD, Comberbach DM, Ghommidh C. A study of continuous ethanol production using a highly flocculent yeast in the gas lift tower fermentor. Chem Eng J 1984;29:9–24.

Bungay HR. Confessions of a bioenergy advocate. Trends Biotechnol 2004;22:67–71.

Cartwright CP, Veazey FJ, Rose AH. Effect of ethanol on activity of the plasma-membrane ATPase in and accumulation of glycine by *Saccharomyces cerevisiae*. J Gen Microbiol 1987;133:857–65.

Casey GP, Ingledew WM. Ethanol tolerance in yeasts. Crit Rev Microbiol 1986;13:219–80.

Chance B, Holmes W, Higgins J, Connelly C. Localization of interaction sites in multi-component transfer systems: theorems derived from analogies. Nature 1958;182:1190–3.

Conway T. The Entner–Doudoroff pathway: history, physiology and macular biology. FEMS Microbiol Rev 1992;103:1–28.

Cysewski GR, Wilke CR. Rapid ethanol fermentations using vacuum and cell recycle. Biotechnol Bioeng 1977;19:1125–43.

D'Amore T, Stewart GG. Ethanol tolerance of yeast. Enzyme Microb Technol 1987;9:322–30.

Daugulis AJ, McLellan PJ, Li J. Experimental investigation and modeling of oscillatory behavior in the continuous culture of *Zymomonas mobilis*. Biotechnol Bioeng 1997;56:99–105.

Decamps C, Norton S, Poncelet D, Neufeld RJ. Continuous pilot plant-scale immobilization of yeast in κ-carrageenan gel beads. AIChE J 2004;50:1599–605.

Desimone MF, Degrossi J, D'Aquino M, Diaz LE. Ethanol tolerance in free and sol–gel immobilized *Saccharomyces cerevisiae*. Biotechnol Lett 2002;24:1557–9.

Devantier R, Scheithauer B, Villas-Boas SG, Pedersen S, Olsson L. Metabolite profiling for analysis of yeast stress response during very high gravity ethanol fermentation. Biotechnol Bioeng 2005;90:703–14.

Ferreira HG. On the intracellular accumulation of ethanol in yeast. Biotechnol Bioeng 1983;25:2263–8.

Fieschko J, Humphrey RE. Effects of temperature and ethanol concentration on the maintenance and yield coefficient of *Zymomonas mobilis*. Biotechnol Bioeng 1983;25:1655–60.

Ge XM, Bai FW. Intrinsic kinetics of continuous ethanol fermentation using a self-flocculating fusant yeast strain SPSC01. J Biotechnol 2006;124:363–72.

Ge XM, Zhao XQ, Bai FW. Online monitoring and characterization of flocculating yeast cell flocs during continuous ethanol fermentation. Biotechnol Bioeng 2005;90:523–31.

Ge XM, Zhang L, Bai FW. Impact of the floc size distributions on observed substrate uptake and product formation rates. Enzyme Microb Technol 2006a;39:289–95.

Ge XM, Zhang L, Bai FW. Impacts of temperature, pH, divalent cations, sugars and ethanol on the flocculating of SPSC01. Enzyme Microb Technol 2006b;39:783–7.

Ghosh A, Chance B. Oscillations of glycolytic intermediates in yeast cells. Biochem Biophys Res Commun 1964;16:174–81.

Ghose TK, Roychoudhury PK, Ghosh P. Simultaneous saccharification and fermentation (SSF) of lignocellulosics to ethanol under vacuum cycling and step feeding. Biotechnol Bioeng 1984;16:377–81.

Gong CS, Chen LF. Ethanol production by a self-aggregating mutant of *Saccharomyces uvarum*. Biotechnol Bioeng Symp 1984;14: 257–68.

Groot WJ, Kraayenbrink MR, Waldram RH, van der Lans RGJM, Luyben KchAM. Ethanol production in an integrated process of

fermentation and ethanol recovery by pervaporation. Bioprocess Eng 1992a;8:99–111.
Groot WJ, Sikkenk CM, Waldram RH, van der Lans RGJM, Luyben KchAM. Kinetics of ethanol production by baker's yeast in an integrated process of fermentation and microfiltration. Bioprocess Eng 1992b;8:39–47.
Guijarro JM, Lagunas R. *Saccharomyces cerevisiae* does not accumulate ethanol against a concentration gradient. J Bacteriol 1984;160:874–8.
Hess B. The glycolytic oscillator. J Exp Biol 1979;81:7–14.
Holzberg I, Finn RK, Steinkraus KH. A kinetic study of the alcoholic fermentation of grape juice. Biotechnol Bioeng 1967;9:413–27.
Huang SY, Chen JC. Analysis of the kinetics of ethanol fermentation with *Zymomonas mobilis* considering temperature effect. Enzyme Microb Technol 1988;10:431–9.
Hu CK, Bai FW, An LJ. Effect of the flocculating of a self-flocculating yeast on its ethanol tolerance and corresponding mechanisms. Chin J Biotechnol 2005;21:123–8.
Hynne F, Dan Ø, SØrensen PG. Full-scale model of glycolysis in *Saccharomyces cerevisiae*. Biophys Chem 2001;91:121–63.
Ikegami T, Yanagishita H, Kitamoto D, Negishi H, Haraya K, Sano T. Concentration of fermented ethanol by pervaporation using silicalite membranes coated with silicone rubber. Desalination 2002;149:49–54.
Ingledew WM. Alcohol production by *Saccharomyces cerevisiae*: a yeast primer, in the alcohol textbook. 3rd ed. UK: Nottingham University Press; 1999.
Jamai L, Sendide K, Ettayebi K, Errachidi F, Hamdouni-Alami O, Tahri-Jouti MA, et al. Physiological difference during ethanol fermentation between calcium alginate-immobilized *Candida tropicalis* and *Saccharomyces cerevisiae*. FEMS Microbiol Lett 2001;204:375–9.
Jirku V. Whole cell immobilization as a means of enhancing ethanol tolerance. J Ind Microbiol Biotechnol 1999;22:147–51.
Jones RP. Biological principles for the effects of ethanol. Enzyme Microb Technol 1989;11:130–53.
Jones ST, Korus RA, Admassu W, Heimsch RC. Ethanol fermentation in a continuous tower fermentor. Biotechnol Bioeng 1984;16:742–7.
Jöbses IML, Egberts GTC, Luyben KCAM, Roels JA. Fermentation kinetics of *Zymomonas mobilis* at high ethanol concentrations: oscillations in continuous cultures. Biotechnol Bioeng 1986;18: 868–77.
Jones AM, Ingledew WM. Fuel ethanol production: appraisal of nitrogeneous yeast foods for very high gravity wheat mash fermentation. Process Biochem 1994a;29:483–8.
Jones AM, Ingledew WM. Fuel alcohol production: assessment of selected commercial proteases for very high gravity wheat mash fermentation. Enzyme Microb Technol 1994b;16:683–7.
Jones AM, Ingledew WM. Fuel alcohol production: optimizing of temperature for efficient very-high-gravity fermentation. Appl Environ Microbiol 1994c;60:1048–51.
Kida K, Yamadaki M, Asano S, Nakata T, Sonoda Y. The effect of aeration on stability of continuous ethanol fermentation by a flocculating yeast. J Ferment Bioeng 1989;68:107–11.
Kida K, Asano S, Yamadaki M, Iwasaki K, Yamaguchi T, Sonoda Y. Continuous high-ethanol fermentation from cane molasses by a flocculating yeast. J Ferment Bioeng 1990;69:39–45.
Kierstan M, Bucke C. The immobilization of microbial cells, subcellular organelles, and enzyme in calcium alginate gels. Biotechnol Bioeng 1977;14:387–97.
Kourkoutas Y, Bekatorou A, Banat IM, Marchant R, Koutinas AA. Immobilization technologies and supporting materials suitable in alcohol beverages production: a review. Food Microbiol 2004;21: 377–97.
Kuriyama H, Ishibashi H, Miyagawa H, Kobayashi H, Mikami E. Optimization of two-stage continuous ethanol fermentation using flocculating yeast. Biotechnol Lett 1993;15:415–20.
Larue F, Lafon-Lafourcade S, Ribereau-Gayon P. Relationship between the inhibition of alcoholic fermentation by *Saccharomyces cerevisiae* and the activities of hexokinase and alcohol dehydrogenase. Biotechnol Lett 1984;6:687–92.
Lee CW, Chang HN. Kinetics of ethanol fermentation in membrane cell recycle fermentor. Biotechnol Bioeng 1987;29:1105–12.
Lee JH, Woodard JC, Pagan RJ. Vacuum fermentation for ethanol production using strains of *Z. mobilis*. Biotechnol Lett 1981;3: 177–82.
Levenspiel O. The Monod equation: a revisit and a generalization to product inhibition situations. Biotechnol Bioeng 1980;22: 1671–87.
Levenspiel O. The tanks-in-series models, in chemical reaction engineering. 3rd ed. New York: John Wiley & Sons; 1999.
Li J, McLellan PJ, Daugulis AJ. Inhibition effects of ethanol concentration history and ethanol concentration change rate on *Zymomonas mobilis*. Biotechnol Lett 1995;17:321–6.
Lin Y, Tanaka S. Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 2006;69:627–42.
Lin YH, Bayrock DP, Ingledew WM. Evaluation of *Saccharomyces cerevisiae* grown in a multi-stage chemostat environment under increasing levels of glucose. Biotechnol Lett 2002;24:449–53.
Limtong S, Nakata M, Funahashi H, Yoshida T, Seki T, Kumnuanta J, et al. Continuous ethanol production by a concentrated culture of flocculating yeast. J Ferment Technol 1984;62:55–62.
Luong JHT. Kinetics of ethanol inhibition in alcohol fermentation. Biotechnol Bioeng 1985;17:280–5.
Madigan MT, Martinko JM, Parker J. Nutrition and metabolism. Brock biology of microbiology. 9th ed. NJ: Prentice-Hall; 2000.
Madsen MF, Dan ØS, SØrensen PG. On the mechanisms of glycolytic oscillations in yeast. FEBS J 2005;272:2648–60.
Madson PW, Monceaux DA. Fuel ethanol production, in the alcohol textbook. 3rd ed. UK: Nottingham University Press; 1999.
McLellan PJ, Daugulis AJ, Li J. The incidence of oscillatory behavior in the continuous fermentation of *Zymomonas mobilis*. Biotechnol Prog 1999;15:667–80.
Nagashima M, Azuma M, Noguchi S, Inuzuka K, Samejima H. Continuous ethanol fermentation using immobilized yeast cells. Biotechnol Bioeng 1984;26:992–9977.
Nagodawithana TW, Steinkraus KH. Influence of the rate of ethanol production and accumulation on the viability of *Saccharomyces cerevisiae* in rapid fermentation. Appl Environ Microbiol 1976;31: 158–62.
Najafpour G, Younesi H, Ismail KSK. Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. Bioresour Technol 2004;92:251–60.
O'Brien DJ, Craig JC. Ethanol production in a continuous fermentation membrane pervaporation system. Appl Microbiol Biotechnol 1996;44:699–704.
O'Brien DJ, Roth LH, McAloon AJ. Concentration of fermented ethanol by pervaporation using silicalite membranes coated with silicone rubber: a preliminary economic analysis. J Membr Sci 2000;166:105–11.
Öztop HN, Öztop AY, Karadag E, Isikver Y, Saraydin D. Immobilization of *Saccharomyces cerevisiae* onto acrylamide–sodium acrylate hydrogels for production of ethyl alcohol. Enzyme Microb Technol 2003;32:114–9.
Pascual C, Alonso A, Garcia I, Roman C, Kotyk A. Effect of ethanol on glucose transport, key glycolytic enzymes, and proton

extrusion in *Saccharomyces cerevisiae*. Biotechnol Bioeng 1988;32:374–8.
Patnaik PR. Oscillatory metabolism of *Saccharomyces cerevisiae*: an overview of mechanisms and models. Biotechnol Adv 2003;21: 183–92.
Porto MM, Lafforgue C, Strehaiano P, Goma G. Fermentation coupled with microfiltration: kinetics of ethanol fermentation with cell cycle. Bioprocess Eng 1987;2:65–8.
Reddy LVA, Reddy OVS. Improvement of ethanol production in very high gravity fermentation by horse gram (*Dolichos biflorus*) flour supplementation. Lett Appl Microbiol 2005;41:440–4.
Reddy LVA, Reddy OVS. Rapid and enhanced production of ethanol in very high gravity (VHG) sugar fermentation by *Saccharomyces cerevisiae*: role of finger millet (*Eleusine coracana* L) flour. Process Biochem 2006;41:726–9.
Richard P. The rhythm of yeast. FEMS Microbiol Rev 2003;27: 547–57.
Richard P, Bakker BM, Teusink B, van Dam K, Westerhoff HV. Acetaladehyde mediates the synchronization of sustained glycolytic oscillations in populations of yeast cells. Eur J Biochem 1996;235:238–41.
Roffler SR, Blanch HW, Wilke CR. *In situ* recovery of fermentation products. Trends Biotechnol 1984;2:129–36.
Rosa MF, Sá-Correia I. Ethanol tolerance and activity of plasma membrane ATPase in *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. Enzyme Microb Technol 1992;14:23–7.
Ryu DDY, Kim YJ, Kim JH. Effect of air supplement on the performance of continuous ethanol fermentation system. Biotechnol Bioeng 1984;26:12–6.
Salguerio SP, Sá-Correia I, Novais JM. Ethanol induced-leakage in *Saccharomyces cerevisiae*: kinetics and relationship to yeast ethanol tolerance and alcohol fermentation productivity. Appl Environ Microbiol 1988;54:903–9.
Saraydin D, Öztop HN, Karadag E, Öztop AY, Isikver Y, Güven O. The use of immobilized *Saccharomyces cerevisiae* on radiation crosslinked acrylamide–maleic acid hydrogel carriers for production of ethyl alcohol. Process Biochem 2002;37:1351–7.
Sprenger GA. Carbohydrate metabolism in *Zymomonas mobilis*: a catabolic highway with some scenic routes. FEMS Microbiol Lett 1996;145:301–7.
Stevsborg N, Lawfors HG, Martin N, Beveridge T. Effect of growth temperature on the morphology and performance of *Z. mobilis* ATCC 29191 in batch and continuous culture. Appl Microbiol Biotechnol 1986;25:116–23.
Swings J, Deley J. The biology of *Zymomonas mobilis*. Bacteriol Rev 1977;41:1–46.
Taylor F, Kurantz MJ, Goldberg N, McAloon AJ, Craig JC. Dry-grind process for fuel ethanol by continuous fermentation and stripping. Biotechnol Prog 2000;16:541–7.
Thomas KC, Ingledew WM. Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mash. Appl Environ Microbiol 1990;56:2046–50.
Thomas KC, Ingledew WM. Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes. J Ind Microbiol 1992;10:61–8.
Thomas KC, Hynes SH, Ingledew WM. Effects of particulate materials and osmoprotectants on very-high-gravity ethanolic fermentation. Appl Environ Microbiol 1994;60:1519–24.
Thomas KC, Hynes SH, Ingledew WM. Practical and theoretical considerations in the production of high concentration of alcohol by fermentation. Process Biochem 1996;31:321–31.
Vane LM. A review of pervaporation for product recovery from biomass fermentation process. J Chem Technol Biotechnol 2005;80:603–29.
Verbelen PJ, Schutter DPD, Delvaux F, Verstrepen KJ, Delvaux FR. Immobilized yeast cell systems for continuous fermentation application. Biotechnol Lett 2006;28:1515–25.
Verstrepen KJ, Derdelinckx G, Verachtert H. Yeast flocculation: what brewers should know. Appl Microbiol Biotechnol 2003;61:197–205.
Wang S, Thomas KC, Sosulski K, Ingledew WM. Grain pearling and very high gravity (VHG) fermentation technologies for fuel alcohol production from rye and triticale. Process Biochem 1999;34:421–8.
Wolf J, Passarge J, Somsen O, Snoep J, Heinrich R, Westerhoff HV. Transduction of intracellular and intercellular dynamics in yeast glycolytic oscillations. Biophys J 2000;78:1145–53.
Xu TJ, Zhao XQ, Bai FW. Continuous ethanol production using self-flocculating yeast in a cascade of fermentors. Enzyme Microb Technol 2005;37:634–40.
You KM, Rosenfield CL, Knipple DC. Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. Appl Environ Microbiol 2003;69:1499–503.
Yuan Z, Medina MA, Boiteux A, Stefan C, Müller, Hess B. The role of fructose 2,6-bisphosphate in glycolytic oscillations in extracts and cells of *Saccharomyces cerevisiae*. Eur J Biochem 1990;192:791–5.