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Using fed-batch fermentation in very high gravity brewing: effects of Tween 80 and ergosterol supplementation on fermentation performance of immobilized yeast in calcium alginate gel

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Abstract: : In very high gravity brewing, fed-batch fermentation was carried out with *Saccharomyces cerevisiae* immobilized in calcium alginate gel. The specific gravity of initial wort and feeding medium originated from 55% malt and 45% maltose syrup adjunct was adjusted to 28°Brix and 41°Bx, respectively. The effect of supplementation of mixture of Tween 80 and ergosterol to the initial wort or to the feeding medium was examined. The results indicated that addition of 0.3% (v/v) Tween 80 and 24ppm ergosterol to the initial wort improved the fermentation performance of the immobilized yeast. Under these conditions, the primary fermentation time reduced 24h and the ethanol concentration was similar in comparison with those of the fed-batch culture without the supplementation.

Key words: ergosterol, fed-batch fermentation, high gravity brewing, immobilization, *Saccharomyces cerevisiae*, Tween 80

Introduction

A major innovation in brewing is very high gravity technology – the fermentation of wort containing 16g or more of dissolved solids per 100g of wort. The use of this technology is advantageous as it allows a greater volume of beer to be produced without expanding existing facilities (Cunningham and Stewart, 1998). On the other hand, the drawbacks of this process include decreased foam stability and flavor matching (Virkajarvi *et al*., 2002); increased stress on yeast, for example: osmotic stress, ethanol stress and nutrient limitation (Cahill and Murray, 2000; Dragone, 2004). Possibility of reducing detrimental factors acting in very high gravity wort could be yeast immobilization or fed-batch fermentation. According to Pátková (2000), Tran *et al*. (2008; 2010), sugar assimilation and ethanol production rates of the immobilized yeast in calcium alginate gel in high gravity wort were significantly higher than those of the free yeast. Meanwhile Vu and Le (2007, 2010) demonstrated that fed-batch technique increased the total substrate content in the fermenter, but always maintained a low substrate concentration for reducing the negative effect of osmotic pressure on the free yeast in high gravity brewing.

Based on the potential results of the previous studies, in this paper, combined application of immobilized yeast in calcium alginate gel and fed-batch fermentation to very high gravity brewing was examined. This study focused on the primary fermentation kinetics. The impacts of Tween 80 and ergosterol supplementation to the initial wort and to the feeding medium on the fermentation performance of the immobilized biocatalyst were then evaluated.

Materials and methods

Materials

Saccharomyces cerevisiae (a lager strain) used in this study was originated from Microorganism collection of Food Microbiology Laboratory, Department of Food Technology, Ho Chi Minh City University of Technology. Sodium alginate was supplied by Biotechnology Center, Nha trang University. The ratio of mannuronic acid to guluronic acid was 1.2. The viscosity of 2% alginate solution at 25°C was 423.6cp. Malt was purchased from Duong Malt Company; the extraction yield was 80%. Hop was supplied by Bach Dang Brewing Company; the alpha acid content was 8% (w/w). High maltose syrup was purchased from Bibica Company; the dissolved solid was 80% (w/w); the dextrose equivalence was 42. Other chemicals used in this study were supplied by Merck (EU).

Inoculum preparation

Yeast cells for immobilization were prepared by the two-stage batch cultivation. In the first stage, yeast was propagated in 100 mL Erlenmeyer Shake-flask containing 15 mL of 8% (w/w) malt wort. In

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the second stage, yeast was multiplicated in 500 mL Erlenmeyer Shake-flask containing 150 mL of 8% (w/w) malt wort. For both stages, precultures were carried out at 30°C and 100 rpm for 24h. Yeast biomass was recovered by centrifugation at 6.000rpm for 20 min and subsequently suspended in a determined volume of sterile water. The obtained yeast suspension was used for immobilization.

Immobilization

A volume of yeast suspension ($1.0x10^8$ cells/mL) was mixed with an equal volume of sterile sodium alginate solution (50 g/L) and homogenized. This mixture was then dropped into a 3% (w/v) calcium chloride solution by a peristaltic pump. The drops solidified upon contact with calcium chloride solution. The residence time of the gel beads in calcium chloride solution was 4 hours at 4°C for increasing the gel strength. The gel beads were then washed in sterile water. Yeast cell concentration in the gel bead was $5.0x10^7$ cells/mL.

Media

Wort was firstly prepared from barley malt by infusion mashing (Briggs *et al*., 2004). The all malt wort was then concentrated under vacuum condition (60°C, 40-140 mbar) and subsequently added by high maltose syrup. The soluble extract in the final wort was originated from 55% barley malt and 45% maltose syrup adjunct. In fed-batch fermentation, the specific gravity of the initial wort and the feeding medium was 28°Bx and 41°Bx, respectively.

Fermentations

Fermentation was realized in stainless steel fermenters at 17°C. The pitching rate was $1.0x10^7$ viable cells/mL of wort. The fermentation was considered as it was completed when the degree of attenuation reached 75%. The degree of attenuation was calculated by the reducing sugar content in the initial wort and in the green beer (Pátková *et al*., 2000).

In the first experiment, batch fermentation was carried out with 28°Bx wort. The objective of this experiment was to determine the suitable moment for adding the feeding medium to the culture.

In the second experiment, fed-batch fermentation was realized. The fermentation was started with 28°Bx wort. After 144 fermenting hours, the feeding medium with specific gravity of 41°Bx was added to the culture and its volume was similar to the culture volume in the fermenter before feeding. 5 cultures were carried out as follows:

- Culture A: The initial wort was supplemented with 0.3% (v/v) Tween 80 and 24ppm ergosterol, the feeding medium was unsupplemented.

- Culture B: The initial wort was supplemented with 0.3% (v/v) Tween 80 and 36ppm ergosterol, the feeding medium was unsupplemented.

- Culture C: The initial wort was unsupplemented, the feeding medium was supplemented with 0.3% (v/v) Tween 80 and 24ppm ergosterol .

- Culture D: The initial wort was unsupplemented, the feeding medium was supplemented with 0.3% (v/v) Tween 80 and 36ppm ergosterol .

- Culture E (control sample): Both initial wort and feeding medium were unsupplemented by Tween 80 and ergosterol.

Analytical methods

Yeast concentration and viability: The calcium alginate gel beads containing yeast cells were dissolved in a 3% (w/v) EthyleneDiamine Tetraacetic Acid disodium salt (EDTA) solution. The yeast concentration was then determined by haemocytometry using Thoma counter chamber (Tran *et al*., 2008).

Reducing sugar and free amino nitrogen (FAN) were measured by spectrophotometric method, using 3,5-dinitrosalicylic acid and ninhydrin, respectively (Jones *et al*., 2007). Ethanol concentration was determined by a method based on distillation and density quantification (AOAC, 1990). Diacetyl was quantified by spectrophotometric method using o-phenylenediamine (European Brewery Convention, 1998).

Statistical analysis

All experiments were carried out in duplicate. The means were subjected to analysis of variance (ANOVA) with a p value <0.05 using STATGRAPHICS © Plus for windows 3.0 (Copyright 1994-1997 by Statistical Graphics Corporation).

Results and Discussions

Kinetics of batch fermentation

The kinetics of batch fermentation on 28°Bx wort are visualized in Figure 1. Yeast cell number in the culture increased quickly during the first days of fermentation because the wort was rich in nutrients. The maximum concentration of cells was obtained at the 96th fermenting hour. After that, cell number reduced slightly. Simultaneously, sugars were assimilated and ethanol was produced by the immobilized yeast. The fermentation kinetics by the fixed yeast in calcium alginate gel in 28°Bx wort in this study was similar to the findings in 24°Bx wort (Tran *et al.,* 2010).

The analysis of variance showed that after the

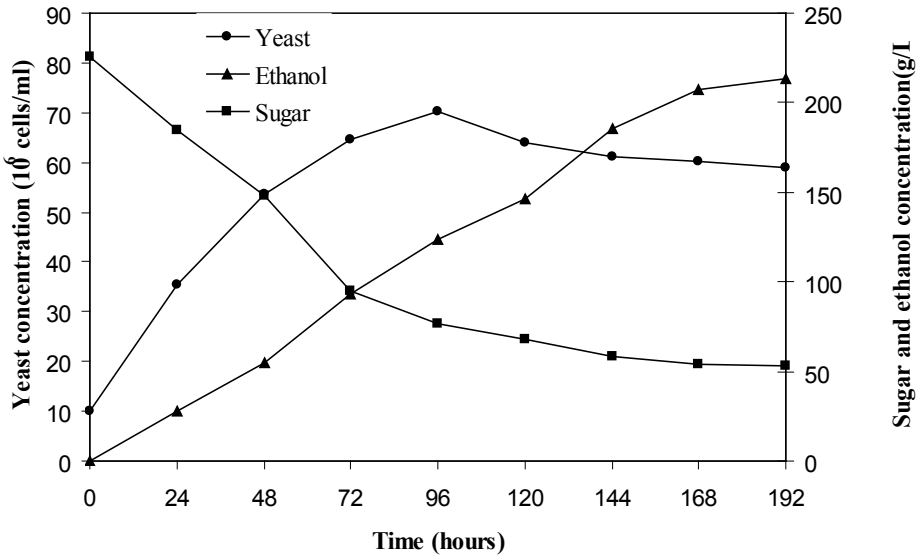

Figure 1. Kinetics of batch fermentation by immobilized yeast in calcium alginate gel

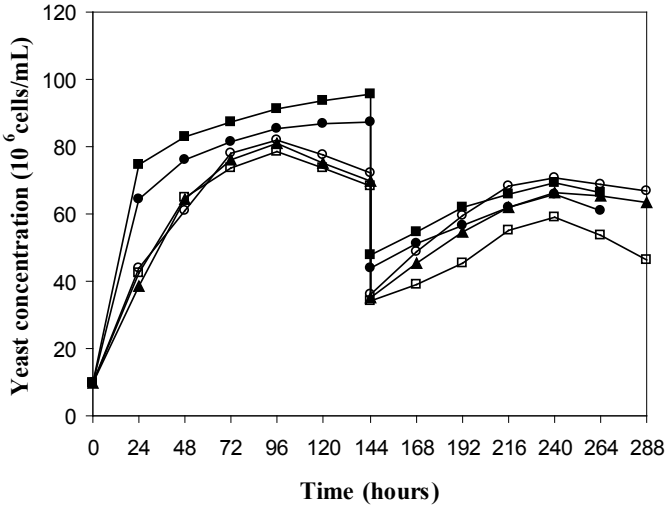

Figure 2. Kinetics of yeast growth during fed-batch fermentation: (✹): culture A, (■): culture B, (▲): culture C, (○): culture D, (□): culture E (control)

144th fermenting hour, sugar content in the culture changed insignificantly. It was due to the formation of some exo-metabolites of yeast in the culture. According to Briggs *et al*. (2004), ethanol, higher alcohols, organic acids… in the fermented wort could decrease the metabolic activities of brewing yeast. In fed-batch fermentation, feeding fresh medium to the culture could dilute the concentration of toxic metabolites for the microorganism. Thus the 144th fermenting hour was chosen as the moment for adding the fresh wort to the culture. At the 144th hour, the specific gravity of the culture was 15°Bx. After adding 41°Bx wort to the culture, the specific gravity of the obtained mixture was 28°Bx.

Effect of Tween 80 and ergosterol supplemention on kinetics of fed-batch fermentation

Lipid in wort, especially unsaturated long-chain fatty acids and sterols play an important role on the growth and metabolism of yeast. These compounds increased the stress tolerance of yeast to such severe conditions as high osmotic pressure and high ethanol level in the culture (Odumeru *et al*., 1992). Casey *et al*. (1983) reported that supplementation with 0.8% yeast extract, 24ppm ergosterol and 0.24% Tween 80 to 27°P wort increased growth of the free yeast, shortened fermetation time (from 15 days to 4 days) and produced 14-16%(v/v) ethanol.

Sterols and unsaturated fatty acids are essential membrane components in yeast; however they are normally present in the wort in sub-optimal quantities (Russell, 1994). This experiment focused on combined supplementation of ergosterol and Tween 80 the main compound of which was oleic acid. 0.3% Tween and 24ppm ergosterol or 0.3% Tween and 36ppm ergosterol was added to the initial wort or to the feeding medium.

The yeast growth in fed-batch fermentation is presented in Figure 2.

The fermentation could be divided into 2 stages: before and after feeding fresh wort to the culture. During the first stage of fermentation, the multiplication of the immobilized yeast in cultures supplemented with Tween 80 and ergosterol (A and B) was more rapid than that in non-supplemented cultures (C, D and E). At the 144th fermenting hour, the yeast cell number in culture A ($8.7x10^7$ cell/mL) and B ($9.6x10^7$ cell/mL) was significantly higher than that in cultures C, D and E (approximately $7.0x10^7$ cells/mL). As a result, adding Tween 80 and ergosterol to the initial wort increased the yeast growth. Similar observation with the free yeast and immobilized yeast in calcium alginate gel in batch fermentation was also noted by different researchers (Nguyen and Le, 2009; Tran *et al*., 2010). It can be mentioned that yeast growth in culture B was faster than that in culture A thanks to a higher content of ergosterol in the initial medium. In addition, reduction in yeast cell number was not observed during the first fermentation stage in cultures A and B. Consequently, supplementation of Tween 80 and ergosterol to wort at the beginning of fermentation prevented autolysis of the immobilized yeast. Casey et al. (1984) also reported that addition of Tween 80, ergosterol and yeast extract to high gravity wort ameliorated the viability of the free yeast during the fermentation.

During the second stage of fermentation, yeast growth continued; the growth rate of yeast in cultures supplemented with Tween 80 and ergosterol in the feeding wort (C and D) was faster than that in cultures A, B and E. Maximum concentration of yeast cells in all cutures were observed at the 240th fermenting hour. However, the peak of cell number in cultures A, B, C and D with Tween 80 and ergosterol addition was significantly higher than that in control sample E. Moreover, the analysis of variance demonstrated that the difference in maximum cell number in cultures A, B, C and D was not significant.

Based on the results in Figure 2, it can be affirmed that the immobilized yeast grew better during the fed-batch fermentation when Tween 80 and ergosterol were added to the initial wort or to the feeding wort. The evolution of sugar and free amino nitrogen concentration in fed-batch culture is given in Figure 3 and 4, respectively.

In general, the concentration of carbon and nitrogen substrates was gradually decreased during the first and second stages of fermentation. It can be noted that the immobilized yeast in cultures supplemented with Tween 80 and ergosterol at the starting of fermentation consumed substrates more rapidly than that in other cultures. Table 1 demonstrates that the sugar and free amino nitrogen uptake rates in cultures A and B were the highest. Although the yeast growth in cultures C and D was better than that in control culture E, the sugar assimilation rate of cultures C, D and E was similar. "Consequently, addition of Tween 80 and ergosterol to the feeding medium could not augmented the carbon substrate uptake rate of the immobilized yeast in fed-batch fermentation."

The kinetics of ethanol formation of immobilized yeast in calcium alginate gel in fed-batch fermentation is illustrated in Figure 5. During both stages of the fermentation, the immobilized biocatalyst in cultures A and B synthesized ethanol faster than that in cultures C, D and E although the final ethanol content in all cultures was similar. The analysis of variance on Table 1 indicated that the ethanol production rate in cultures

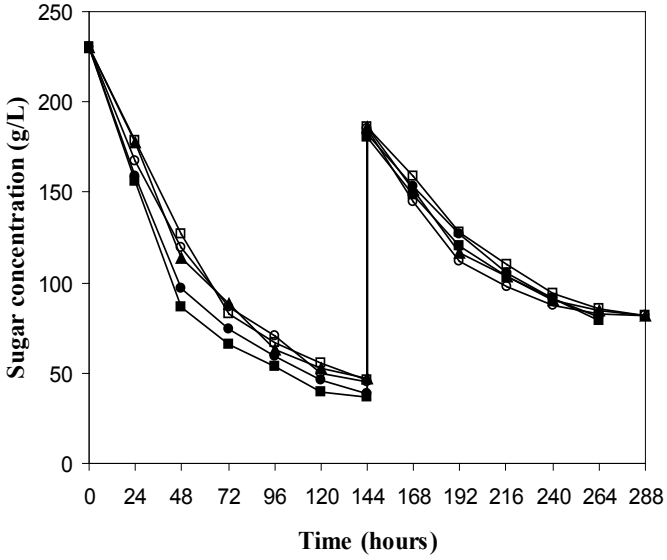

Figure 3. Sugar assimilation of immobilized yeast during the fed-batch fermentation (✹): culture A, (■): culture B, (▲): culture C, (○): culture D, (□): culture E (control).

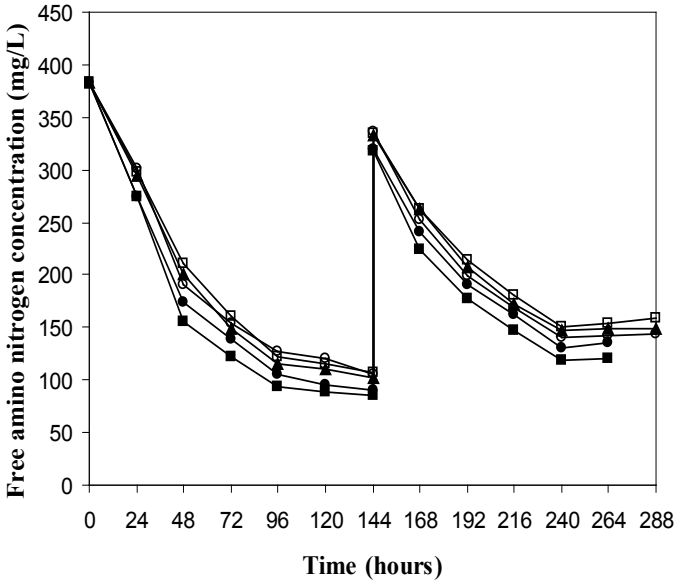

Figure 4. Evolution of free amino nitrogen during the fed-batch fermentation (✹): culture A, (■): culture B, (▲): culture C, (○): culture D, (□): culture E (control).

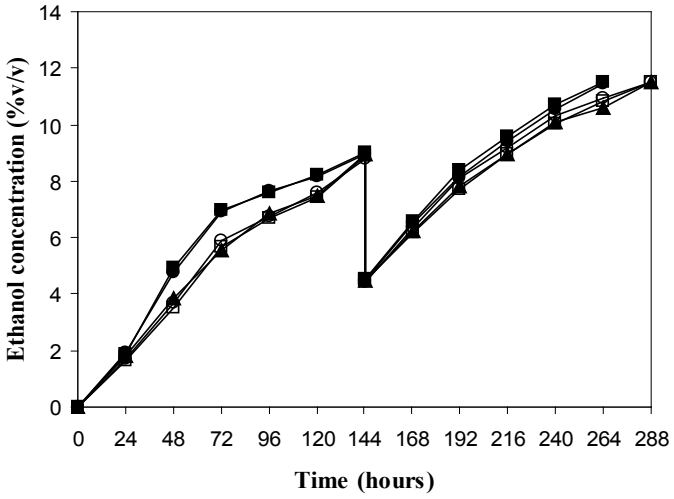

Figure 5. Kinetics of ethanol production during the fed-batch fermentation (✹): culture A, (■): culture B, (▲): culture C, (○): culture D, (□): culture E (control).

Table 1. Effect of Tween 80 and ergosterol on fermentation characteristics by immobilized yeast in very high gravity brewing using fed-batch culture

Culture	A	B	C	D	E
Sugar uptake rate (g/L.h)	0.75^{b}	0.75^{b}	0.68^{a}	0.68^{a}	0.68^{a}
FAN uptake rate (g/L.h)	0.97^{b}	1.01^{c}	0.84^{d}	0.88^{e}	0.80^{a}
Ethanol production rate (g/L.h)	0.34^{b}	0.35^{b}	0.32^{a}	0.32^{a}	0.32^{a}
Fermentation time (h)	264^{b}	264^{b}	288^{a}	288^{a}	288^{a}
Dilution ratio of green beer to reach the ethanol concentration of 5% (v/v)	2.29^{a}	2.30^{a}	2.30^{a}	2.30^{a}	2.30^{a}

Each value represents the mean of two independent samples. Different letters in each row mean significant difference ($P<0.05$).

Table 2. Some physico-chemical properties of green beer after dilution to reach the ethanol concentration of 5%

Culture	A	B	C	D	E
Reducing sugars (g/L)	35.1[b]	34.2[b]	35.7[c]	35.6[c]	36.3[a]
FAN (g/L)	104.5[b]	104.2[b]	102.9[a]	104.8[c]	102.1[a]
Diacetyl (mg/L)	0.26[b]	0.27[c]	0.25[d]	0.28[e]	0.23[a]
pH	4.62[b]	4.63[b]	4.65[c]	4.65[a]	4.68[a]

Each value represents the mean of two independent samples. Different letters in each row mean significant difference ($P<0.05$).

A and B with addition of Tween 80 and ergosterol at the beginning of fermentation was higher than that in other cultures. In high gravity brewing, brewers are interested in dilution ratio of the green beer. The higher the dilution ratio, the higher the economic efficiency of the production. In this experiment, the dilution ratio of the green beer to 5% (v/v) ethanol concentration of all cultures was equivalent.

Table 1 show that the fermentation time in cultures A and B was 24 hours shorter than that in cultures C, D and E. An interesting result should be noted that supplementing Tween 80 and ergosterol to the medium at the starting of fermentation increased the metabolic activities of the immobilized yeast and reduced the fermentation time. Nevertheless, adding the same nutrients to the feeding medium just improved the yeast growth but had no influence on fermentation time.

Table 2 presents some physico-chemical characteristics of the green beer after being diluted to 5% ethanol concentration. The residual content of reducing sugars, free amino nitrogen and pH value of all cultures were in conventional ranges for the green beer (Briggs *et al*., 2004). However, diacetyl content in cultures A, B, C and D was a little higher than that in control culture E. Diacetyl is an unfavorable compound in beer because of its butter flavor. The formation of diacetyl in brewing was closely related to the yeast growth (Boulton and Quain, 2001). According to Boulton and Quain (2001), high yeast growth facilitated the biosynthesis of diacetyl. Nevertheless brewers can prevent high diacetyl concentration in the final product by addition of alpha acetolactate decarboxylase preparation to the green beer during the maturation.

It can be noted that culture A supplemented with lower ergosterol level had lower diacetyl concentration than culture B. Accordingly, the appropriate content of Tween 80 and ergosterol added to the initial wort were 0.3% (v/v) and 24ppm, respectively.

Conclusion

Supplementing of Tween 80 and ergosterol to wort at the beginning of fermentation improved the growth of the immobilized yeast in fed-batch culture and reduced the fermentation time; meanwhile adding such nutrients to the feeding medium just ameliorated the yeast growth but had no effect on fermentation time. Reduction in fermentation time could increase the productivity of the breweries. Combined application of immobilized yeast and fed-batch fermentation was therefore a potential solution to overcome certain disadvantages in very high gravity brewing.

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