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Using fed-batch fermentation in high-gravity brewing: effects of nutritional supplementation on yeast fermentation performance

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Abstract: To intensify the brewing process and increase the fermenter productivity, many breweries throughout the world have been embracing high-gravity brewing. However, increasing original wort concentration can cause a negative effect on yeast fermentation performance. This study focused on the application of fed-batch fermentation in high-gravity brewing. High gravity worts of 24°Bx (using 30% high maltose syrup adjunct) supplemented with yeast extract or mixture of yeast extract and Tween 80 were used as feeding-media in fed-batch cultures. As a results, the nutrients supplemented to feeding media improved the yeast fermentation performance. Especially, the supplementation of 0.25% (w/v) yeast extract and 0.8% (v/v) Tween 80 to the feeding-medium not only reduced the primary fermentation time, but also increased the ethanol concentration of the fed-batch culture in comparison with the fed-batch culture without this supplementation to the feeding-medium.

Keywords: Fed-batch fermentation, high gravity brewing, *Saccharomyces cerevisiae*, yeast extract, Tween 80

Introduction

The traditional brewing is generally performed by fermenting worts of 11 – 12°Bx specific gravity to produce beers of 4 – 5% (v/v) ethanol. With the need to produce good quality beers in a short time and in a least-expensive way, many breweries have been switching to high gravity brewing, a technology in which worts containing 16 g or more of dissolved solids per 100 g are fermented. The use of this technology increases plant efficiency and capacity, reduces energy, labour and capital costs, as well as increases ethanol yields per unit of fermentable extract (Blieck *et al.*, 2007). Moreover, it can help improve beer stability, produce smoother taste and greater flexibility to the final product (Casey *et al.*, 1984; Pátková *et al.*, 2000; Almeida *et al.*, 2001; Reilly *et al.*, 2004).

However, this process still has some drawbacks. When the wort concentration increases, the yeasts are exposed to such severe conditions as the increasing of osmotic pressure and the toxicity of produced ethanol (Pátková *et al.*, 2000; Erten *et al.*, 2007), resulting in a decrease in viability of the yeast and slow or stuck fermentations (Almeida *et al.*, 2001); nutrient limitations, especially concerning dissolved oxygen and assimilable nitrogen (Casey *et al*. 1984; Dragone *et al.*, 2003; Dragone *et al.*, 2004). These problems decrease the possibility of reusing the yeast and the stability of beer foam, increase the contamination during the fermentation (Almeida *et al.*, 2001).

The arising problems have been solved by adding nutritional supplements, using higher pitching rates, higher fermentation temperatures (Casey *et al.*, 1984), more efficient aeration than in conventional brewing (Lolodo *et al.*, 1999) and applying immobilised yeast (Pátková *et al*., 2000). One of the possibilities to reduce the detrimental factors acting in high-gravity brewing could be fed-batch fermentation (Vu and Le, 2007). Fed-batch is defined as a technique in microbial processes where one or more nutrients are supplied to the bioreactor during cultivation and in which the products remain in the containment until the end of the run (Yamanè and Shimizu, 1984). This technique helps to increase the total substrate content in the fermenter, but always maintain a low substrate concentration for reducing the negative effect of

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osmotic pressure on yeast during fermentation.

In the recent paper, we focused on the primary fermentation in high-gravity brewing using fed-batch technique. When the initial specific gravity of wort was 16°Bx, we found that the 108th fermenting hour was suitable for adding feeding-medium to the culture (Vu and Le, 2007). This study continued focusing on the primary fermentation in brewing and examined the impact of yeast extract and Tween 80 added to the feeding-media on the yeast performance in fed-batch culture.

Materials and Methods

Materials

Yeast used in this study was a lager strain of *Saccharomyces cerevisiae*, supplied by Foster Tien Giang Ltd. Company, Vietnam. The pre-inoculum was obtained from yeast cultures maintained on malt-agar slants at 4°C. The inoculum propagation was carried out in the 10 °Bx wort.

Barley malt (79.2% extraction yield, 4.5% moisture) was originated from Australia and supplied by Duong Malt Co., Ltd, Viet Nam. Hop (8% α-acid) was supplied by Bach Dang Brewing Company, Vietnam. High Maltose Syrup (HMS, 80% dissolved solids, 42 Dextrose Equivalent) was purchased from Bien hoa Sugar Company, Vietnam. Yeast extract (Merck AG, Germany) was used as nitrogen supplement. Tween 80 (Shantou Xilong Chemical Factory, Guangdong, China) served as a source of unsaturated fatty acid. Other chemicals used in this study were originated from several chemical suppliers in China.

Wort production

In batch operation, we fermented the 20°Bx wort. In fed-batch operation, the fermentation was started as a batch process with 16°Bx wort. At the 108th fermenting hour when the specific gravity of the culture reached 8°Bx, the 24°Bx wort (called feeding-medium) was added to the culture. The culture volume in the fermenters before feeding and the feeding-medium volume were similar. The specific gravity of the culture in the fermenter after feeding was 16°Bx.

The 16°Bx, 20°Bx worts and 24°Bx feeding-media were obtained by adding HMS to all-malt worts, and the ratio of HMS adjunct was fixed at 30%. The all-malt worts were prepared by infusion mashing (Kunze, 2004).

Only the 24°Bx feeding-medium used in the fed-batch control culture was prepared by vacuum concentration at 60°C from the all-malt wort.

Fermentations

Fermentation was performed in stainless steel fermenters at 17°C. The pitching rate was 10 x 10^6 viable cells mL^{-1}.

In batch control sample, 3L of 20°Bx wort (30% HMS adjunct) was fermented. 20°Bx is the average specific gravity of 1.5L of 16°Bx wort and 1.5L of 24°Bx feeding-medium.

In fed-batch operation, we examined 5 following samples:

- Fed-batch culture 1: 1.5L of 16°Bx wort (30% HMS adjunct) was fermented. After 108 fermenting hours, the culture was added with 1.5L of feeding-medium 1. Feeding-medium 1 was 24°Bx wort (30% HMS adjunct). The specific gravity after feeding was 16°Bx.
- Fed-batch culture 2: 1.5L of 16°Bx wort (30% HMS adjunct) was fermented. After 108 fermenting hours, the culture was added with 1.5L of feeding-medium 2. Feeding-medium 2 was 24°Bx wort (30% HMS adjunct) supplemented with 0.25% (w/v) yeast extract. The specific gravity after feeding was 16°Bx.
- Fed-batch culture 3: 1.5L of 16°Bx wort (30% HMS adjunct) was fermented. After 108 fermenting hours, the culture was added with 1.5L of feeding-medium 3. Feeding-medium 3 was 24°Bx wort (30% HMS adjunct) supplemented with 0.4% (w/v) yeast extract. The specific gravity after feeding was 16°Bx.
- Fed-batch culture 4: 1.5L of 16°Bx wort (30% HMS adjunct) was fermented. After 108 fermenting hours, the culture was added with 1.5L of feeding-medium 4. Feeding-medium 4 was 24°Bx wort (30% HMS adjunct) supplemented with 0.25% (w/v) yeast extract and 0.4% (v/v) Tween 80. The specific gravity after feeding was 16°Bx.
- Fed-batch culture 5: 1.5L of 16°Bx wort (30% HMS adjunct) was fermented. After 108 fermenting hours, the culture was added with 1.5L of feeding-medium 5. Feeding-medium 5 was 24°Bx wort (30% HMS adjunct) supplemented with 0.25% (w/v) yeast extract and 0.8% (v/v) Tween 80. The specific gravity after feeding was 16°Bx.

We also carried out a fed-batch control sample where the feeding-medium was the 24°Bx all-malt wort.

Analytical methods

During the fermentation, the evolution of total number of yeast cell, wort specific gravity, reducing sugar, free amino nitrogen, ethanol and diacetyl contents were examined. The number of yeast cell was determined by using an Improved Neubauer Haemocytometer at x40 magnification with a light microscope. After removing yeast biomass by centrifugation (6000 rpm, 15 minutes, 4°C), the specific gravity of the culture was measured by a refractometer. Concentration of reducing sugars was quantified by spectrophotometric method using dinitrosalicylic acid reagent (Miller, 1959). Free Amino Nitrogen (FAN) content was measured by spectrophotometric method, using ninhydrin reagent (EBC, 1998). Ethanol concentration was determined by a method based on distillation and density quantification (AOAC, 1990). Concentration of diacetyl was determined by spectrophotometric method using O – phenylendiamin reagent (EBC, 1998). The sugar uptake rate (g/L.h) was calculated as the ratio of the reducing sugar content (g/L) consumed by yeast to the fermentation time (h). The fermentation time (h) was determined when 92% of reducing sugar content was assimilated by the yeast.

Statistical treatment

All experiments were carried out in duplicate. The data 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 Discussion

Effect of yeast extract supplemented to feeding-medium on kinetics of fed-batch fermentation

Using 30% HMS adjunct could change the balanced ratio of carbon to assimilable nitrogen in wort. Yeast extract has been considered as a traditional nitrogen source for wort supplementation in brewing (Dragone *et al*., 2004; Casey *et al*., 1984). In this study, yeast extract was therefore added to the feeding-medium for examining its effect on yeast fermentation performance.

Yeast growth during the fermentation is presented in Figure 1. At the first stage of fermentation, yeast cell number in all cultures increased quickly because the wort was rich in oxygen and nutrients. However, during the first 36 hours, the growth rate of yeast in the batch control culture was slower than that in fed-batch cultures. Besides, the maximum concentration of yeast in the batch control culture was 1.2×10^8 cells/mL (at the 84th fermenting hour), lower than that in fed-batch cultures before feeding (approximately 1.3×10^8 cells/mL at the 108th fermenting hour). This proved that higher osmotic pressure in batch culture (20°Bx in comparison with 16°Bx in fed-batch samples) had inhibited yeast growth. This observation is in agreement with the results of Le and Pham (2007) who examined yeast growth in 16°P, 18°P, 20°P and 22°P worts.

In fed-batch fermentation, after fresh wort feeding, the growth rate of yeast in fed-batch culture 1 was lower than that in fed-batch culture 2, 3 and

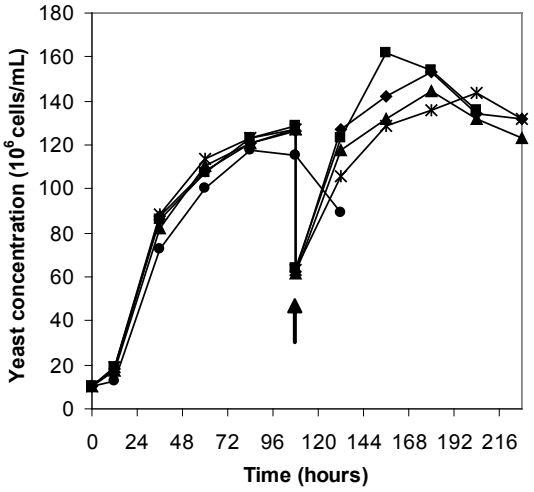

Figure 1. Effect of yeast extract supplemented to the feeding-medium on the kinetics of yeast growth during the fermentation: (●) batch control culture; (∗) fed-batch culture 1; (▲) fed-batch culture 2; (◆) fed-batch culture 3; (■) fed-batch control culture

the fed-batch control culture. The highest maximum concentration of yeast was obtained in the fed-batch control culture (approximately 1.6×10^8 cells/mL) at the 156th fermenting hour (Figure 1). Fed-batch culture 2 and 3 both achieved the maximum yeast concentration at the 180th fermenting hour. The growing time of yeast in fed-batch culture 1 was the longest. The maximum yeast concentration obtained in fed-batch culture 3 (approximately 1.5×10^8 cells/mL) was higher than that in fed-batch culture 1 and 2 (approximately 1.4×10^8 cells/mL). This may be due to the higher concentration of yeast extract supplemented to feeding-medium 3.

In general, the maximum yeast concentration after feeding in all fed-batch cultures was always higher than that before feeding. The increase in yeast concentration after feeding played a very important role in enhancing fermentation rate of yeast, which resulted in increasing rate of ethanol and other metabolite production.

The fermentation characteristics are shown in Table 1. The fermentation time of fed-batch culture 1 was the longest (204 hours). When adding yeast extract to the feeding-medium or using all-malt feeding-medium, the culture time decreased by as much as 11.8% as compared to that of fed-batch culture 1. The batch control culture lasted 156 hours, which was 24 hours shorter than that in fed-batch culture 2 and 3.

Although the total specific gravity of worts for fermentation in the batch control culture and fed-batch cultures was the same (20°Bx), the sugar contents assimilated by yeast in fed-batch cultures were higher than that in the batch control culture (Table 1). This fact is very important, because the higher the sugar content consumed by yeast, the higher the final ethanol content in green beer.

The results from table 1 also show that the sugar uptake rates in the batch control culture and in fed-batch culture 1 were the lowest (approximately 0.84 g/L.h). When adding yeast extract to feeding-media, the sugar uptake rate in fed-batch culture 2 and 3 was improved to 0.95 g/L.h, which was 13.1% higher than that in the batch control culture and fed-batch culture 1. Of all samples, the sugar uptake rate in the fed-batch control culture was the highest (Table 1). These results indicate that 20°Bx wort where 30% of the original extract came from HMS in batch control culture caused high osmotic pressure and nutrient deficiency, which affected the sugar uptake rate of yeast. Although yeast cells in fed-batch culture 1 were not exposed to high osmotic pressure, they were still impacted by nutrient limitation, and this would inhibit the fermentation rate.

As mentioned in previous articles (Casey *et al.*, 1984; O'Connor-Cox and Ingledew, 1989),

Table 1. Effect of yeast extract on fermentation characteristics in high gravity brewing using fed-batch cultures

	Batch control culture	Fed-batch culture 1	Fed-batch culture 2	Fed-batch culture 3	Fed-batch control culture
Fermentation time (h)	156^{b}	204^{c}	180^{a}	180^{a}	180^{a}
Sugar content assimilated by yeast (g/L)	130.62^{c}	171.89^{b}	171.63^{b}	170.17^{b}	183.87^{a}
Sugar uptake rate (g/L.h)	0.84^{c}	0.84^{c}	0.95^{b}	0.95^{b}	1.02^{a}
Ethanol concentration in green beer (% v/v)	7.96^{c}	8.49^{ab}	8.50^{ab}	8.41^{b}	8.57^{a}
Diacetyl content in green beer (mg/L) after being diluted to ethanol concentration of 5% (v/v).	0.37^{a}	0.33^{a}	0.37^{a}	0.55^{b}	0.59^{b}
Volume of final beer (L) after diluting 3 L of green beer to reach the ethanol concentration of 5 % (v/v)*.	4.78^{c}	5.09^{ab}	5.10^{ab}	5.05^{b}	5.14^{a}

Each value represents the mean of two independent samples. Different letters in each row mean significant difference ($P<0.05$).
* It was supposed that the ethanol content in the green beer did not augment during the secondary fermentation.

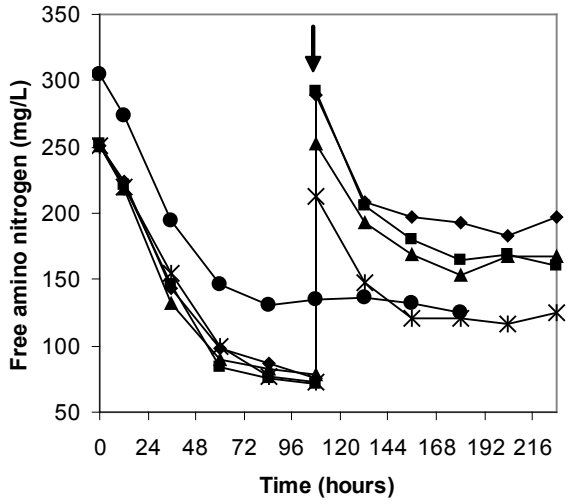

Figure 2. Kinetics of free amino nitrogen assimilation during the fermentation: (●) batch control culture; (∗) fed-batch culture 1; (▲) fed-batch culture 2; (◆) fed-batch culture 3; (■) fed-batch control culture

brewing adjuncts like high maltose syrup make little contribution to the nitrogen content of wort, serving mainly to dilute all of the non-carbohydrate components while increasing the wort gravity. Thus, yeast extract was supplemented to feeding-media 2 and 3 in order to increase the FAN concentration. The kinetics of FAN concentration in the cultures is shown in Figure 2. However, it can be noted that the total FAN contents consumed by yeasts in fed-batch culture 1, 2, 3 were equivalent (Figure 5). This means that although being provided with more FAN content, yeast cells in fed-batch culture 2 and 3 hardly ever consumed more. Just when using all-malt feeding-medium, FAN concentration consumed by yeast increased, though the FAN concentrations after feeding in fed-batch culture 3 and the fed-batch control culture were the same (approximately 300 mg/L, Figure 2).

According to O'Connor-Cox and Ingledew (1989), wort amino acids represent the major source of assimilable nitrogen for brewing yeasts. Feeding-medium in the fed-batch control sample was originated from all-malt wort, thus it could contain a balanced C/N ratio and other growth factors like vitamins for the yeast growth in comparison with feeding-media 2 and 3. That may the reason why FAN content consumed by yeast in fed-batch control culture was about 13% higher than that in fed-batch culture 1, 2 and 3.

However, as mentioned before, the yeast growth rate after feeding as well as the sugar uptake rate in fed-batch culture 2 and 3 was faster than that in fed-batch culture 1. This could be explained that yeast extract contained not only FAN, but also other substances which were advantageous for fermentation. According to previous researches by O'Connor-Cox *et al.* (1989) and Jones (1985), stimulatory effects of yeast extract on yeast performance are attributed to other components, not the assimilable nitrogen level. Yeast extract is a source of vitamins such as biotin and pantothenate, and Jones (1985) attributed its stimulatory effects to the cofactor supply. According to Jones (1985), biotin promotes yeast growth. Therefore, it is reasonable to conclude that for most yeast strains, one of the limiting nutrients supplied by yeast extract is biotin. This vitamin is heat-stable so that its loss during medium sterilization is minimal (Jones, 1985). For these reasons, we assumed that, vitamin source, especially biotin from yeast extract improved yeast fermentation performance in fed-batch culture 2 and 3. Besides, yeast cells in fed-batch culture 3 received more yeast extract than that in fed-batch culture 2, which resulted in faster growth rate of yeast.

The result from Figure 5 shows that the total FAN content consumed by yeasts in fed-batch culture 1, 2 and 3 was 57.6% higher than that in the batch control culture. The total FAN concentration consumed in the fed-batch control culture was 78.1% higher than that in the batch control culture. This means that using fed-batch technique would enhance not only the amount of sugar content but also FAN content for yeast growth.

The fermentation time of the batch culture was shorter than that of fed-batch cultures (Table 1). However, the final ethanol content obtained in fed-batch cultures was always higher than that in the batch culture, which resulted in higher volume of final beer

after being diluted to reach the ethanol concentration of 5% (v/v). As stated in table 1, fed-batch culture 1, 2 and 3 had the similar beer volume, which was 6.4% higher than that in the batch control culture. The fed-batch control culture reached the highest volume in final beer. Thus, when supplementing yeast extract to feeding-media or using all-malt feeding-medium, nutrients such as FAN and essential vitamins like biotin may promote yeast fermentation performance, which resulted in higher sugar uptake rate and producing more ethanol content. Alfenore *et al*. (2002) reported that the assimilation of biotin conditioned the growth rate and the ethanol production of *Saccharomyces cerevisiae.* In addition, according to O'Connor-Cox and Ingledew (1989), under non-limiting nitrogen conditions, the amount of carbon channeled toward sterol production will be minimal. Thus, the carbon flow through the glycolytic pathway should be maximal. Therefore, the ethanol productivity is greatly enhanced under conditions where assimilable nitrogen is rich.

Diacetyl is one of the most important by – products in alcoholic fermentation and it is a key compound in beer maturation. Its formation is closely related to amino acid metabolism (Kunze, 2004). At low levels, it gives beer a slick mouthfeel; at higher levels, the flavor becomes buttery, which decreases the sensory properties of the final product. Our results show that, after diluting the green beer to reach the ethanol concentration of 5% (v/v), the diacetyl contents in the batch control and fed-batch culture 1 and 2 were the same and lower than those in fed-batch culture 3 and the fed-batch control culture (Table 1). Low diacetyl level in green beer is an advantage in brewing because the maturation time will be shorter.

From these results, we supposed that the supplementation of 0.25% (w/v) yeast extract to feeding-medium (fed-batch culture 2) was more efficient, because the ethanol content in the green beer was higher as well as the diacetyl content was lower, which helped to produce more final beer with a shorter maturation time. The fed-batch control culture also gave high final ethanol content, but the feeding-medium should be prepared by wort vacuum concentration, which would increase energy cost.

Effect of yeast extract and Tween 80 supplemented to feeding-medium on kinetics of fed-batch fermentation

Unsaturated fatty acid has been demonstrated to play an important role in the alcohol tolerance of yeasts (Casey *et al*., 1984; Mishra and Prasad, 1989). Watson (1982) proved that in a medium rich in oleic acid $C_{18:1}$, cells typically had a high percentage of $C_{18:1}$ residue in their phospholipids, which resulted in producing higher ethanol concentration and retaining their viability after prolonged exposure to high ethanol concentration. Commercial Tween 80 is rich in oleic acid. Thus, this experiment focused on the combination of yeast extract and Tween 80 supplemented to the feeding-medium used in fed-batch culture.

From the previous experiment results, we found that supplementing 0.25% (w/v) yeast extract to feeding-medium was more effective. For further investigation, we continued adding Tween 80 with the content of 0.4% (v/v) or 0.8% (v/v) to the feeding-medium in order to determine the effect of both supplements on yeast fermentation performance.

The kinetics of yeast growth during the fermentation is shown in figure 3. After fresh wort feeding, the maximum yeast concentrations in fed-batch culture 4 and 5 were 1.6×10^8 and 1.5×10^8 cells/mL, respectively; and both reached at the 180^{th} fermenting hour. We found that the yeast growth rate and maximum yeast concentration in fed-batch culture 4 were both higher than those in fed-batch culture 5 after feeding (statistical analysis showed that the differences were significant).

Our results also indicated that after feeding fresh wort, the ethanol production rate in fed-batch culture 4, 5 and the fed-batch control culture was faster than that in fed-batch culture 1 (Figure 4). After 180 fermenting hours, the final ethanol concentrations obtained in fed-batch culture 4 and 5 were 8.41% (v/v) and 8.73% (v/v), respectively (Table 2). This indicates that with the same reducing sugar content (approximately 173 g/L, Table 2), yeast cells in fed-batch culture 4 consumed sugar primarily for increasing biomass (Figure 3), thus the ethanol content obtained in fed-batch culture 4 would be lower than that in fed-batch culture 5. The final ethanol content in fed-batch culture 5 was the highest (Table 2), which was 10% higher than that in batch control culture.

Besides, it can be noted that, when adding both yeast extract and Tween 80 to the feeding-media, the FAN content consumed by yeast increased. The results from Figure 5 showed that, FAN contents consumed by yeasts in fed-batch culture 4 and 5 were the same and 5.7% higher than those in fed-batch culture 2 and 3. The fermentation time of fed-batch culture 4 and 5 was shortened by 24 hours, compared with that of fed-batch culture 1 (Table 2). In addition, the sugar uptake rate of fed-batch culture 4 and 5 was higher than that of fed-batch culture 1 and the batch control culture.

In summary, it can be supposed that simultaneous adding of yeast extract and Tween 80 to feeding media

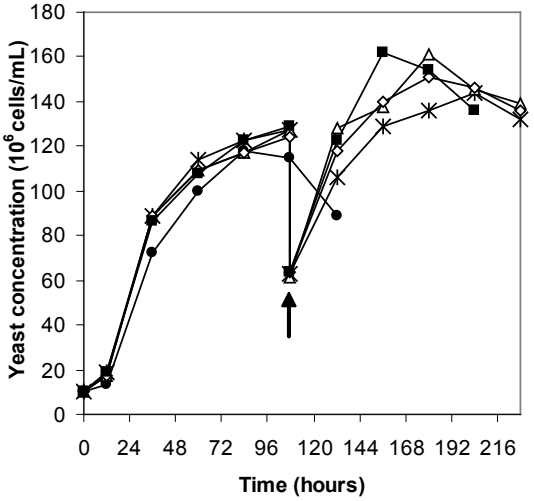

Figure 3. Effect of yeast extract and Tween 80 supplemented to the feeding-medium on the kinetics of yeast growth during the fermentation: (●) batch culture; (∗) fed-batch culture 1; (△) fed-batch culture 4; (◇) fed-batch culture 5; (■) fed-batch control culture

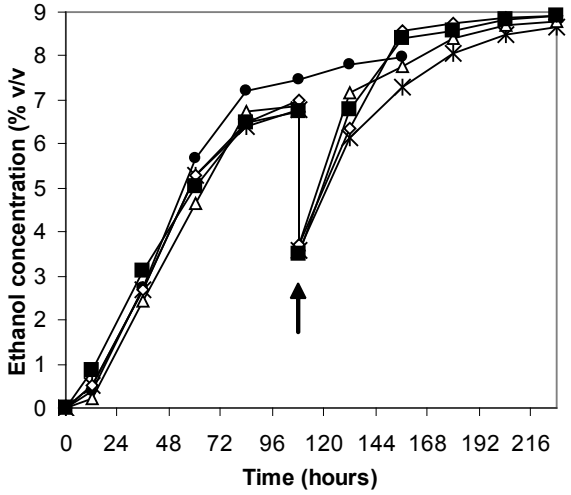

Figure 4. Kinetics of ethanol production during the fermentation: (●) batch culture; (∗) fed-batch culture 1; (△) fed-batch culture 4; (◇) fed-batch culture 5; (■) fed-batch control culture

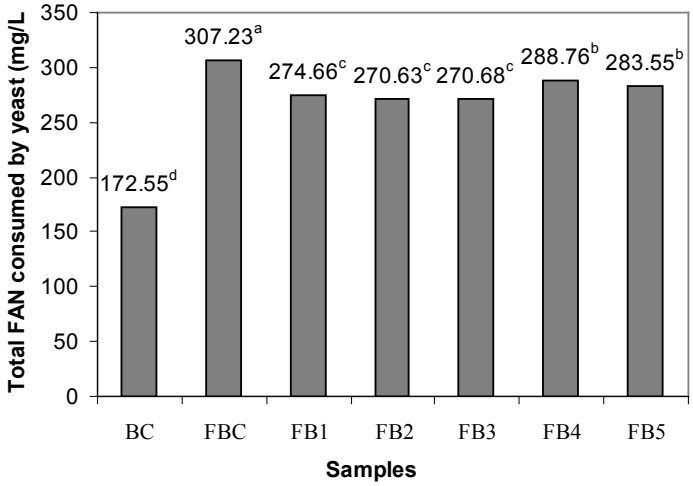

Figure 5. Total FAN consumed by yeast during the fermentation. BC: batch culture; FBC: fed-batch control culture; FB1: fed-batch culture 1; FB2: fed-batch culture 2; FB3: fed-batch culture 3; FB4: fed-batch culture 4; FB5: fed-batch culture 5. Each value represents the mean of two independent samples.
Different letters above the values mean significant difference ($P<0.05$)

Table 2. Effect of yeast extract and Tween 80 on fermentation characteristics in high gravity brewing using fed-batch cultures

	Batch control culture	Fed-batch culture 1	Fed-batch culture 4	Fed-batch culture 5	Fed-batch control culture
Fermentation time (h)	156^{b}	204^{c}	180^{a}	180^{a}	180^{a}
Sugar content assimilated by yeast (g/L)	130.62^{c}	171.89^{b}	172.38^{b}	173.93^{b}	183.87^{a}
Sugar uptake rate (g/L.h)	0.84^{c}	0.84^{c}	0.96^{b}	0.97^{b}	1.02^{a}
Ethanol concentration in green beer (% v/v)	7.96^{d}	8.49^{bc}	8.41^{c}	8.73^{a}	8.57^{b}
Diacetyl content in green beer (mg/L) after being diluted to ethanol concentration of 5% (v/v).	0.37^{a}	0.33^{a}	0.55^{b}	0.52^{b}	0.59^{b}
Volume of final beer (L) after diluting 3 L of green beer to reach the ethanol concentration of 5 % (v/v) *.	4.78^{d}	5.09^{bc}	5.05^{c}	5.24^{a}	5.14^{b}

Each value represents the mean of two independent samples. Different letters in each row mean significant difference ($P<0.05$).
* It was supposed that the ethanol content in the green beer did not augment during the secondary fermentation.

4 and 5 would provide yeast cells in fed-batch culture 4 and 5 not only with more FAN, vitamins but also oleic acid. On the one hand, yeast would consume FAN for the synthesis of cellular protein. According to O'Connor-Cox *et al.* (1989), the ability to improve ethanol tolerance is related to protein synthesis. Protein components of cell membrane may play an important role in yeast ethanol tolerance levels and adaptation. On the other hand, oleic acid from Tween 80 would enrich more oleic acid in the cytoplasmic membrane of yeast. These facts may help yeasts in fed-batch culture 4 and 5 increase biomass and synthesize ethanol with higher rate than those in fed-batch culture 1 after feeding fresh wort (Figure 4 and figure 5). This result is in agreement with what Casey *et al*. (1984) reported: both assimilable nitrogen (yeast extract) and unsaturated acids supplemented to wort were required to successfully ferment high gravity worts in a batch process.

The diacetyl content in green beer after being diluted to the ethanol concentration of 5% (v/v) is shown in Table 2. Fed-batch culture 4, 5 and the fed-batch control culture had higher diacetyl concentration than fed-batch culture 1 and batch control culture (0.55 mg/L compared with 0.37 mg/L).

In this experiment, although the diacetyl content in fed-batch culture 5 was higher than that in the batch culture and fed-batch culture 1, the ethanol content in fed-batch culture 5 was the highest, which resulted in the highest volume of beer after diluting green beer to 5% (v/v) of ethanol content. The fed-batch control culture had the same diacetyl content as in fed-batch culture 5, but the ethanol content was lower (8.57% (v/v) compared with 8.73% (v/v). Moreover, the cost for vacuum concentration of wort was quite high. Thus, we concluded that fed-batch culture 5 was more efficient than fed-batch culture 1, 4 and the fed-batch control culture.

Conclusion

In this study, batch operation with 20°Bx wort (30% HMS adjunct) finished after 156 hours of fermentation and reached the ethanol content of 7.96% (v/v). When operating fed-batch culture 1 with unsupplemented feeding-medium, although the total specific gravity was also 20°Bx, the ethanol content increased as much as 6.7% as compared with the batch control culture. This proved that using fed-batch technique maintained a low substrate concentration during the fermentation for reducing the negative effect of osmotic pressure on yeast. Supplementing 0.25% yeast extract to the feeding-medium in fed-batch culture 2 not only reduced the fermentation time as much as 11.8% but also produced the same ethanol content as well as diacetyl concentration in comparison with fed-batch culture 1. Especially, when adding simultaneously 0.25% (w/v) yeast extract and 0.8% (v/v) Tween 80 to feeding-medium, the yeast fermentative performance was improved and the ethanol content in the green beer was the highest (8.73% (v/v)) in comparison with that of other fed-batch cultures. The fed-batch control culture also obtained high ethanol concentration, however the cost of production energy, equipment investment and labour would increase. In conclusion, it can be said that fed-batch fermentation with feeding-medium supplemented with nutrients is a very promising strategy for production of beer using high-gravity brewing.

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