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Using high pitching rate for improvement of yeast fermentation performance in high gravity brewing

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Abstract: One of the promising methods to improve yeast fermentation performance in high gravity brewing is to increase pitching rate in the bioreactor. In this study, fermentation with four different pitching rates (1.5×10^7, 4.5×10^7, 7.5×10^7, and 10.5×10^7 viable cells/ml wort) was carried out to investigate the impact of pitching rate on yeast fermentation performance and beer quality from 24°Bx wort. The obtained results showed that higher pitching rate resulted in higher maximum yeast cell number in the culture, higher sugar uptake and ethanol production rates, but higher diacetyl level in the green beer as well. The results also indicated that this method itself was even more efficient than a method of supplementing nutrients (Tween 80 and yeast extract) to wort, or a combination of these two methods in respect of the sugar uptake, ethanol production rates; ethanol, and diacetyl concentrations in the green beer.

Keywords: high pitching rate, high gravity brewing, nutritional supplementation, *Saccharomyces cerevisiae*

Introduction

High gravity brewing can be described as a procedure which employs wort at higher than normal extract (Erten *et al*., 2007). Traditionally, brewing worts of 12°P are fermented to produce beers of 5% (v/v) ethanol (Bliek *et al*., 2006). In high gravity brewing, wort gravity can reach up to 16 – 18°P (Casey *et al*., 1984) or even higher McCaig *et al*., 1992; Pátková *et al*., 2000), resulting in higher ethanol concentration in the green beer. After fermentation, the product is diluted, usually with oxygen free water, in order to obtain beer with regular ethanol content (5%) or desired alcohol content (Bliek *et al*., 2006). The diluted process is often performed at a later stage in the processing and before packaging (Erten *et al*., 2007).

High gravity brewing has been remarkably developed over the past few years due to a number of benefits: increased brewing capacity, hence more efficient use of existing plant facilities; reduced energy, labor, cleaning, and effluent costs; improved physical and flavor stability of beer; more alcohol per unit of fermentable extract due to reduced yeast growth; higher adjunct rates; smoother taste; and greater flexibility (Thomas *et al*., 1996; Erten *et al*., 2007). For example, with the use of 15°P wort, energy consumption can lower by as much as 14% and an increase in manpower productivity of 25-30% (Hackstaff, 1978) or the brewery capacity increases by 50% for 18°P wort (Bliek *et al*., 2006). However, this technology still exists some problems: decreased foam stability of finished beer, problems of flavor match, and a negative effect on yeast performance due to high osmotic pressure and ethanol concentration (Pátková *et al*., 2000; Erten *et al*., 2007), leading to lower fermentation rate as well as longer fermentation time. Nutrient limitation, especially dissolved oxygen and assimilable nitrogen is also noted (Casey *et al*., 1984). Moreover, a combination of high gravity brewing with other modern practices, such as the use of tall cylindroconical fermenters, results in increased hydrostatic pressure, carbon dioxide level and decreased oxygen level (Bliek *et al*., 2006).

Many authors have suggested various methods to overcome these drawbacks such as higher fermentation temperature, nutritional supplementation (Casey *et al*., 1984), immobilised yeast (Pátková *et al*., 2000), mutant yeast strain (Bliek *et al*., 2006), more efficient aeration than conventional brewing (Casey *et al*, 1984; O'Connox Cox and Ingledew, 1989; Jones *et al*., 2007), and higher pitching rate (Heyse and Piendl, 1973; Casey *et al*., 1984, 1985; O'Connox – Cox and Ingledew, 1991; Edelen *et al*., 1996; Erten *et al*., 2007; Verbelen *et al*., 2008). Among these solutions, nutritional supplementation is considered

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as a popular method because it is effective and quite simple to operate. With supplementing yeast extract, ergosterol, and oleic acid to wort, it was possible to ferment wort up to 31% dissolved solids and produced beers containing 16.2% (v/v) ethanol (Casey *et al*., 1984).

Besides, high pitching rate has been recently considered as a potential method in high gravity brewing thanks to some typical advantages such as shortening fermentation time, increasing fermentation rate, and increasing degree of attenuation. In traditional lager brewing, pitching rate often ranges from 5–20 million cells/mL wort (Erten *et al*., 2007). However, in high gravity brewing, this rate should be increased to ensure the complete fermentation. Inoculation rate was reported to increase four or five – fold higher than the normal one and a variety of outcomes have been observed. A research by Verbelen *et al.* (2008) pointed out that the maximum cell number in the culture increased with higher pitching rate but new yeast cell mass synthesis was the same regardless of the pitching rate, whereas a study by O'Connox Cox and Ingledew (1991) stated that the yeast population appeared stable during fermentation when using high pitching rate (8.0×10^7cells/ml). According to these two authors, limited yeast growth was seen at high pitching rate but the fermentation rate and degree of attenuation were still higher than those of the control sample (1.5×10^7cells per ml). Erten *et al.* (2007) and Verbelen *et al.* (2008) showed that the ethanol concentration was independent of pitching rate; in contrast, the study by O'Connox Cox and Ingledew (1991) presented an increase in the ethanol content with increasing pitching rate. In terms of flavor, Erten (2007) found lower diacetyl level at higher pitching rate whereas Verbelen (2008) reported the contradictory result. It is noted that although these results did indicate that high pitching rate is clearly advantageous in high gravity brewing, the initial gravity of all worts in the experiments above was not over 15 – 16°P.

The aim of this paper was to study the effects of pitching rate on fermentation with very high gravity wort – 24°Bx. We also compared the effects of using high pitching rate method with supplementing nutrients to wort, the common method in high gravity brewing, as well as evaluated the combined effects of the two methods above.

Materials and Methods

Wort

24°Bx wort was prepared by adding high maltose syrup to an all-malt wort and the ratio of high maltose syrup adjunct was 30%. High maltose syrup (80% dissolved solids, 42 Dextrose Equivalent) was supplied by Bien Hoa Confectionery Joint Stock Company, Viet Nam. All–malt wort was produced from barley malt using infusion mashing method (Kunze, 2004). Barley malt (extraction yield 79.2%) originated from Australia and supplied by Duong Malt Co., Ltd, Viet Nam.

Chemicals

In some experiments, yeast extract (Merck and Co., Inc) and Tween 80 (0.6% free oleic acid) (Shantou Xilong Chemical Factory Guangdong) were added to high gravity wort as sources of assimilable nitrogen and unsaturated fatty acid.

Yeast and Ferfentation conditions

Lager brewing strain of *Saccharomyces cerevisiae* used in this study originated from Microorganism collection of Food Microbiology Lab, Department of Food Technology, Ho Chi Minh City University of Technology. The stock culture was maintained on malt – agar slants at 4°C. Yeast propagation was performed in the 10°Bx all – malt wort in an incubator at 30°C. The required inoculum size was prepared by centrifuging the culture above at 6000 rpm at 4°C for 15 min. All fermentations were carried out in duplicate in a bioreactor containing 2L of sterile 24°Bx wort. Initial content of dissolved oxygen (prior inoculation) was 8 ppm. The primary fermentation was conducted at 17°C and completed when 85% of the reducing sugars had been consumed.

Fermentation analysis

Samples were daily removed in order to determine total yeast cell number, yeast viability, pH, specific gravity, free amino nitrogen, reducing sugar, ethanol, and diacetyl concentration. Yeast cell number was quantified by using Thoma Haemocytometry. Viable cells were determined by using methylene blue staining (ASBC, 1992). The specific gravity was measured by a refractometer. Reducing sugars were 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) assimilated by yeast to the fermentation time (h). The ethanol production rate (g/L.h) was calculated as the

ratio of the ethanol content produced by yeast to the fermentation time (h).

Statistical analysis

The data was analyzed for statistical significance by Analysis of Variance (ANOVA). Multiple Range Test with the Least Significant Difference ($LSD_{0.05}$) was applied in order to determine which means are significantly different from which others by using STATGRAPHICS © Plus for windows 3.0.

Results and Discussion

Effect of pitching rate on yeast fermentation performance and diacetyl content in the green beer

In this experiment, samples with four different pitching rates were used: 1.5×10^7 (control sample), 4.5×10^7, 7.5×10^7, and 10.5×10^7 viable cells/ml wort.

Fermentation performance

The fermentation time of four samples with different pitching rates is illustrated in Table 1. As expected, increasing pitching rate did remarkably reduce the fermentation time in high gravity brewing. With pitching rate of 4.5×10^7, 7.5×10^7, and 10.5×10^7 cells/ml, the fermentation time reduced by 18%, 27%, and 39%, respectively, as compared to that of the control sample. Decrease in fermentation time increases the fermenter capacity and lower the energy, labor, and capital costs (Le *et al*., 2007).

The sugar uptake and the ethanol production rates are also given in Table 1. It was observed that these two fermentation characteristics were significantly improved with increasing pitching rate in high gravity brewing as well. The results from Table 1 demonstrated that in cultures with 4.5×10^7, 7.5×10^7, and 10.5×10^7 cells/ml, the sugar uptake rate increased 24%, 43%, and 67%, respectively, as compared to that of the control sample. In terms of the ethanol production rate, the same trend as the sugar consumption rate of yeast was also recognized. The ethanol production rates were 32%, 52%, and 73% higher than that of the control sample.

Besides, the increase in pitching rate from 1.5×10^7 cells/ml to 7.5×10^7 cells/mL led to augment ethanol concentration in the green beer from 9.20% (v/v) to 10.05% (v/v). However, the ethanol content in the green beer dropped when pitching rate reached up to 10.5×10^7 cells/ml (Table 1). The result was not in accordance with studies carried out by Erten *et al.* (2007) and Verbelen *et al*. (2008). These authors reported that the ethanol concentration in the green beer from 16°P and 15°P all-malt wort was appeared unaffected by pitching rate.

Yeast cell number and yeast viability

Maximum yeast cell number in the culture and net yeast growth (the difference between the maximum cell number per unit of volume and the cell number added at pitching), (Edelen *et al*., 1996) are visualized in Figure 1. It was seen that the higher the pitching rate, the higher the maximum cell number occurred in the culture. The result showed that maximum cell number in the culture with the highest pitching rate was 1.36 times higher than that of the control sample. Our result was also in agreement with the findings of Verbelen *et al*. (2008), Erten *et al*. (2007) in which 16°P and 15°P all-malt worts were used.

Although the yeast cell number in the culture increased with increasing pitching rate, according to Figure 1, the net yeast growth decreased when pitching rate was above 4.5×10^7 cells/ml. The net growth in the culture of the control sample and the culture pitched with 4.5×10^7 cells/ml was nearly similar. Yet when the pitching rates of 7.5×10^7 cells/ml, and 10.5×10^7/ml were used, the net growth reduced by 1.2 and 1.4 times, respectively, than that of the control sample. Therefore, the higher pitching rate, the less new cell number occurred in the culture. Because the initial oxygen content was the same in all cultures, it was supposed that with the increase in pitching rate, the amount of oxygen that every yeast cell received would be reduced. This could limit yeast

Table 1. Effect of four different pitching rates on fermentation characteristics in high gravity brewing

	Pitching rate ^{x}(10^6 viable cells/ml)*			
	15	45	75	105
Fermentation time (hours)	132 ± 6.00 ^{a}	108 ± 3.00 ^{b}	96 ± 3.00^c	84 ± 3.00^d
Ethanol concentration in the green beer (% v/v)	9,20 ± 0.04^a	9,41± 0.01^b	10,05 ± 0.07^c	9,63 ± 0.06^d
Sugar uptake rate (g/L.h)	1,20 ± 0.04^a	1,51 ± 0.06^b	1,72 ± 0.07^c	2,00 ± 0.08^d
Ethanol production rate (g/L.h)	0,55 ± 0.03^a	0,69 ± 0.07^b	0,83 ± 0.05^c	0,88 ± 0.07^d
Diacetyl concentration in the green beer (mg/L)	0,39 ± 0.05^a	0,45 ± 0.01^b	0,47 ± 0.02^c	0,53 ± 0.02 ^{d}

*Values within rows followed by the different letters are significantly different (P=0.05).

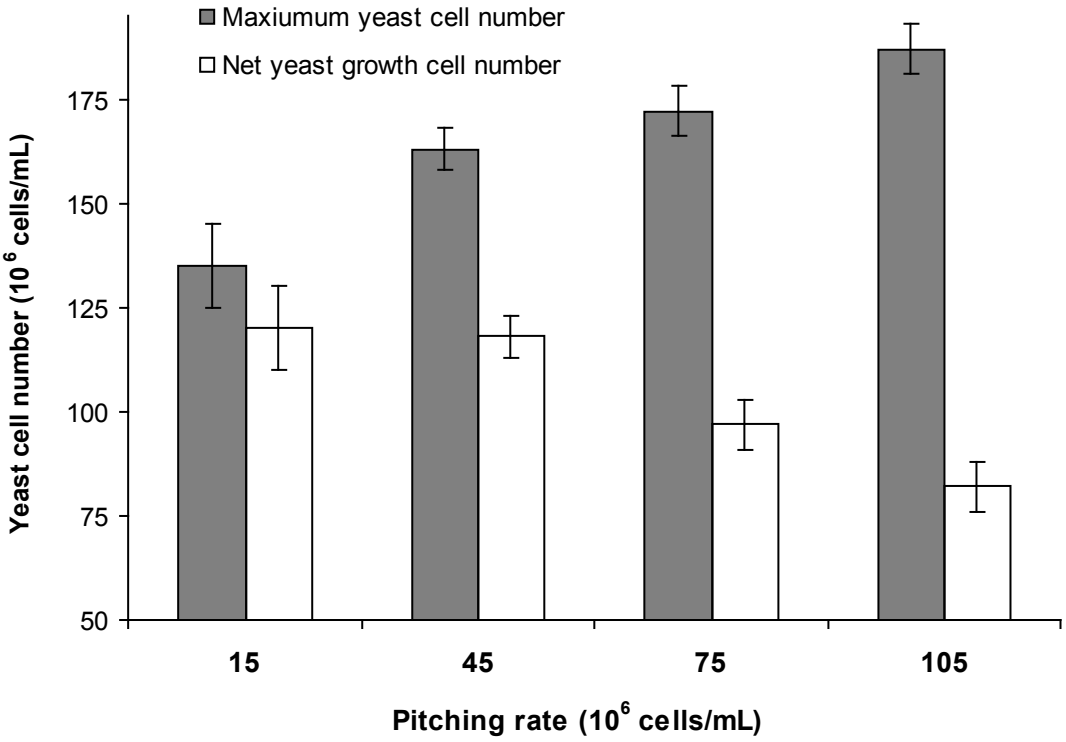

Figure 1. Maximum yeast cell number and net yeast growth (the difference between the maximum cell number per unit of volume and the cell number added at pitching) with different pitching rates in high gravity brewing.

cell division in the medium when higher pitching rate was employed. However, Verbelen *et al*. (2008) reported that the same number of new yeast cells was generated in the culture when fermenting wort with a lower gravity (15°P) than that of this study.

Accordingly, the net yeast growth also helped explain the different ethanol concentrations in the green beer as mentioned above. The ethanol concentration augmented when pitching rate increased from 1.5×10^7 cells/mL to 7.5×10^7 because fewer new cells were produced in the cultures. The fewer new cells in the culture, the more amount of sugar yeast converted to ethanol, and the less amount of sugar yeast consumed to build new cell structure. However, ethanol content dropped with the highest pitching rate of 105×10^7 cells/ml and the net yeast growth was lower as compared to that in the culture with the pitching rate of 7.5×10^7 cells/ml. This was probably that yeasts used sugars to transfer to energy storage substances of available cells than build new cell structure at very high pitching rate of 10.5×10^7 cells/mL.

The percentage of viable cells at the end of all the fermentations remained above 82% in the four samples.

Diacetyl content in the green beer

Diacetyl is one of the most important by–products in alcoholic fermentation. High diacetyl level in beer causes a butter flavor that decreases the sensory properties of the final product (Kunze, 2004). Hence, in this experiment, diacetyl content in the green beer was examined.

Table 1 presents diacetyl level in the green beer of four samples with different pitching rates. The higher the pitching rate, the higher the concentration of diacetyl occurred in the culture. This phenomenon was probably owing to higher production of α-acetolactate during the fermentation and a shorter fermentation time that resulted in incomplete reduction of diacetyl (Verbelen *et al.*, 2008). Higher diacetyl content in the green beer will lead to longer maturation time. However, Verbelen *et al*. (2008) suggested several methods to accelerate the maturation such as using immobilised yeast to speed up the maturation stage, supplementing of α-acetolactate decarboxylase to the wort, or applying genetically manipulated yeast strains.

In contrast, Erten *et al*. (2007) fermented 16°P all-malt wort and reported that at lower pitching rate, diacetyl concentration in the green beer was higher.

Based on the results of this experiment, it was concluded that using high pitching rate could significantly enhance yeast fermentation performance. With the gravity wort of 24°Bx, pitching rate of 7.5×10^7 cells/ml was considered as the suitable inoculation size in respect of both ethanol production rate and ethanol concentration in the green beer.

Table 2. Fermentation time and diacetyl concentration in the green beer of four samples in high gravity brewing.

	Sample[x]			
	S1	S2	S3	S4
Fermentation time (hours)	132 ± 6.00[a]	108 ± 3.00[b]	96 ± 3.00[c,d]	96 ± 3.00[c,d]
Diacetyl concentration in the green beer (mg/L.h)	0,39 ± 0.05[a]	0,7± 0.04[b]	0,47 ± 0.02[c]	0,88 ± 0.04[d]

*Values within rows followed by the different letters are significantly different (P=0.05).
S1: Control sample with pitching rate of 1.5×10^7 viable cells/ml, S2: sample supplemented with 0.5% w/v yeast extract and 0.4% v/v Tween 80 and pitching rate of 1.5×10^7 viable cells/ml, S3: sample with high pitching rate of 7.5×10^7 cells/ml. S4: sample supplemented with 0.5% w/v yeast extract and 0.4% v/v Tween 80 and high pitching rate of 7.5×10^7 cells/ml

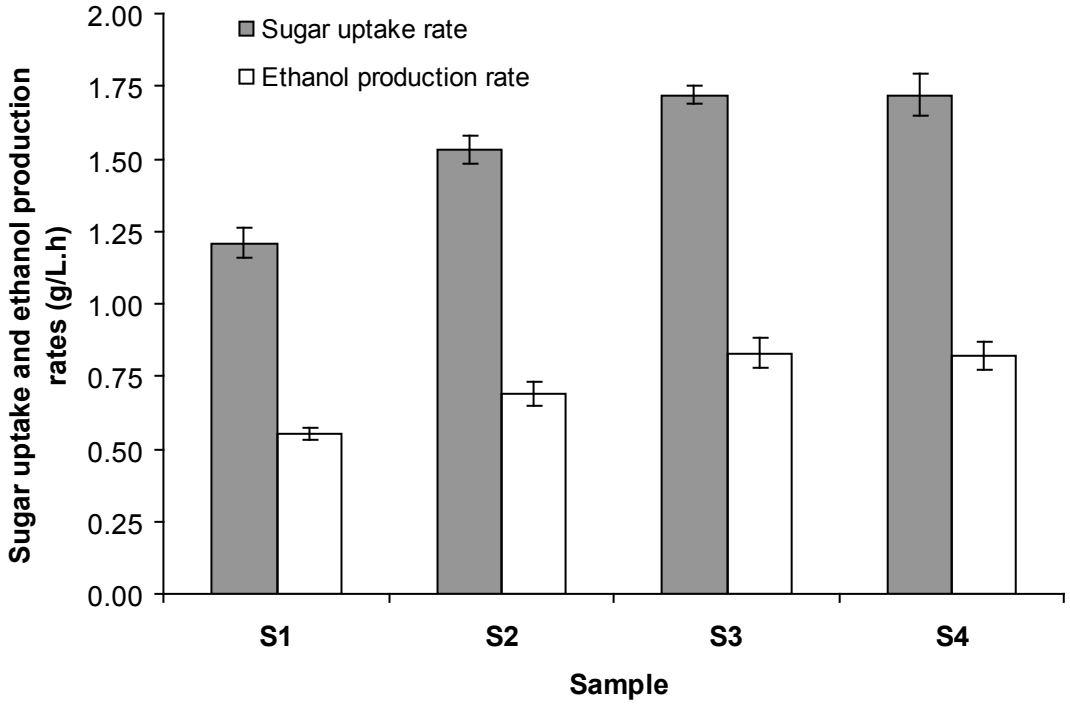

Figure 2. Yeast sugar uptake and ethanol production rates (g/L.h) in high gravity brewing. S1: Control sample with pitching rate of 1.5×10^7 viable cells/mL, S2: sample supplemented with 0.5% w/v yeast extract and 0.4% v/v Tween 80 and pitching rate of 1.5×10^7 viable cells/ml, S3: sample with high pitching rate of 7.5×10^7 cells/ml. S4: sample supplemented with 0.5% w/v yeast extract and 0.4% v/v Tween 80 and high pitching rate of 7.5×10^7 cells/ml.

Hence, 7.5×10^7 cells/ml was chosen for the next experiment.

Comparison of using high pitching rate and nutritional supplementation for improving yeast fermentation performance in high gravity brewing

As mentioned above, besides using high pitching rate, supplementing nutrients to wort prepared with high ratio of adjunct is one of the popular methods to solve the drawbacks in high gravity brewing. In this experiment, we compared the effects of these two methods in order to evaluate the influence of them on yeast fermentation performance. The purpose of this experiment was to find out whether using high pitching rate is more advantageous than supplementing nutrients to the medium. The combined effect of these two methods was also examined. Four samples were therefore studied. The first was the control sample S1 with normal pitching rate (1.5×10^7 cells/ml). The second sample S2 was supplemented with 0.5% (w/v) yeast extract and 0.4% (v/v) Tween 80 as sources of free amino nitrogen (FAN), unsaturated fatty acid and the pitching rate was 1.5×10^7 cells/ml. The third sample S3 was not supplemented with nutrients for yeast and the pitching rate was 7.5×10^7 cells/ml. High pitching rate (7.5×10^7 cells/ml) and nutritional

supplementation (0.5% w/v yeast extract and 0.4% v/v Tween 80) were used in the fourth sample S4.

Fermentation performance

The results, as expected, revealed that using high pitching rate or/and supplementing nutrients to wort shortened the fermentation time in high gravity brewing. The fermentation time of sample S2, S3, and S4 reduced by 18%, 27%, and 27%, respectively, as compared to that of the control sample (Table 2). It was noted that the fermentation time of high pitching rate cultures (S3 and S4) was shorter than that of sample S2 in which the culture was supplemented with nutrients but normal pitching rate was used.

The results from Figure 2 demonstrated that the sugar uptake and ethanol production rates in the high pitching rate cultures with or without nutritional supplementation (sample S3 and S4) were significantly improved over that seen in supplemented medium alone (sample S2) as well. The sugar uptake and ethanol production rates of high pitching rate (sample S3 and S4) were increased by 13% and 20%, respectively, than those of sample S2. However, it seemed that the combination of using high pitching rate and supplementing nutrients to the medium (sample S4) brought about no effectiveness for improving the sugar uptake rate as well as ethanol production rate as compared to using high pitching rate alone. In both samples S3 and S4, yeast assimilated sugars at the same rate and the fermentation completed at the same time. This phenomenon suggested that when worts were pitched at high levels, there was no need to supplement assimilable nitrogen or unsaturated fatty acid to wort prepared with high – sugar adjunct ratio. This is especially important because in some kinds of beers, supplementing nitrogen to wort is not allowed.

At the end of the primary fermentation, the ethanol concentrations in sample S1, S2, S3 and S4 were 9.20%, 9.45%, 10.05%, and 9.90% (v/v), respectively. Therefore, the highest amount of ethanol was obtained in sample S3 and S4 with high pitching rate. This is a crucial note because the higher the ethanol level in the green beer, the higher the volume of final product was obtained.

In summary, between the method of using high pitching rate and the method of supplementing nutrients to wort, the application of high pitching rate showed striking advantages over the nutritional supplementation to the medium in respect of the fermentation time, the sugar uptake rate as well as the ethanol production rate and the ethanol concentration in the green beer.

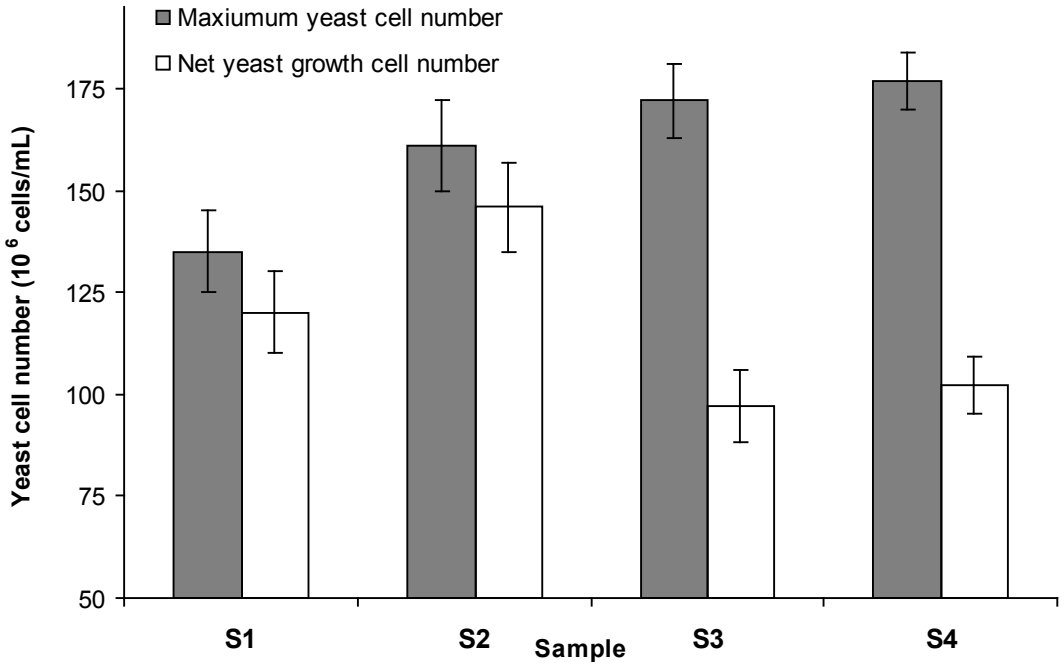

Figure 3. Total maximum yeast cell numbers and the net yeast growth (the difference between the maximum cell number per unit of volume and the cell number added at pitching) in high gravity brewing. S1: Control sample with pitching rate of 1.5×10^7 viable cells/ml, S2: sample supplemented with 0.5% w/v yeast extract and 0.4% v/v Tween 80 and pitching rate of 1.5×10^7 viable cells/ml, S3: sample with high pitching rate of 7.5×10^7 cells/ml. S4: sample supplemented with 0.5% w/v yeast extract and 0.4% v/v Tween 80 and high pitching rate of 7.5×10^7 cells/ml.

Yeast cell number and yeast viability

Figure 3 presents maximum yeast cell number in the culture and net yeast growth of sample S1, S2, S3, and S4. The maximum cell numbers were for 13.5x107, 16.1x107, 17.2x107, 17.7x107 cells/ml for S1, S2, S3, and S4, respectively. With the normal pitching rate (sample S1 and S2), supplementing suitable nutrients to the medium led to augment considerably the maximum cell number (sample S2) in the culture. With higher pitching rate, however, non-significant difference in maximum cell number in the culture between sample S3 (unsupplemented wort) and sample S4 (supplemented wort) was observed.

Although the maximum cell number of culture S2, S3, and S4 was nearly similar, the net yeast growth in the cultures with high pitching rate (sample S3 and S4) was lower than that in the culture with normal pitching rate (sample S1 and S2). Therefore, it was confirmed that the higher pitching rate, the less new cell number occurred in the culture. Moreover, with the use of high pitching rate, nutritional supplementation did not help increase the cell number in the culture as it did in the case of normal pitching rate.

In this experiment, FAN utilization of yeasts was also determined (Figure 4). FAN uptake level of sample S2 was 21% higher than that of the control sample. This was due to higher FAN content in the medium of sample 2. Although both sample S3 and sample S4 had the same pitching level of 7.5×10^7 viable cells/ml as well as the maximum cell number in the culture and the net yeast growth, the FAN consumption of sample S3 was 36% lower than that of sample S4. Hence, it was supposed that instead of using FAN for cell division, FAN supplemented in culture S4 was probably consumed to increase dry weight of yeast cells. Accordingly, this could interpret the reason why nutritional supplementation caused no increase in yeast cell number of culture S4 as mentioned above.

Diacetyl concentration in the green beer

Diacetyl concentration in the green beer of four samples is presented in Table 2. Table 2 indicated that the diacetyl level in sample S2 and S4, which were supplemented with nitrogen and unsaturated fatty acid, is higher than that in sample S1 and S3. Many authors have affirmed that diacetyl formation is closely related to the valine biosynthesis (Kunze, 2004). Based on this theory, higher diacetyl level in the green beer of sample S2 and S4 was probably due to the higher content nitrogen used for yeast cell

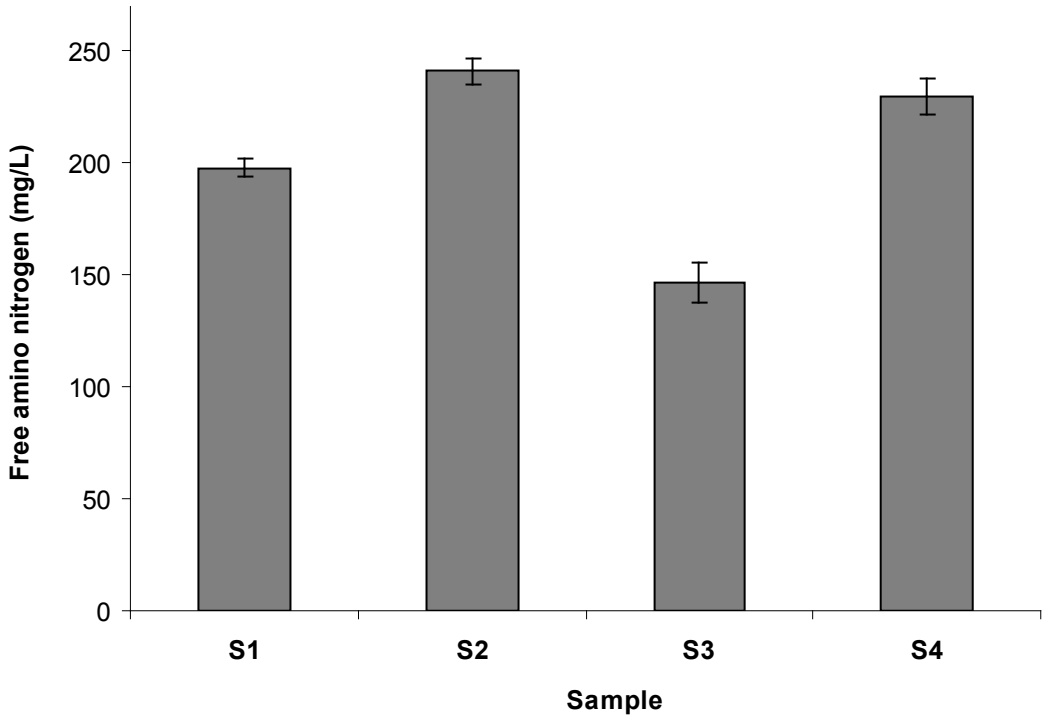

Figure 4. FAN uptake content (mg/L) in high gravity brewing. S1: Control sample with pitching rate of 1.5×10^7 viable cells/ml, S2: sample supplemented with 0.5% w/v yeast extract and 0.4% v/v Tween 80 and pitching rate of 1.5×10^7 viable cells/ml, S3: sample with high pitching rate of 7.5×10^7 cells/ml. S4: sample supplemented with 0.5% w/v yeast extract and 0.4% v/v Tween 80 and high pitching rate of 7.5×10^7 cells/ml.

division or increase in yeast biomass. Therefore, with regard to diacetyl content, the use of high pitching rate without supplementing nutrients to the medium was better than the nutritional supplementation and combination of these two methods.

Conclusion

It has been proved that high pitching rate is beneficial in many aspects in high gravity brewing. In this study, with increasing pitching rate, the fermentation time, the sugar uptake, and ethanol production rates as well as ethanol concentration in the green beer were significantly improved although the initial wort gravity was up to 24°Bx. This method was also found more effective than the supplementation of nutrients to the medium. Especially, the use of high pitching level helped decrease the dependence of yeast growth on nutrients. The results from this study manifested that when supplementing nutrients to high pitching rate wort, the improvement of yeast fermentation performance was less effective as compared to using high pitching rate wort without nutritional supplementation. In addition, supplementing nutrients to high pitching rate wort created higher diacetyl content in the green beer.

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