EFFECTS OF HIGH PITCHING RATE AND NUTRITIONAL SUPPLEMENTION ON YEAST FERMENTATION PERFORMANCE IN VERY HIGH GRAVITY BREWING

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ABSTRACT: *In this study, the 30°Bx wort with 30% maltose syrup adjunct was used for very high gravity brewing. Increase in pitching rate from 25×10^6 cells/mL to 125×10^6 cells/mL shortened the primary fermentation time and augmented the level of ethanol and diacetyl in the green beer. The suitable pitching rate was 75×10^6 cells/mL. Under this condition, the fermentation time reduced 44.2% and the ethanol concentration in the green beer increased 13.7% in comparison with those in the culture with conventional pitching rate; the ethanol yield achieved maximum of 44.1%. Combination of high pitching rate and nutritional supplementation to 30°Bx wort reduced the fermentation time 8.7% and maintained the similar ethanol content in the green beer and the similar ethanol yield in comparison with the high pitching rate culture.*

Keywords: *pitching rate, Tween 80, very high gravity brewing, yeast extract.*

1. INTRODUCTION

The high-gravity brewing technology has become widespread throughout the brewing industry during the past 35 years. This method of beer production has many advantages such as: increased brewery capacity by 20–30% without additional expenditures for facilities, reduced cost of energy and labor [1], improved physical and flavor stability of beer, more alcohol per unit of fermentable extract due to reduced yeast growth, higher adjunct rates [2]. High-gravity brewing also offers a flexibility of the beer type (products with different sugar or alcohol levels) [1]. Many researches were carried out to increase wort gravity in high gravity brewing [3,4]. However, the obtained results showed that the yeast viability was rapidly reduced within 24 hours after pitching, the fermentation rates decreased and the ethanol yield was lower when the wort gravity was more than 24°P [3]. In high gravity wort, the yeasts are exposed to severe conditions such as high osmotic pressure and toxicity of produced ethanol [1], nutrient limitation such as assimilable nitrogen and fatty acids [4].

These arising problems have been solved with high pitching rates [5], nutrient supplementation to wort [4], more efficient aeration than in conventional brewing [6]… Among those solutions, high pitching rates are advantageous in many respects [5]. Attenuation begins more rapidly, and viability losses that occur immediately after pitching are not apparent. Moreover, the profiles of volatile flavor in the beer produced were relatively unaffected by increased inoculum size [4].

There have been various studies on the effects of pitching levels on beer fermentation, the wort gravity varied from 15 to 24oP [2,7]. This study focused on the effects of pitching rate on yeast fermentation performance in very high gravity wort (30oBx). In addition, combined application of high pitching rate and nutritional supplementation to 30oBx wort was also examined.

2. MATERIALS AND METHODS

2.1. Materials

Wort: 30^{o}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. All–malt wort was produced from barley malt by infusion mashing. Barley malt (extraction yield: 79.2%) was originated from Australia and supplied by Duong Malt Co., Ltd.

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: Lager brewing strain of *Saccharomyces cerevisiae* used in this study was originated from Microorganism collection of Food Microbiology Laboratory, Department of Food Technology, Ho Chi Minh City University of Technology. Yeast propagation was performed in the 16^{o}Bx all – malt wort in an incubator at 30^{o}C. The required inoculum size was prepared by centrifuging the culture above at 6000 rpm at 4°C for 15 min.

2.2. Fermentation

Fermentation was carried out in a bioreactor containing 2L of sterile 30^{o}Bx wort. Initial content of dissolved oxygen (prior to inoculation) was 8 ppm. The primary fermentation was conducted at 17^{o}C and completed when 80% of the reducing sugars had been consumed.

Effects of high pitching rate on yeast fermentation performance

In this experiment, five pitching rates (25×10^6, 50×10^6, 75×10^6, 100×10^6 and 125×10^6 cells/mL) were used.

Combined high pitching rate and nutritional supplementation to wort in very high gravity brewing: Optimization of supplemented nutrients for yeast by response surface methodology

Assimilable nitrogen (yeast extract) and unsaturated fatty acid (Tween 80) were added to the 30^{o}Bx wort as supplemented nutrients for brewing yeast. The experiment was carried out according to a randomised, quadratic central composite circumscribed response surface design with 2 independent variables and 5 levels. Two variables included yeast extract level (X_1) and Tween 80 level (X_2). Ethanol concentration in the green beer (Y) was the dependent variable. The experimental design is shown in Table 2. The complete design consisted of 12 experimental points including 4 factorial points, 4 axial points and 4 center

points and the experiment was carried out in a random order. The software Modde version 5.0 was used to generate the experimental planning and to process data. The pitching rate was fixed at 75×10^6 cells/mL.

2.3. Fermentation analysis

Samples were daily removed in order to determine total yeast cell number, yeast viability, reducing sugar, ethanol, and diacetyl concentration. Yeast cell number was quantified by using Thoma Haemocytometry. Viable cells were determined by using methylene blue staining [8]. Reducing sugars were quantified by spectrophotometric method using dinitrosalicylic acid reagent [9]. Free amino nitrogen (FAN) content was measured by spectrophotometric method, using ninhydrin reagent [8]. Ethanol concentration was determined by a method based on distillation and density quantification [9]. Diacetyl was determined by spectrophotometric method using O–phenylendiamin reagent [8].

Maximum specific growth rate was calculated by a method reported elsewhere [10]. 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).

2.4. Statistical analysis

All fermentations were realized in duplicated. The data was analyzed for statistical significance by Analysis of Variance using the soflware Statgraphics plus version 3.2.

3. RESULTS AND DISCUSSION

3.1. Effect of pitching rate on yeast fermentation performance

Fermentation performance

Fig. 1 shows the kinetics of sugar assimilation. The concentration of reducing sugars in the cultures with high pitching rates decreased significantly faster than that in the culture with conventional pitching rate (25×10^6 cells/mL). Table 1 demonstrates that the sugar uptake rate in the cultures with pitching rate of 50×10^6, 75×10^6, 100×10^6 and 125×10^6 cells/mL increased 35.8%, 78.8%, 92.7% and 122.6%, respectively, in comparison with that in the control. This observation was in agreement with the results of Erten (2007) who examined the effect of inoculum size on sugar uptake rate on the 16^oP wort [2].

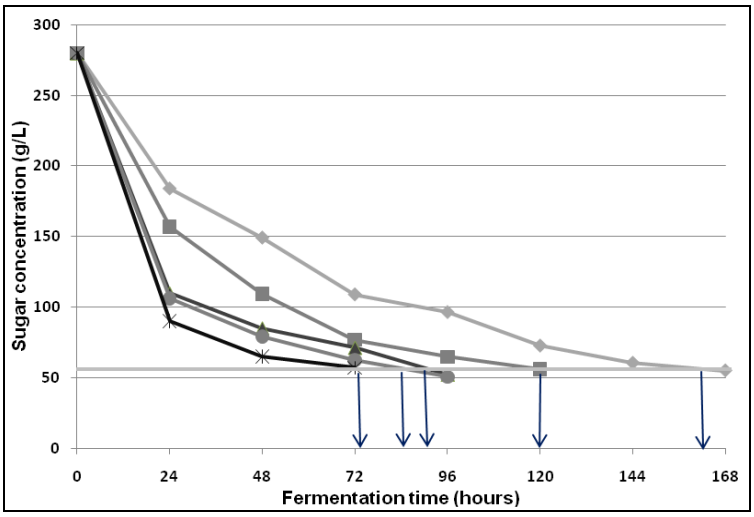

Fig 1. Sugar assimilation during the fermentation
(◆) 25×10^6 cells/mL, (■) 50×10^6 cells/mL, (▲) 75×10^6 cells/mL, (●) 100×10^6 cells/mL, (∗) 125×10^6 cells/mL

The higher the pitching rate, the higher the sugar uptake rates and the shorter the fermentation time. The time required to reach a fermentation degree of 80% was approximately 163 hours for the conventional inoculum size (25×10^6 cells/mL), 120h for two-fold higher pitching rate, 91h for the three-fold higher pitching rate, 85h for the four-fold higher pitching rate and 74 h for the highest pitching rate (125×10^6 cells/mL). Noteworthy, when the pitching rate increased from 25×10^6 to 75×10^6 cells/mL, a reduction of the fermentation time of 60% was achieved (Table 1).

Similarly, the ethanol production rate increased 53.1%, 103.8%, 118% and 154% when the pitching rate augmented two-, three-, four- and five-fold, respectively higher than the conventional value. A slightly increase in ethanol concentration in the green beer was also observed in the culture with increased inoculum size.

The ethanol yield was improved when the pitching rate increased from 25×10^6 to 75×10^6 cells/mL. At higher pitching rates, the analysis of variance showed that the ethanol yield decreased slightly. The highest ethanol yield reached 44.1% in the culture with the pitching rate of 75×10^6 cells/mL. According to Suihko et al. 1993, as the original wort gravity increased, more fermentable extract was metabolized to ethanol rather than utilized for yeast growth [11].

Yeast growth

Fig. 2 illustrates the kinetics of yeast growth in the cultures with different pitching rates. Increase in pitching rate augmented the maximum cell number in the culture. The results show that maximum cell number in the culture with the highest pitching rate (125×10^6 cells/mL) was 68.3% higher than that in the culture with the conventional pitching rate. However, the net growth in the cultures with

high pitching rate (50×10^6, 75×10^6, 100×10^6 and 125×10^6 cells/mL) was not significantly different from that in the culture with conventional pitching rate. Hence, the same amount of new yeast cells was generated during the fermentation in all examined cultures. This means that at the end of fermentation, the yeast population in the cultures with high pitching rate had a higher percentage of 'older' cells. Our result was also in agreement with the findings of Verbelen et al. (2008) who carried out high gravity brewing with 15°P all-malt wort [7].

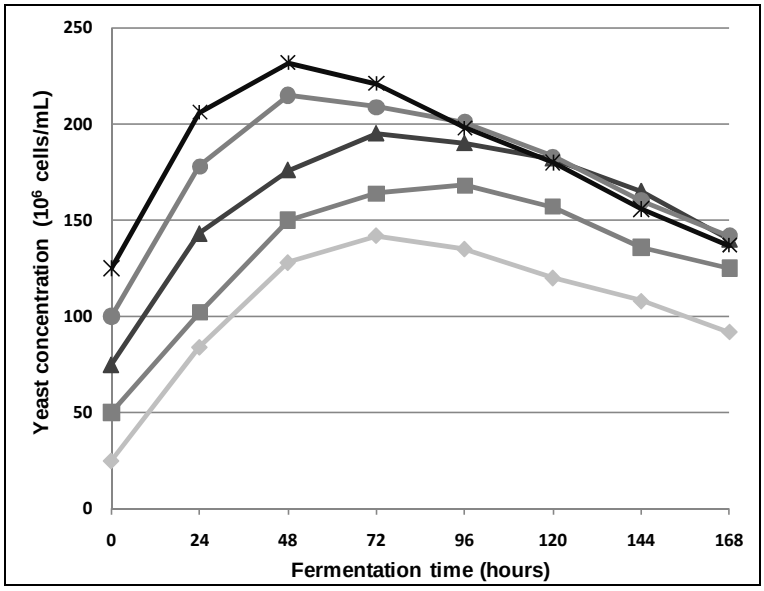

Fig 2. Kinetics of yeast growth during the fermentation.

(◆) 25×10^6 cells/mL, (■) 50×10^6 cells/mL, (▲) 75×10^6 cells/mL, (●) 100×10^6 cells/mL, (∗) 125×10^6 cells/mL

Table 1 also presents that the maximum specific growth rate of yeast at the conventional pitching rate was the highest ($0.065h^{-1}$). When the pitching rate increased two-, three-, four- and five-fold higher than the conventional value, the maximum specific growth rate decreased 52.3%, 64.6%, 63.1% and 72.3%, respectively. At the beginning of the fermentation, the yeast viability was approximately 98%. The percentage of viable cells at the end of the fermentation remained above 86% in the four cultures with high pitching rate. In the control, the percentage of viable cells only reached 71% (Table 1).

Diacetyl content in the green beer

Diacetyl can cause a 'buttery' off-flavour above its threshold of 80 ppb. Diacetyl is formed by extracellular oxidative decarboxylation of alpha acetolactate - an intermediate in the biosynthesis pathway of valine from pyruvate. Subsequently, yeast cells assimilate and reduce diacetyl to the flavor inactive compounds such as acetoin and 2, 3-butanediol [12].

Diacetyl in the green beer was present in dramatically higher amounts with high pitching rate particularly when the inoculum size was 100×10^6 cells/mL or higher (Table 1). This was probably due to higher production alpha acetolactate. Moreover, the shorter fermentation time likely resulted in incomplete reduction of diacetyl to acetoin and 2,3-butanediol [12].

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

	Pitching rate (x 10^6 cells/mL)				
	25	50	75	100	125
Fermentation time (hours)	163^a	120^b	91^c	85^d	74^e
Cell viability after fermentation (%)	71^a	86^b	97^c	94^d	97.5^c
Maximum specific growth rate (h^{-1})	0.065^a	0.031^b	0.023^c	0.024^c	0.018^d
Sugar uptake rate (g/L.h)	1.37^a	1.86^b	2.45^c	2.64^d	3.05^e
Ethanol production rate (g/L.h)	0.53^a	0.81^b	1.08^c	1.15^d	1.34^e
Ethanol concentration in the green beer (% v/v)	10.98^a	12.36^b	12.48^c	12.45^c	12.64^d
Ethanol production yield (%)	38.6^a	43.5^b	44.1^b	43.7^b	43.9^b
Diacetyl concentration in the green beer (mg/L) after being diluted to ethanol concentration of 5% (v/v)	0.31^a	0.53^b	0.59^b	0.81^c	0.97^d

Different letters in each row mean significant difference (P< 0.05)

In summary, increase in inoculum size shortened the fermentation time and increased the level of ethanol and diacetyl in the green beer. For 30^oBx wort, the appropriate pitching rate was 75×10^6 cells/mL, which allows both a significant reduction of fermentation time and an enhancement in ethanol concentration in the green beer. As a result, 75×10^6 cells/mL was chosen for the next experiment.

3.2. Combined high pitching rate and nutritional supplementation to wort in very-high gravity brewing: optimization of supplemented nutrients for yeast by response surface methodology

Based on the previous results of Dragone et al. (2003) [13] and Casey et al. (1984) [4], yeast extract level of 0.8% w/v and Tween 80 level of 0.24% v/v were chosen as the central conditions of the central composite rotary design. Table 3 show the ethanol concentration in the green beer for each run obtained from the experimentation.

Table 2. Independent variables and their levels in the response surface design

Independent variables	Symbol	Range and levels				
		$-2^{1/2}$	-1	0	+1	$+2^{1/2}$
Yeast extract concentration % (w/v)	X_1	0.52	0.6	0.8	1.0	1.08
Tween 80 concentration % (v/v)	X_2	0.16	0.18	0.24	0.3	0.32

Table 3. Optimization of nutrient supplementation for yeast: experimental design and results

Exp.No.	X_1	X_2	**Response, Y% (v/v)**
1	-1	-1	12.42
2	+1	-1	12.40
3	-1	+1	12.54
4	+1	+1	12.50
5	$-2^{1/2}$	0	12.48
6	$+2^{1/2}$	0	12.43
7	0	$-2^{1/2}$	12.38
8	0	$+2^{1/2}$	12.52
9	0	0	12.58
10	0	0	12.60
11	0	0	12.59
12	0	0	12.61

X_1: yeast extract concentration, X_2: Tween 80 concentration, Y: ethanol concentration in the green beer

In order to establish the fitted model, multiple regression analysis was performed on the experimental data and the final predictive equation obtained is as given below:

$$\mathbf{Y = 12.60 - 0.02\ X_1 + 0.05\ X_2 - 0.06\ X_1^2 - 0.07\ X_2^2} \quad (1)$$

where Y, X_1 and X_2 were the ethanol concentration in the green beer (% v/v), the yeast extract concentration (% w/v) and the Tween 80 concentration (% v/v), respectively.

Table 4 presents ANOVA of the fitted model. According to the ANOVA table, the regression model is significant at the considered confidence level ($P < 0.05$) since a satisfactory correlation coefficient was obtained and the F-value was higher than the F listed value.

The effect of each variable on the response is presented in Table 5 for a 95% confidence level. The Tween 80 level had a stronger effect on ethanol concentration than the yeast extract level in the medium. Besides, no interaction between the two variables on the ethanol concentration was observed.

Fig. 3 presents the three-dimensional response surface plot according to Eq. 1. From the model, the optimal conditions were the yeast extract concentration of 0.77% w/v and the Tween 80 concentration of 0.26% v/v, at

which the model predicted a maximum response of 12.61% v/v.

Finally, two cultures were realized and compared: one culture with high pitching rate (75×10^6 cells/mL) and one culture with high pitching rate (75×10^6 cells/mL) and nutritional addition (0.77% w/v yeast extract and 0.26% v/v Tween 80 were supplemented to the 30°Bx wort).

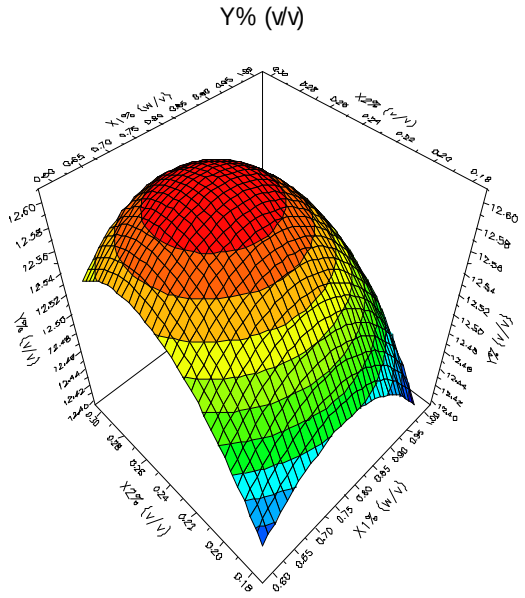

Fig 3. Response surface plot for maximizing ethanol concentration in the green beer Y% (v/v), X_1: yeast extract concentration % (w/v), X_2: Tween 80 concentration % (v/v)

Table 4. Analysis of variance of the regression model in experiments of supplemented nutrients

Source of variation	DF	SS	MS	F
Regression	5	1708.21	311.50	161.2
Residual	6	10.11	1.82	
Total	11	1718.32	156.21	
Listed F-value*				6.4

SS: sum of squares, DF: degrees of freedom, MS: mean square, F: F-value,* F-value at 95% of confidence level.

Table 5. Estimated effect of independent variables on ethanol concentration in the green beer

Factor*	Effect	Standard error	P
X_1	-0.020	0.0033	0.004035
X_2	00520	0.0033	1.45E-005
$X_1 \times X_1$	-0.060	0.0039	9.53E-006
$X_2 \times X_2$	-0.070	0.0039	9.54E-006

X_1: yeast extract concentration % (w/v), X_2: Tween 80 concentration % (v/v), P indicates significance of linear regressions. * Significant factor at 95% of confidence level.

Table 6 shows that the combination of high pitching rate and nutritional supplementation shortened the fermentation time 8.7% in comparison with the culture with high pitching rate. In addition, the combined method improved yeast viability (99%) at the end of the fermentation. However, the ethanol concentration in the green beer as well as the ethanol yield in the two cultures were not significantly different.

It can be affirmed that the combination of high pitching rate and nutritional supplementation to the very high gravity brewing wort reduced notably the fermentation time and still achieved high ethanol concentration in the green beer and high ethanol yield in very high gravity brewing.

Table 6. Fermentation characteristics of high gravity brewing

	Sample with high pitching rate	Sample with high pitching rate and nutrient addition*
Fermentation time (h)	92[a]	84[b]
Cell viability (%)	96[a]	99[b]
Ethanol concentration in the green beer (% v/v)	12.48[a]	12.61[a]
Ethanol production yield (%)	49.1[a]	49.6[a]
Diacetyl concentration in the green beer (mg/L) after being diluted to ethanol concentration of 5% (v/v)	0.43[a]	0.51[b]

** 0.77% w/v yeast extract and 0.26% v/v Tween 80 was added to 30^{o}Bx wort prior to fermentation.*

Different letters in each row mean significant difference (P< 0.05)

4. CONCLUSION

In 30^{o}Bx wort, increase in pitching rate led to a faster fermentation rate as well as a higher ethanol concentration in the green beer. However, high pitching rate resulted in low yeast growth and high diacetyl content in the culture. The combination of high pitching rate and nutritional addition to the wort reduced fermentation time and increased the yeast viability.

ẢNH HƯỞNG CỦA GIẢI PHÁP SỬ DỤNG TỈ LỆ GIỐNG CẤY CAO VÀ BỔ SUNG CHẤT DINH DƯỠNG ĐẾN HOẠT TÍNH LÊN MEN CỦA NẤM MEN TRONG QUÁ TRÌNH LÊN MEN BIA NỒNG ĐỘ RẤT CAO

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TÓM TẮT: *Nghiên cứu này sử dụng dịch nha với độ chất khô 30^{o}Bx và được bổ sung thế liệu là sirô maltose để thực hiện quá trình lên men bia nồng độ rất cao. Khi tăng tỉ lệ giống cấy từ 25 triệu lên 125 triệu tế bào/mL, thời gian lên men sẽ rút ngắn, hàm lượng ethanol và diacetyl trong bia non sẽ tăng lên. Tỉ lệ giống cấy thích hợp là 75 triệu tế bào/mL. Khi đó, thời gian lên men giảm 44.2%, nồng độ ethanol trong bia non tăng 13.7% so với mẫu đối chứng sử dụng tỉ lệ giống cấy theo phương pháp truyền thống; hiệu suất sinh tổng hợp ethanol đạt giá trị cực đại là 44.1%. Khi kết hợp giải pháp sử dụng tỉ lệ giống cấy cao với giải pháp bổ sung chất dinh dưỡng vào dịch nha 30^{o}Bx thì thời gian lên men sẽ giảm 8.7% so với trường hợp chỉ sử dụng tỉ lệ giống cấy cao, còn nồng độ ethanol trong bia non và hiệu suất sinh tổng hợp ethanol thu được sẽ không thay đổi.*

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