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Improvement of Bioethanol Production from Sweet Sorghum Juice under Very High Gravity Fermentation: Effect of Nitrogen, Osmoprotectant, and Aeration

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Abstract: To improve ethanol production fermentation efficiency from sweet sorghum juice under a very high gravity (VHG, 280 g/L of total sugar) condition by *Saccharomyces cerevisiae* NP01, dried spent yeast (DSY), yeast extract, and glycine concentrations were optimized using an L_9 (3^4) orthogonal array design. The results showed that the order of influence on the ethanol concentration (P_E) was yeast extract > glycine > DSY. The optimal nutrient concentrations for ethanol production were determined as follows: yeast extract, 3; DSY, 4; and glycine, 5 g/L. When a verification experiment under the projected optimal conditions was done, the P, ethanol yield ($Y_{p/s}$), and ethanol productivity (Q_p) values were 120.1 g/L, 0.47, and 2.50 g/L·h, respectively. These values were similar to those of the positive control experiment with yeast extract supplementation at 9 g/L. The yeast viability under the optimal condition was higher than that of the control experiment. To improve sugar utilization and ethanol production, aeration at 2.5 vvm for 4 h was applied under the optimal nutrient supplementation. The P, $Y_{p/s}$, and Q_p values were significantly increased to 134.3 g/L, 0.50, and 2.80 g/L·h, respectively.

Keywords: very high gravity (VHG); nitrogen source; osmoprotectant; aeration; sweet sorghum; *Saccharomyces cerevisiae*

1. Introduction

Bioethanol is an alternative fuel that has been of much interest for most of the past 50 years [1]. Ethanol can be blended with gasoline or can be used alone in dedicated engines. Additionally, it can be used in advanced flexi-fuel hybrid engines [2]. Thus, a strong need exists for efficient ethanol production using low-cost raw materials and production processes. Ethanol can be produced from numerous biomass feedstocks. In addition to the main raw materials, i.e., sugarcane, corn grain, tapioca starch, and sugarcane molasses, sweet sorghum (*Sorghum bicolor* (L.) Moench) juice is a promising alternative feedstock for bioethanol production. Its stalks contain high levels of fermentable sugars. This hardy crop can be cultivated in nearly all regions of the world, including tropical areas [3].

The very high gravity (VHG) fermentation process has been introduced as a technology for ethanol production to improve its cost-effectiveness. This process involves the preparation of a mash and

its fermentation to completion. The mash contains at least 270 g/L of dissolved solids [4]. Normally, ethanol fermentations at high sugar levels or under VHG conditions are sluggish, and sugar is rarely completely fermented. This may be due to an increase in the osmotic pressure and fermentation time, which has an adverse effect on microbial cells. It has been reported that *Saccharomyces cerevisiae*, can better utilize sugars in broth culture, as well as tolerate higher ethanol concentrations with adequate nutrient supplementation [4–7]. VHG fermentations use a yeast strain that can withstand greater osmotic stresses and higher ethanol concentrations. Therefore, supplementation of the media with an osmoprotectant can improve the prospects for cell survival. It was reported that glycine (a poor nitrogen source) might serve as an osmoprotectant. It indeed has a high stimulatory effect on yeast growth and fermentation [8]. Additionally, sugar consumption increased when glycine was added to a VHG ethanolic fermentation employing *S. cerevisiae* NCYC 1324.

Laopaiboon et al. [4] and Nuanpeng et al. [9] showed that sweet sorghum juice strain KKU 40 was suitable raw material for ethanol production. VHG fermentation from sweet sorghum juice required only nitrogen supplementation to improve ethanol production. Nitrogen sources, such as yeast extract, are widely used at laboratory scale to promote yeast growth, ethanol yield, and fermentation efficiency for VHG fermentation [4,9–11]. Gomez-Flores et al. [12] reported that yeast extract could increase sugar consumption because it consisted of adenine, lactose, and trehalose, that improve protein synthesis and cell growth [13]. However, yeast extract is relatively expensive and thus are not suitable for large scale fermentations. Suwanapong et al. [14] showed that dried spent yeast (DSY) is a suitable nitrogen source for VHG ethanol fermentations from sweet sorghum juice. However, in fermentation with DSY supplementation (21 g/L), the resulting ethanol yield and productivity were 7% and 27% lower than when 9 g/L of yeast extract was added to the medium. Therefore, incorporating DSY with a small amount of yeast extract in the presence of an osmoprotectant should result in equivalent ethanol yield and substrate utilization to a fermentation supplemented with yeast extract at a level of 9 g/L under VHG conditions.

In this research, an orthogonal array design (OAD) is applied to study the interactions of parameters that play fundamental roles in these types of fermentations [15,16]. The number of test conditions required can be sizably reduced using OAD. The results of the OAD produce an optimal set of conditions, and it arranges the various factors in the order of their influence for effective optimization of experimental conditions [17,18].

Numerous research studies reported the optimum aeration rate during fermentation to promote yeast growth and ethanol production. Arshad et al. [19] indicated that aeration (0.2–0.6 vvm) improved viable cell growth. However, the final ethanol concentration (P_E) decreased at higher aeration rates. Jayus et al. [20] found that an aeration rate of 0.3 vvm for 4 h increased the number of yeast cells and the P_E values simultaneously increased from 102.9 g/L to 120.9 g/L compared with an unaerated culture. Khongsay et al. [7] found that an aeration rate of 2.5 vvm for 4 h during VHG fermentation from sweet sorghum juice increased the yeast cell population and the P_E value, as well as reduced the fermentation time.

In the present study, the main factors affecting a VHG ethanol fermentation from sweet sorghum juice by *S. cerevisiae* NP01 were investigated. First, the yeast extract, DSY, and glycine levels in the sweet sorghum juice were optimized using L_9 (3^4) OAD. Then, verification experiments with these parameters at the optimal conditions were done. Finally, an aeration rate of 2.5 vvm, aeration time of 4 h, and an agitation rate of 200 rpm [7] were used to improve ethanol production in a batch VHG fermentation under the optimal conditions.

2. Materials and Methods

2.1. Microorganism and Inoculum Preparation

S. cerevisiae NP01 was inoculated into yeast extract malt extract (YM) medium and incubated at 30 °C with an agitation rate of 150 rpm for 15 h [21]. Then, the yeast was transferred into a medium

containing 360 mL of sweet sorghum juice (total sugar concentration of 150 g/L) and incubated under the same conditions for 12 h.

2.2. Raw Materials and Nutrient Supplements

Sweet sorghum juice (*cv*. KKU 40) extracted from the plant stalks was obtained from the Faculty of Agriculture, Khon Kean University, Thailand. The juice contained total soluble solids of 17 °Bx. Due to storage problems and the need to prevent bacterial contamination, the sweet sorghum juice was concentrated to a high sugar concentration of 68 °Bx and stored at 4 °C for further experiments. The compositions of the sweet sorghum juice were analyzed as previously reported [22]. It contained 160.56 g/L of total sugar, which consisted of sucrose, 112.39; glucose, 24.08, and fructose, 22.48 g/L.

DSY was donated by the Beerthip Brewery (1991) Co., Ltd., Bang Baan, Phra Nakhon Sri Ayutthaya, Thailand. The protein content of this DSY was 50.12% dry weight [21]. The yeast extract used as a nitrogen source (protein content, 74.50% DCW) was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India [21], and the glycine used as an osmoprotectant was purchased from BDH, England.

2.3. Ethanol Production Medium

An ethanol production (EP) medium was made from the concentrated sweet sorghum juice in Section 2.2 by diluting with distilled water to obtain 280 g/L of total sugar. Yeast extract, DSY, and glycine were added into the EP medium, as shown in Table 1. The medium was transferred into a 500 mL air-locked Erlenmeyer flask (working volume of 400 mL) before sterilization by autoclave [4].

Table 1. The L_9 (3^4) orthogonal test and the experimental results.

Run	*A*	*B*	*Blank*	*C*	P_E (g/L)	Q_p (g/L·h)	$Y_{p/s}$ (g/g)
1	1	4	1	1	107.5 ± 0.1 ^{a,b}	2.24 ± 0.00 ^{a,b}	0.46 ± 0.00 ^{d}
2	1	12	2	3	106.9 ± 0.3 ^{a}	2.23 ± 0.01 ^{a}	0.44 ± 0.00 ^{b}
3	1	20	3	5	108.2 ± 0.4 ^{b,c}	2.25 ± 0.01 ^{b,c}	0.43 ± 0.00 ^{e}
4	3	4	2	5	119.8 ± 0.2 ^{f}	2.50 ± 0.00 ^{f}	0.47 ± 0.00 ^{e}
5	3	12	3	1	113.3 ± 0.2 ^{e}	2.36 ± 0.00 ^{e}	0.45 ± 0.00 ^{e}
6	3	20	1	3	112.7 ± 0.4 ^{e}	2.35 ± 0.01 ^{e}	0.44 ± 0.00 ^{a,b}
7	5	4	3	3	109.5 ± 0.2 ^{c,d}	2.28 ± 0.00 ^{c,d}	0.42 ± 0.00 ^{a}
8	5	12	1	5	111.2 ± 0.1 ^{d,e}	2.32 ± 0.00 ^{d,e}	0.43 ± 0.00 ^{e}
9	5	20	2	1	105.5 ± 0.4 ^{a}	2.20 ± 0.01 ^{a}	0.46 ± 0.00 ^{e}

A: yeast extract (g/L); *B*: DSY (g/L) and *C*: Glycine (g/L); P_E: ethanol concentration; Q_p: ethanol productivity, and $Y_{p/s}$: ethanol yield; $^{a, b, c, d, e, f}$: Data superscripted by the same letter within the same column are not significantly different using Duncan's multiple range test at a 0.05 level. The experiments were performed in triplicate, and the results are expressed as mean ±SD.

2.4. Orthogonal Experiment Design

To investigate the influence of yeast extract (*A*), DSY (*B*), and glycine (*C*) levels on ethanol fermentation, the L_9 (3^4) orthogonal design in Table 1 was used. Three levels of each parameter, *A* (1, 3, and 5 g/L), *B* (4, 12, and 20 g/L), and *C* (1, 3, and 5 g/L), were set. The effects of each parameter on the characteristic properties were estimated using ANOVA [23,24].

2.5. Fermentation Conditions

Batch ethanol fermentations under various conditions were performed in 500-mL air-locked flasks. The initial yeast cell concentration in the sterile EP medium was 5×10^7 cells/mL [25]. Temperature and agitation rate were controlled at 30 °C and 100 rpm, respectively. Samples were collected for analysis at regular time intervals. Additional experiments under optimal conditions were done to validate the results of the orthogonal tests. These fermentations were done in a 2-L bioreactor (Biostat® B, B. Braun Biotech, Germany) with a final working volume of 1 L. It was autoclaved at 110 °C for 40 min [4].

Additionally, EP media containing 9 g/L yeast extract and without nutrient supplementation were used for ethanol fermentation as positive and negative control treatments, respectively.

2.6. Effect of Aeration on Ethanol Production

Ethanol fermentation under optimal conditions (Section 2.4) was done as previously described (Section 2.5). The agitation rate, aeration rate, and aeration time were 200 rpm, 2.5 vvm, and 4 h, respectively [7]. Samples were taken at regular time intervals for chemical analyses.

2.7. Analytical Methods

The number of viable yeast cells was determined using a hemacytometer with methylene blue staining [26], and level of total sugar in the fermentation broth was determined in terms of total carbohydrates using a phenol sulfuric acid method [27]. Ethanol (P_E, g/L) and glycerol concentrations (P_G, g/L) were analyzed using gas chromatography and high performance liquid chromatography (HPLC), respectively [4,28]. The ethanol yield ($Y_{p/s}$) and volumetric ethanol productivity (Q_p, g/L·h) were determined, according to Laopaiboon et al. [29]. Fermentable nitrogen was measured by formol titration (modified from Zoecklein et al., [26]).

3. Results and Discussion

3.1. The Results of Orthogonal Experiments

Figure 1 shows the main parameter changes during ethanol fermentation of Run 1 (Table 1). No lag phase was observed, indicating that the yeast cells were very active. Total sugar concentration decreased from 273.6 to 38.0 g/L after 56 h, whereas viable cells increased from 4.1×10^7 to 3.1×10^8 cells/mL in 12 h and slightly decreased after 36 h to 2.1×10^8 cells/mL at 72 h. The lower cell concentrations might have been due to high ethanol concentrations. This is in agreement with Bai et al. [30], who reported that during ethanol fermentation, yeast cells were negatively impacted by high ethanol concentrations and its corresponding inhibition of yeast cell growth, particularly under VHG conditions.

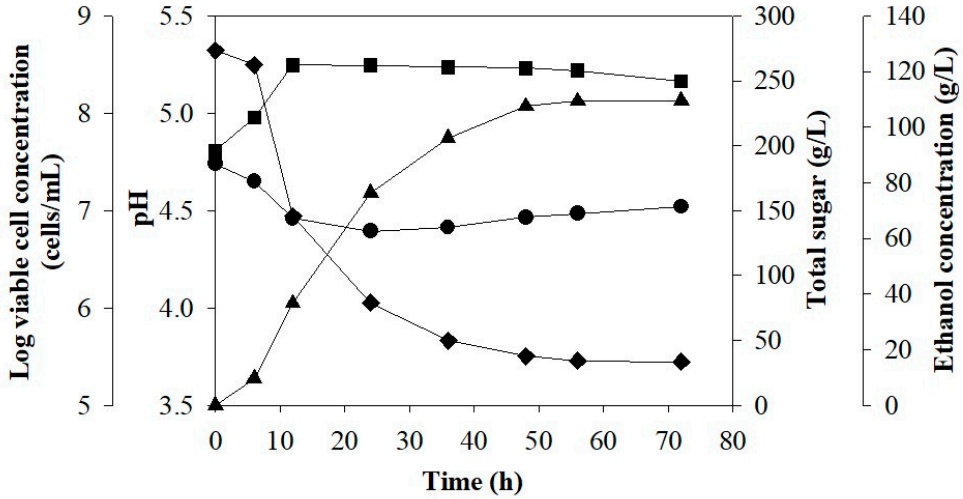

Figure 1. Ethanol fermentation profiles of Run 1 from the sweet sorghum juice supplemented with yeast extract (1 g/L), DSY (4 g/L), and glycine (1 g/L) by *S. cerevisiae* NP01 (■ = log viable cells, ● = pH, ◆ = total sugar and ▲ = ethanol).

The pH of the broth slightly decreased from 4.7 to 4.4 over the first 12 h. The ethanol concentration (P_E) significantly increased to 107.5 g/L at 48 h, while the ethanol volumetric productivity (Q_p) and ethanol yield ($Y_{p/s}$) were 2.24 g/L·h and 0.46 g/g, respectively. The main parameter changes during the

ethanol fermentation of Runs 2 to 9 were similar to those of Run 1 (data not shown). The P_E, Q_p, and $Y_{p/s}$ values of Runs 1 to 9 are shown in Table 1.

3.2. Impact of Multi-Factors on Ethanol Concentration

The P_E values of Runs 1 to 9 range from 105.5 to 119.8 g/L (Table 1). Range analysis was used to elucidate the importance of factors (yeast extract, factor *A*; DSY, factor *B*; and glycine, factor *C*) in the orthogonal experimental runs (Table 2). A larger range (*R*) value of a factor represents a greater effect on the final P_E value. The highest (7.74) and lowest range values (1.67) were found on factors *A* and *B*, respectively. This indicates that yeast extract had a greater influence on ethanol production than glycine and DSY, respectively. The optimal concentrations of yeast extract, DSY, and glycine for improving the P_E value were 3, 4 and 5 g/L, respectively ($A_2B_1C_3$). ANOVA was applied to determine the order of influence of the three parameters under study on the final *P* level. An *F*-value of 178.95 implies model significance, as there is only a 4.66% likelihood that these values could arise due to noise. The prob ($F < 0.05$) value indicates significance. According to the data, the order of influence ($F_{yeast\ extract}$ = 364.03, $F_{glycine}$ = 108.64, and F_{DSY} = 64.18) is the same as the magnitude of the *R* values. The correlation between the predicted values and experimental data resulted in an R^2 value of 0.998 (99.8%), confirming an excellent fit [31].

Table 2. The range analysis of L_9 (3^4) orthogonal experimental runs of ethanol concentration.

	***A*: Yeast Extract**	***B*: DSY** [a]	**Blank**	***C*: Glycine**
K_1	645.04 [b]	673.64	652.72	652.44
K_2	691.50	662.74	618.46	658.24
K_3	652.54	652.70	653.90	678.40
k_1	107.51 [c]	111.61	108.79	108.74
k_2	115.25	110.46	103.08	109.07
k_3	108.76	108.78	108.98	113.07
R	7.74 [d]	1.67	5.91	4.33
Q	A_2	B_1	-	C_3

[a] dried spent yeast; [b] K_i^A = Σ the amount of target ethanol concentration at A_i; [c] $K_i^A = K_i^A/3$; [d] R_i^A = max $\{K_i^A\}$ − min $\{K_i^A\}$.

Figure 2 shows the P_E values using these three factors at various levels ranging from 107.5 (k_1) to 115.3 (k_2) g/L as the yeast extract increased from 1 to 5 g/L. A maximal P_E value, 115.3 g/L (k_2), was obtained using 3 g/L of yeast extract. A higher yeast extract concentration, 5 g/L, did not improve ethanol production owing to limitations of yeast metabolism. For the DSY concentrations tested, *S. cerevisiae* NP01 showed the highest P_E value, 111.6 g/L (k_1), at a concentration of 4 g/L. The ethanol concentration did not increase with increasing levels of DSY in the medium. The results using DSY are similar to those of yeast extract, indicating a limitation in the yeast metabolism. Bafrncová et al. [6] showed that excess assimilable nitrogen did not result in increased rates of ethanol production or reduce fermentation time. Additionally, a maximal P_E value, 113.1 g/L (k_3), was obtained at a glycine concentration of 5 g/L. Thomas et al. [8] showed that the stimulatory effect of glycine on yeast growth increased with concentrations up to 40 mM or 0.53 mg/L under VHG condition using *S. cerevisiae* (uvarum) NCYC 1324. However, they reported that further increases in the glycine concentration did not improve fermentation or growth. Thomas et al. [8] reported that under VHG fermentation, glycine was not used as a nitrogen source for yeast growth, but rather, it acted as an osmoprotectant.

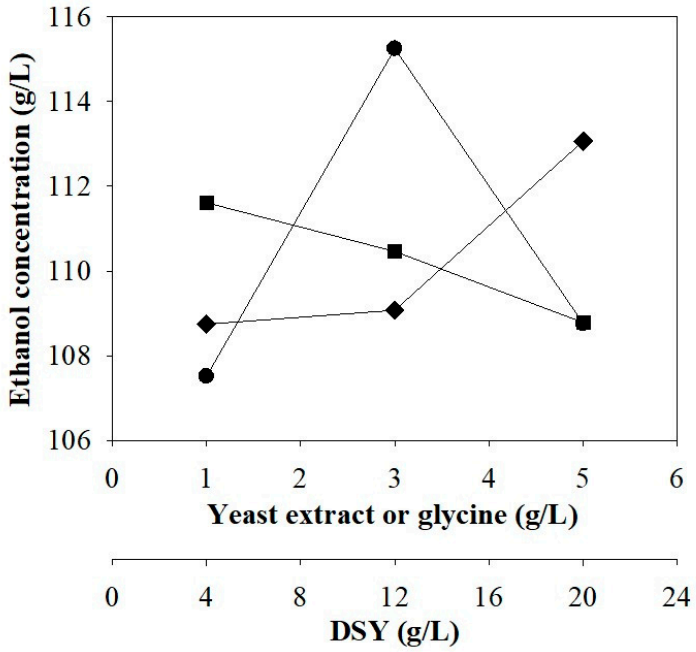

Figure 2. Ethanol concentrations at various levels and factors in the orthogonal array design (OAD) test (● = yeast extract, ■ = DSY, ◆ = glycine, DSY = dried spent yeast).

3.3. The Verification Experiments

From the results of the multi-factors on the P_E values, the optimal condition for improving P_E values from fermentation of sweet sorghum juice by *S. cerevisiae* NP01 under VHG conditions was determined as $A_2B_1C_3$, or yeast extract at 3 g/L, DSY at 4 g/L, and glycine at 5 g/L. The model was used to predict the results of experiments in a 2-L bioreactor under optimal conditions. The results of these verification experiments are shown in Figure 3. A maximal P_E value, 120.0 g/L, was obtained at 48 h. At this time, the viable cell numbers were 3.0×10^8 cells/mL, and 18.6 g/L of total sugar remained. The calculated Q_p and $Y_{p/s}$ values were 2.50 g/L·h and 0.47 g/g, respectively. The results of P_E, Q_p, and $Y_{p/s}$ in the 2-L bioreactor were similar to those in the flask culture under optimal conditions (Run 4, Table 1), indicating that the fermentations are comparable.

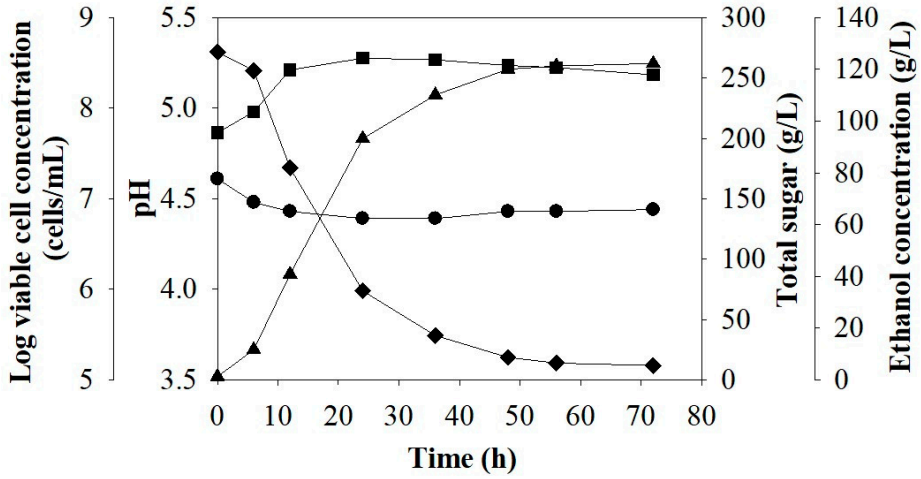

Figure 3. Ethanol fermentation from the sweet sorghum juice under the optimal conditions (yeast extract, 3 g/L; DSY, 4 g/L, and glycine, 5 g/L) by *S. cerevisiae* NP01 in a 2-L bioreactor (■ = log viable cells, ● = pH, ◆ = total sugar, ▲ = ethanol).

3.4. Comparison of Ethanol Fermentation from Sweet Sorghum Juice with and without Nutrient Supplementation

The control treatments under the same condition without nutrient supplementation (negative control experiment) and with 9 g/L of yeast extract (positive control experiment) were also determined in a 2-L bioreactor (Figure 4). Table 3 summarizes the important parameters of the batch ethanol fermentation from sweet sorghum juice under no supplementation, 9 g/L of yeast extract, and the optimal nutrient conditions. The P_E and $Y_{p/s}$ values under the optimal conditions were similar to those with yeast extract supplementation, but the fermentation time of the former was shorter than the latter, resulting in a higher Q_p under the optimal conditions. However, these values were significantly higher than those under no nutrient supplementation. The percentage of sugar consumed under the optimal conditions and yeast extract supplementation were also similar. They were approximately 25% to 27% higher than with no supplementation. These results indicate that supplementation with a combination yeast extract and DSY (as a nitrogen source) and glycine (as an osmoprotectant) significantly promoted sugar consumption and ethanol production efficiency (P_E and Q_p) from the sweet sorghum juice under VHG fermentation. This might have resulted from better yeast cell growth, cell viability, and/or adequate nutrients under optimal supplementation (Figure 4). These results show that the optimal condition, $A_2B_1C_3$, is best for ethanol fermentation. The $Y_{p/s}$ values under all conditions tested did not markedly differ, implying that the metabolic pathway of ethanol conversion by *S. cerevisiae* NP01 was the same. The $Y_{p/s}$ values ranged from 0.47 to 0.49. This implied that by-products, such as glycerol, were produced under these conditions. By-product determination showed a lower glycerol concentration under optimal conditions. The yeast viability under all conditions tested gradually diminished after 36 h (Figure 4), presumably due to ethanol accumulation in the broth [32]. However, the yeast viability under optimal conditions was higher than with yeast extract and without nutrient supplementation. These results might be due to the presence of glycine and its role as an osmoprotectant [8].

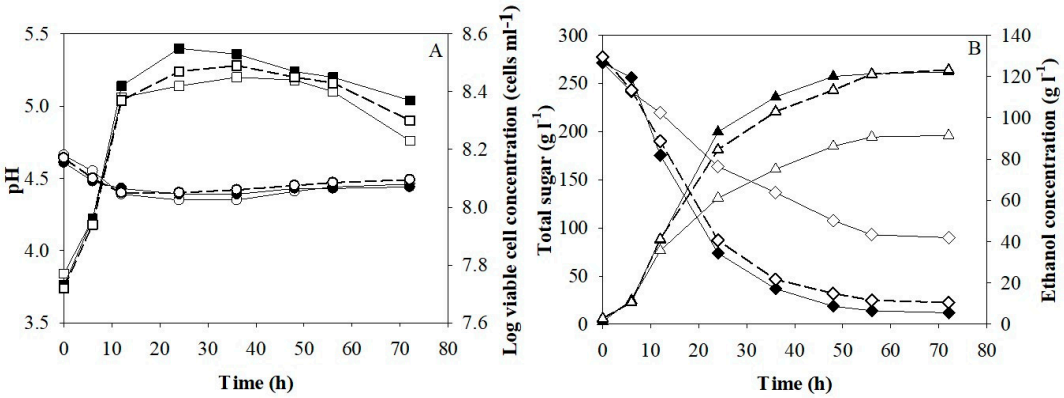

Figure 4. Ethanol fermentation in 2-L fermenter from the sweet sorghum juice with nutrient supplements at the optimal concentrations (yeast extract, 3; DSY, 4, and glycine, 5 g/L) (closed symbols, solid lines), without nutrient supplements (open symbols, solid lines), and with 9 g/L of yeast extract (open symbols, dashed lines). (**A**) pH (●○) and log viable cells (cells/mL, ■□) and (**B**) total sugar (g/L, ♦◊) and ethanol (g/L, ▲△).

Table 3. Fermentation parameters of ethanol fermentation from the sweet sorghum juice with and without nutrient supplements.

Supplement	P_E (g/L)	Q_p (g/L·h)	$Y_{p/s}$ (g/g)	P_G (g/L)	*SC* (%)	*t* (h)
No supplement	90.7 ± 0.2 [a]	1.62 ± 0.01 [a]	0.49 ± 0.01 [a]	11.8 ± 0.2 [a]	65.8 ± 1.1 [a]	56
Yeast extract (9 g/L)	121.8 ± 0.8 [b]	2.18 ± 0.01 [b]	0.47 ± 0.00 [b]	12.2 ± 0.2 [b]	91.1 ± 1.1 [b]	56
Optimal ($A_2B_1C_3$)	120.1 ± 0.1 [b]	2.50 ± 0.00 [c]	0.47 ± 0.00 [b]	10.8 ± 0.2 [c]	93.13 ± 1.9 [b]	48

P_E: ethanol concentration; Q_p: ethanol productivity; $Y_{p/s}$: ethanol yield; P_G: glycerol concentration; *SC*: sugar consumption and *t*: fermentation time. [a, b, c]: Data superscripted with the same letter within the same column are not significantly different using Duncan's multiple range test at the level of 0.05. The experiments were performed in triplicate, and the results are expressed as mean ±SD.

Table 4 shows the amount of fermentable nitrogen utilized in the experiments as well as in the positive and negative control experiments. In the orthogonal experiments, utilization of fermentable nitrogen in the medium increased with the ethanol concentration. The fermentable nitrogen concentrations of the nine runs in the orthogonal array design were much lower when supplementing with yeast extract. Even though the P_E values of the juice supplemented with 9 g/L of yeast extract (the control, 121.1 g/L) and *R4* (119.8 g/L) were very close, the fermentable nitrogen utilized in the control (317.5 mg/L) was much higher than of *R4* (249.3 mg/L), suggesting the fermentation was not limited by the availability of fermentable nitrogen, even with no nutrient supplementation. This implies that nitrogen utilization by the yeast under these conditions depends on yet unknown factors.

Table 4. Fermentable nitrogen during ethanol production from the sweet sorghum juice under various nutrient supplements.

Condition	Fermentable Nitrogen (mg/L)			P_E (g/L)
	Initial	Final	Utilized	
Positive control (no supplement)	301.4 ± 1.2	119.4 ± 0.9	182.1 ± 2.0	90.8 ± 0.9
Negative control (Yeast extract 9 g/L)	637.9 ± 0.8	320.4 ± 1.2	317.5 ± 2.0	121.1 ± 1.6
R_1 [a]	341.1 ± 1.2	129.3 ± 0.9	211.8 ± 2.2	107.5 ± 0.1
R_2	361.2 ± 0.6	155.0 ± 0.9	206.2 ± 1.6	106.9 ± 0.3
R_3	476.0 ± 0.4	260.8 ± 0.4	215.2 ± 0.7	108.2 ± 0.4
R_4	352.2 ± 1.1	102.9 ± 0.4	249.3 ± 1.5	119.8 ± 0.2
R_5	381.7 ± 1.6	142.1 ± 0.8	239.6 ± 2.5	113.3 ± 0.2
R_6	491.5 ± 1.1	256.4 ± 1.6	235.1 ± 2.6	112.7 ± 0.4
R_7	391.8 ± 2.0	172.0 ± 0.3	219.8 ± 2.3	109.5 ± 0.2
R_8	402.5 ± 1.8	162.2 ± 1.1	240.4 ± 2.9	111.2 ± 0.1
R_9	501.6 ± 0.7	301.9 ± 0.5	199.8 ± 1.2	105.5 ± 0.4

P_E: ethanol concentration, [a] R_i = no. of run. The experiments were performed in triplicate, and the results were expressed as mean ±SD.

3.5. Effects of Aeration on Ethanol Fermentation

Under optimal nutrient conditions ($A_2B_1C_3$) for ethanol production from sweet sorghum juice, the sugar in the fermentation was not completely utilized with 18.6 g/L of total sugar remaining in the broth at the end of the process. Aeration was applied to improve sugar utilization. Khongsay et al. [7] reported that the highest ethanol production efficiency from sweet sorghum juice supplemented with 9 g/L yeast extract under a VHG condition (290 g/L of total sugar) was achieved with agitation at 200 rpm, aeration at 2.5 vvm, and an aeration time of 4 h. This optimal aeration condition was applied with optimal nutrient conditions ($A_2B_1C_3$).

Figure 5 shows the profiles of viable yeast cells, pH, residual total sugar, and ethanol levels during ethanol production from sweet sorghum juice by *S. cerevisiae* NP01 in a medium containing 280 g/L of total sugar using optimal aeration [7] and nutrient conditions. The initial total sugar level was 277.0 g/L

with an initial cell concentration of 2.0×10^7 cells/mL. The P_E (134.3 g/L), $Y_{p/s}$ (0.50), and Q_p (2.80 g/L·h) values were obtained after 48 h (including 4 h of aeration) (Table 5). The results showed that under optimal nutrient conditions with appropriate agitation, aeration, and aeration time, the efficiencies of ethanol production in terms of P_E, $Y_{p/s}$, and Q_p values were approximately 6% to 12% higher than those under the same conditions with no aeration. The highest viable yeast cell numbers under aeration were approximately 14% higher than those with no aeration (Figure 6). These results indicated that aeration for only 4 h could significantly promote yeast growth and markedly improve the fermentation efficiencies. Hammond et al. [33] showed that the oxygen present earlier in the fermentation was rapidly utilized for membrane synthesis. The glycerol concentration or P_G under aeration was 2 g/L lower than that with no aeration. Alfenore et al. [34] reported the beneficial effects of aeration in the form of better control of by-product production. Glycerol was the primary by-product. It was greatly reduced from 12 g/L (no aeration) to 4 g/L (aeration at 0.2 vvm throughout the fermentation). Reddy and Reddy [10] reported that higher glycerol content indicates more microbial stress. Cot et al. [35] reported that high ethanol concentrations resulted in the loss of membrane integrity, denaturation of membrane proteins, and inactivation of some intracellular metabolic products. Aeration may protect the cells from the unfavorable effects of high ethanol concentrations, improving membrane functionality, resulting in higher viable yeast cell counts, P_E, and Q_p values. Additionally, Yue et al. [32] reported that large amounts of residual fermentable sugars often remain at the end of fermentation. Some of them were converted into glycerol, which is involved in the osmotic regulation of the cells. The results of our study indicated that the aeration rate had a positive effect on sugar utilization and ethanol production in terms of P_E, Q_p, and $Y_{p/s}$ (Table 5). Adequate amounts of essential nutrients, appropriate aeration, and agitation time promoted sugar utilization of *S. cerevisiae* NP01, leading to an improvement of ethanol production [7].

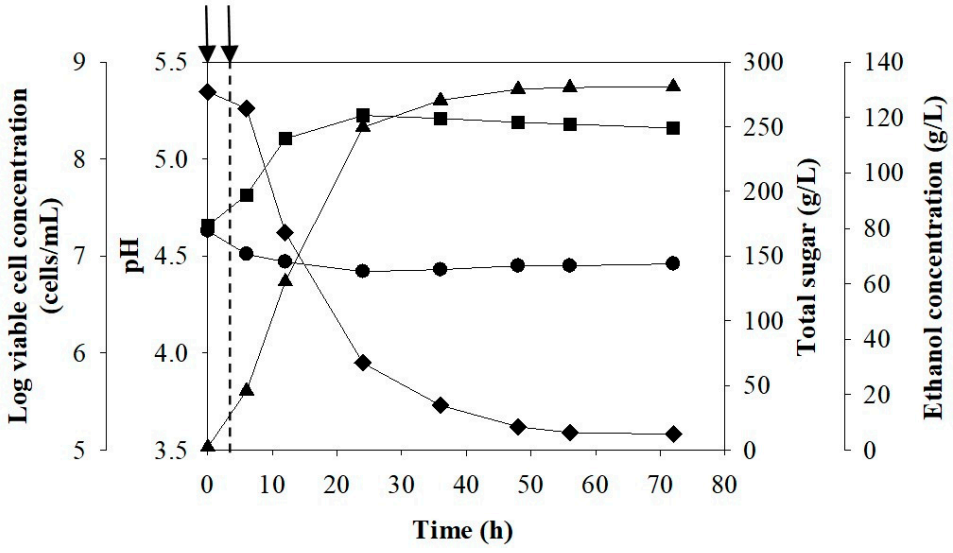

Figure 5. Fermentation profiles during ethanol production by *S. cerevisiae* NP01 from the sweet sorghum juice supplemented with yeast extract (3 g/L), DSY (4 g/L), and glycine (5 g/L) at an agitation rate, 200 rpm; aeration rate, 2.5 vvm, and aeration time, 4 h. The arrows indicate the start and the end of aeration. (■ = log viable cells, ● = pH, ◆ = total sugar, ▲ = ethanol).

Table 5. Fermentation parameters of ethanol production from the sweet sorghum juice under optimal nutrient conditions ($A_2B_1C_3$) with and without aeration.

Aeration	P_E (g/L)	$Y_{p/s}$ (g/g)	Q_p (g/L·h)	P_G (g/L)	*SC* (%)	*t* (h)
No	120.1 ± 0.1 [a]	0.47 ± 0.00 [a]	2.50 ± 0.00 [a]	10.8 ± 0.2 [a]	93.1 ± 1.9 [a]	48
2.5 vvm for 4 h at agitation rate of 200 rpm	134.3 ± 0.2 [b]	0.50 ± 0.00 [b]	2.80 ± 0.00 [b]	8.7 ± 0.1 [b]	96.8 ± 0.7 [b]	48

P_E: ethanol concentration; $Y_{p/s}$: ethanol yield Q_p: ethanol productivity; P_G: glycerol concentration; *SC*: sugar consumption, and *t*: fermentation time. [a, b]: Means superscripted with the same letter within the same column are not significantly different using Duncan's multiple range test at a 0.05 level. The experiments were performed in triplicate, and the results are expressed as mean ±SD.

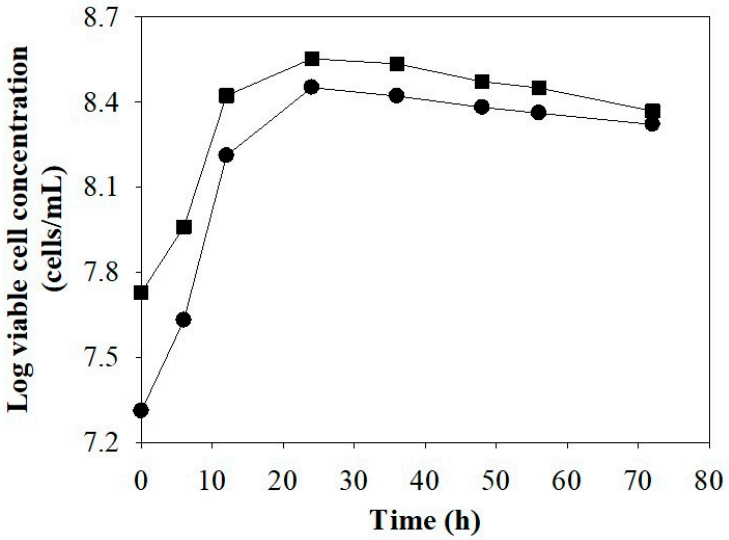

Figure 6. Viable *S. cerevisiae* NP01 cells during ethanol production from the sweet sorghum juice supplemented with yeast extract (3 g/L), DSY (4 g/L), and glycine (5 g/L) with (■) and without (●) aeration.

The effects of aeration on ethanol fermentation have been reported in serval studies. Table 6 compares the ethanol production efficiency under VHG fermentations (280 to 300 g/L of sugar) with various aerated controls. The P_E and Q_p values ranged from 120.9 to 140.2 g/L and 1.77 to 3.36 g/L h, respectively. The differing results might have been due to variations in the raw materials, yeast strain, and operating conditions. The P_E and Q_p values in our study were relatively high compared to similar research studies, indicating that optimal nutrient and aeration conditions were achieved. However, the cost-effectiveness of the process should be further studied.

Table 6. Comparison of very high gravity (VHG) ethanol fermentation under aeration in various studies.

Medium, Sugar Concentration (g/L)	Aeration	P_E (g/L)	Q_p (g/L·h)	t (h)	References
SSJ, 280 [a]	0.05 vvm, 12 h	126.3	2.11	48	Deesuth et al. [36]
SSJ, 290 [b]	2.5 vvm, 4 h	132.8	2.55	52	Khongsay et al. [7]
Molasses, 300 [c]	0.3 vvm, 4 h	120.9	3.36	36	Jayus et al. [20]
SSJ, 298 [d]	−150 mV [g]	140.2	2.92	48	Khongsay et al. [37]
Glucose, 298 [e]	−150 mV [g]	131.0	1.77	72	Liu et al. [38]
SSJ, 280 [f]	2.5 vvm, 4 h	134.3	2.80	48	This study

P_E: ethanol concentration; Q_p: ethanol productivity; and t: fermentation time. SSJ: sweet sorghum juice. [a] Supplemented with DSY 13.5 g/L, Zn^{2+} at 0.01 g/L, Mg^{2+} at 0.05 g/L, and Mn^{2+} at 0.04 g/L. [b] Supplemented with yeast extract at 9 g/L. [c] Supplemented with 100 ppm $(NH_4)_2HPO_4$. [d] Supplemented with yeast extract at 9 g/L and urea at 16 mM. [e] Supplemented with yeast extract at 6 g/L and peptone at 8 g/L. [f] Supplemented with yeast extract 3 g/L, dried spent yeast at 4 g/L, and glycine at 5 g/L. [g] Control redox potential at −150 mV by applying aeration at 0.82 vvm.

4. Conclusions

Sweet sorghum juice is found to be a suitable substrate for VHG ethanol fermentation. The results of our study clearly indicate that available nitrogen, an osmoprotectant, and aeration are essential for high levels of ethanol production from sweet sorghum juice under VHG fermentation. The amount of yeast extract used for ethanol production can be reduced by the addition of a low-cost nitrogen source (DSY). Based on the statistical analysis of ethanol production from sweet sorghum juice by *S. cerevisiae* NP01 in the current study, the optimal fermentation conditions require yeast extract at 3 g/L, dried spent yeast at 4 g/L, and glycine at 5 g/L. Under these conditions, the P_E, $Y_{p/s}$, and Q_p values were 120.1 g/L, 0.47, and 2.50 g/L·h, respectively. Appropriate aeration (2.5 vvm for 4 h) markedly improved ethanol production. The P_E, $Y_{p/s}$, and Q_p values were increased to 134.3 g/L, 0.50, and 2.80 g/L·h, respectively.

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