Research article

Improvement of ethanol production from sweet sorghum juice under batch and fed-batch fermentations: Effects of sugar levels, nitrogen supplementation, and feeding regimes

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A B S T R A C T

Background: Fermentation process development has been very important for efficient ethanol production. Improvement of ethanol production efficiency from sweet sorghum juice (SSJ) under normal gravity (NG, 160 g/L of sugar), high gravity (HG, 200 and 240 g/L of sugar) and very high gravity (VHG, 280 and 320 g/L of sugar) conditions by nutrient supplementation and alternative feeding regimes (batch and fed-batch systems) was investigated using a highly ethanol-tolerant strain, Saccharomyces cerevisiae NP01.
Results: In the batch fermentations without yeast extract, HG fermentation at 200 g/L of sugar showed the highest ethanol concentration (P_E, 90.0 g/L) and ethanol productivity (Q_E, 1.25 g/L·h). With yeast extract supplementation (9 g/L), the ethanol production efficiency increased at all sugar concentrations. The highest P_E (112.5 g/L) and Q_E (1.56 g/L·h) were observed with the VHG fermentation at 280 g/L of sugar. In the fed-batch fermentations, two feeding regimes, i.e., stepwise and continuous feedings, were studied at sugar concentrations of 280 g/L. Continuous feeding gave better results with the highest P_E and Q_E of 112.9 g/L and 2.35 g/L·h, respectively, at a feeding time of 9 h and feeding rate of 40 g sugar/h.
Conclusions: In the batch fermentation, nitrogen supplementation resulted in 4 to 32 g/L increases in ethanol production, depending on the initial sugar level in the SSJ. Under the VHG condition, with sufficient nitrogen, the fed-batch fermentation with continuous feeding resulted in a similar P_E and increased Q_P by 51% compared to those in the batch fermentation.

1. Introduction

Bioethanol is an alternative energy source that is both renewable and environmentally friendly. It can be produced from agricultural raw materials such as corn grain, cassava, sugar cane, sugar cane molasses, and sweet sorghum, among others. Sweet sorghum, Sorghum bicolor (L.) Moench, is a potential alternative feedstock for bioethanol production because the juice from its stalks contains high levels of fermentable sugars, mainly sucrose, fructose, and glucose, and it has short life cycle of only 100–120 d. Moreover, it can be cultivated at almost all temperatures in tropical areas [1,2].

Saccharomyces cerevisiae is widely used in industrial ethanol production [3]. In addition to yeast strains, nutrients, and environmental conditions, the ability of yeast to produce ethanol also depends on the initial sugar concentration of the fermentation medium. In ethanol fermentation, 1 mol of glucose can be converted to 2 mol of ethanol and 2 mol of carbon dioxide. Therefore, a medium containing a higher sugar concentration will give a higher ethanol concentration. Typically, sugar concentrations for ethanol fermentation are divided into normal gravity (NG) (<180 g/L of sugar), high gravity (HG) (180–240 g/L of sugar), and very high gravity (VHG) conditions (≥250 g/L of sugar) [4,5]. However, high sugar concentrations or VHG conditions cause an increased osmotic pressure, which has negative effects on yeast cells. Bafrncová et al. [6] reported that under appropriate environmental and nutritional conditions, S. cerevisiae could produce and tolerate high ethanol concentrations.

Fermentation process development has been very important for efficient ethanol production [7,8]. Ethanol fermentation can be performed in batch, fed-batch, and continuous modes. The batch fermentation is a closed culture system. Biomass and substrate are added into fermenter without removal of media during fermentation, and products are harvested at the end of the fermentation. The batch

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mode has disadvantages, particularly when microorganisms are either slow growing or strongly affected by substrate inhibition [9]. The fed-batch mode is started as a batch mode with a small amount of biomass and substrate in the fermenter. Then, a feeding medium is fed, stepwise or continuously, to the fermenter when most of the initially added substrate has been consumed. This process can increase the total substrate content in the fermenter while maintaining a low substrate concentration during fermentation to reduce the negative effects of osmotic pressure on yeast. The advantages of this process include reduction of substrate inhibition, higher productivity, shortened fermentation time, and reduction of toxic effects of the medium components, which are present at high concentrations [10]. Stepwise feeding of fed-batch fermentation was previously demonstrated to be effective in enhancing ethanol production and yield from sweet sorghum juice (SSJ) under HG conditions [8]. In the current study, stepwise and continuous feedings were examined under VHG conditions to determine if these regimes could enhance fermentation efficiency at very high initial sugar concentrations.

Ethanol produced by yeast is toxic to the yeast itself. To achieve high-level ethanol production, yeast strains that can produce and tolerate high ethanol concentration should be used. *S. cerevisiae* NP01 and *S. cerevisiae* ATCC 4132 are considered robust ethanol-producing strains because of their ability to produce high ethanol titers under HG and VHG conditions [2,11]. However, their ethanol tolerance has not been examined. In the current study, the ability of these yeast strains to tolerate ethanol at various concentrations was tested. Improvement of ethanol production efficiency from SSJ under NG, HG, and VHG conditions by nutrient supplementation and alternative feeding regimes (batch and fed-batch systems) was subsequently investigated.

2. Materials and methods

2.1. Microorganisms

S. cerevisiae NP01 (accession number KP866701) was isolated from Loog-pang (Chinese yeast cake) for Sato (Thai rice wine) making and was identified by gene sequencing analysis using the D1/D2 domain of 26S rDNA [5], and *S. cerevisiae* ATCC 4132 was isolated from molasses distillery yeast. The yeasts were inoculated into 100 mL of yeast extract and malt extract (YM) medium (containing yeast extract, 3 g/L; malt extract, 3 g/L; peptone, 5 g/L; and glucose, 10 g/L) and incubated at 200 rpm and 30°C for 18 h. Then, the cultures (10% inoculum size) were transferred into 350 mL of SSJ containing 100 g/L

of sugar [12] and incubated under the same conditions. After 15 h, the cells were harvested and used as inocula for ethanol fermentations.

2.2. Raw materials and ethanol production medium

Sweet sorghum *cv*. KKU40 was obtained from the Division of Agronomy, Faculty of Agriculture, Khon Kaen University, Thailand. To prevent bacterial contamination and improve storage stability after extraction, the juice (17 °Bx) was heated to approximately 90°C to concentrate to 65 °Bx, cooled, and stored at 4°C until use. It was diluted with distilled water to 160, 200, 240, 280, and 320 g/L of sugar and optionally supplemented with 9 g/L of yeast extract [13] before use as an ethanol production (EP) medium.

2.3. Ethanol tolerance

S. cerevisiae NP01 or *S. cerevisiae* ATCC 4132 was inoculated into 50 mL of SSJ containing 100 g/L of sugar to attain an initial cell concentration of ~5 × 10^7 cells/mL. Then ethanol was added to the cultures at 0, 6, 9, 12, 15, and 18% (v/v). The setup was incubated at 30°C and 100 rpm for 24 h. The yeast viability was measured at regular time intervals. The yeast strain that showed higher ethanol tolerance was used in subsequent experiments.

2.4. Batch ethanol fermentation

EP media with and without 9 g/L of yeast extract were transferred into 500-mL air-locked Erlenmeyer flasks with a working volume of 400 mL and autoclaved at 110°C for 28 min [2]. The active cells of the more ethanol-tolerant strain were inoculated into sterile EP media to obtain an initial cell concentration of ~5 × 10^7 cells/mL. The fermentation was performed at 30°C with an agitation rate of 100 rpm. The samples were withdrawn at regular time intervals for analyses.

2.5. Fed-batch ethanol fermentation

Two feeding regimes for the fed-batch fermentation were used under VHG conditions. The first regime was stepwise feeding. Here, the fermentation was first performed in batch mode with sterile EP medium using 50% of the total working volume [8,14]. After 12 or 24 h, an equal volume of fresh sterile EP medium was carefully added into the flasks. The second regime was continuous feeding. Here, the other half of fresh EP medium was fed continuously at flow rates of 1X
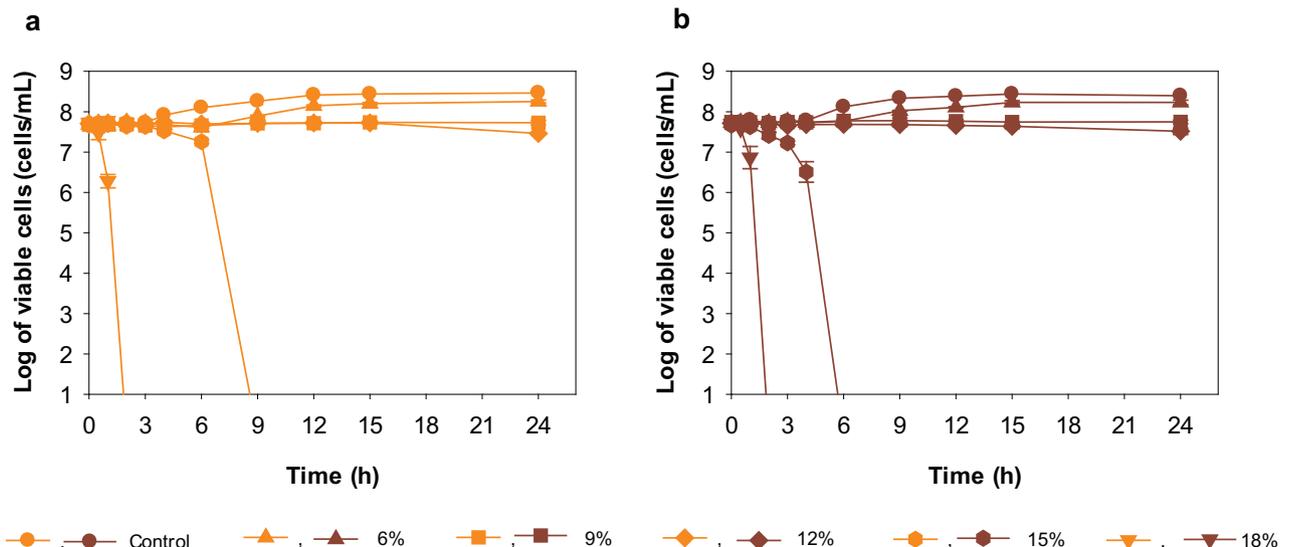

Fig. 1. Time profiles of cell survival of *S. cerevisiae* NP01 (a) and *S. cerevisiae* ATCC 4132 (b) in the presence of ethanol at different concentrations.
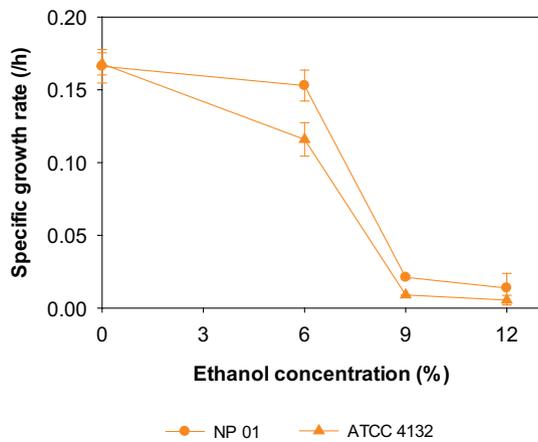
Fig. 2. Comparison of the specific growth rates of *S. cerevisiae* NP01 and *S. cerevisiae* ATCC 4132 in the presence of ethanol at different concentrations.

(10 g sugar/h), 2X (20 g sugar/h), and 4X (40 g sugar/h) to achieve final total sugar concentrations in the range of a VHG condition. During the fed-batch fermentation, samples were taken at regular time intervals for analyses.

2.6. Analytical methods

The viable yeast cell numbers were determined by a direct counting method using hemocytometer and methylene blue staining. The fermentation broth was centrifuged at 13,000 rpm for 10 min to remove solid particles. The supernatant was decanted, and its sugar content was determined using a phenol sulfuric acid method [15]. Ethanol concentration (P_E, g/L) was analyzed by gas chromatography [2]. The ethanol yield ($Y_{E/S}$) was calculated as the actual amount of ethanol produced and expressed as g ethanol per g of sugars utilized (g/g). The volumetric ethanol productivity (Q_E, g/L·h) was calculated by dividing ethanol concentration produced (P_E, g/L) by fermentation time at which the highest ethanol concentration was attained. Nitrogen in the fermentation broth was analyzed using a microwell ninhydrin assay to determine free amino nitrogen (FAN) [16]. Glycerol, the main by-product during ethanol fermentation, was quantified by HPLC according to Sirisantimethakom et al. [17].

The sugar consumption rate (g/L·h) in batch fermentations under NG, HG, and VHG conditions was calculated for use in fed-batch fermentations. It was determined from the sugars consumed during the first 24 h of incubation.
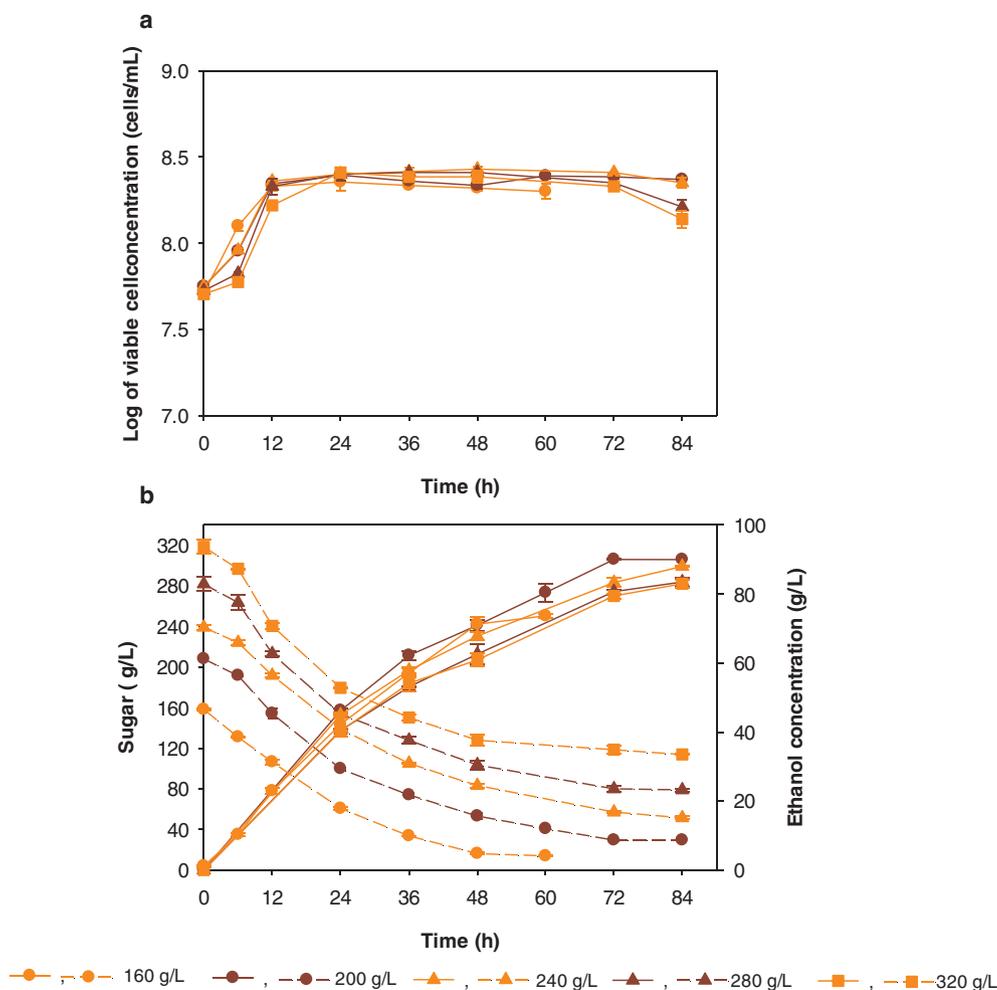

Fig. 3. Batch culture profiles of viable cells (a), sugar (b: dashed lines), and ethanol (b: solid lines) during ethanol fermentation from SSJ containing 160–320 g/L of sugar without nutrient supplementation.

Table 1
Fermentation parameters of batch ethanol production from SSJ containing 160–320 g/L of sugar with and without 9 g/L of yeast extract supplementation.

Initial sugar (g/L)	Fermentation parameter[*]						
	S_C (%)	P_E (g/L)	Q_E (g/L·h)	$Y_{E/S}$ (g/g)	t (h)	FAN$_{initial}$ (mg/L)	FAN$_{consumed}$ (%)
160	89.8 ± 0.3[g]	71.3 ± 0.3[a]	1.49 ± 0.13[d]	0.50 ± 0.04[f]	48	183.0 ± 0.3[a]	81.0 ± 0.4[h]
200	88.8 ± 0.8[f]	90.0 ± 0.1[d]	1.25 ± 0.01[c]	0.50 ± 0.00[f]	72	190.1 ± 0.2[b]	73.0 ± 0.8[g]
240	78.5 ± 0.1[c]	88.0 ± 0.2[c]	1.05 ± 0.00[b]	0.47 ± 0.01[d]	84	199.4 ± 2.5[c]	64.7 ± 1.1[f]
280	72.0 ± 0.6[b]	83.2 ± 1.3[b]	0.99 ± 0.02[a]	0.41 ± 0.01[a]	84	208.0 ± 0.6[d]	63.6 ± 0.7[e]
320	64.2 ± 1.0[a]	83.0 ± 0.0[b]	0.99 ± 0.00[a]	0.42 ± 0.00[a]	84	220.8 ± 0.6[e]	65.3 ± 0.4[f]
160 + YE	91.1 ± 0.2[h]	70.9 ± 0.8[a]	1.97 ± 0.10[f]	0.48 ± 0.03[e]	36	516.6 ± 0.9[f]	59.0 ± 0.6[d]
200 + YE	93.2 ± 0.8[i]	93.8 ± 1.2[e]	1.95 ± 0.02[f]	0.45 ± 0.03[c]	48	529.0 ± 0.3[g]	55.5 ± 1.1[c]
240 + YE	93.0 ± 0.4[i]	102.2 ± 0.9[f]	2.13 ± 0.04[g]	0.45 ± 0.00[c]	48	538.3 ± 2.2[h]	54.4 ± 0.7[b]
280 + YE	86.9 ± 0.2[e]	112.5 ± 0.7[g]	1.56 ± 0.01[e]	0.46 ± 0.00[c,d]	72	544.2 ± 2.5[i]	54.0 ± 1.3[a]
320 + YE	81.6 ± 0.4[d]	112.0 ± 0.1[g]	1.56 ± 0.00[e]	0.44 ± 0.00[b]	72	560.3 ± 1.6[j]	53.4 ± 1.6[a,b]

The experiments were performed in triplicate and the results were expressed as mean ± SD.
a, b, c, d, e, f, g, h, i and j: values with same letter within the same column are not significantly different using Duncan's multiple range test at 0.05 level of significance.

[*] S_C = sugar consumption, P_E = ethanol concentration, Q_E = ethanol productivity, $Y_{E/S}$ = ethanol yield, FAN$_{initial}$ = initial FAN concentration, FAN$_{consumed}$ = FAN consumption, t = fermentation time and YE = 9 g/L of yeast extract.

3. Results and discussion

3.1. Ethanol tolerance

When the NP01 and ATCC 4132 strains were subjected to ethanol at the same concentrations, cell survival of both strains was similar (Fig. 1). The yeast could grow in SSJ containing 100 g/L of sugar in the presence of up to 6% ethanol. However, the growth at 6% ethanol was lower than that in the absence of ethanol. The highest viable cell concentration with no ethanol addition was 2.5 to 2.9 × 10⁸ cells/mL, whereas it was 1.7 to 1.8 × 10⁸ cells/mL in the presence of 6% ethanol at 24 h. No growth was observed at 9% and 12% ethanol for both NP01 and ATCC 4132 after 24 h. The viable cell counts of both NP01 and ATCC 4132 under these two conditions were relatively constant
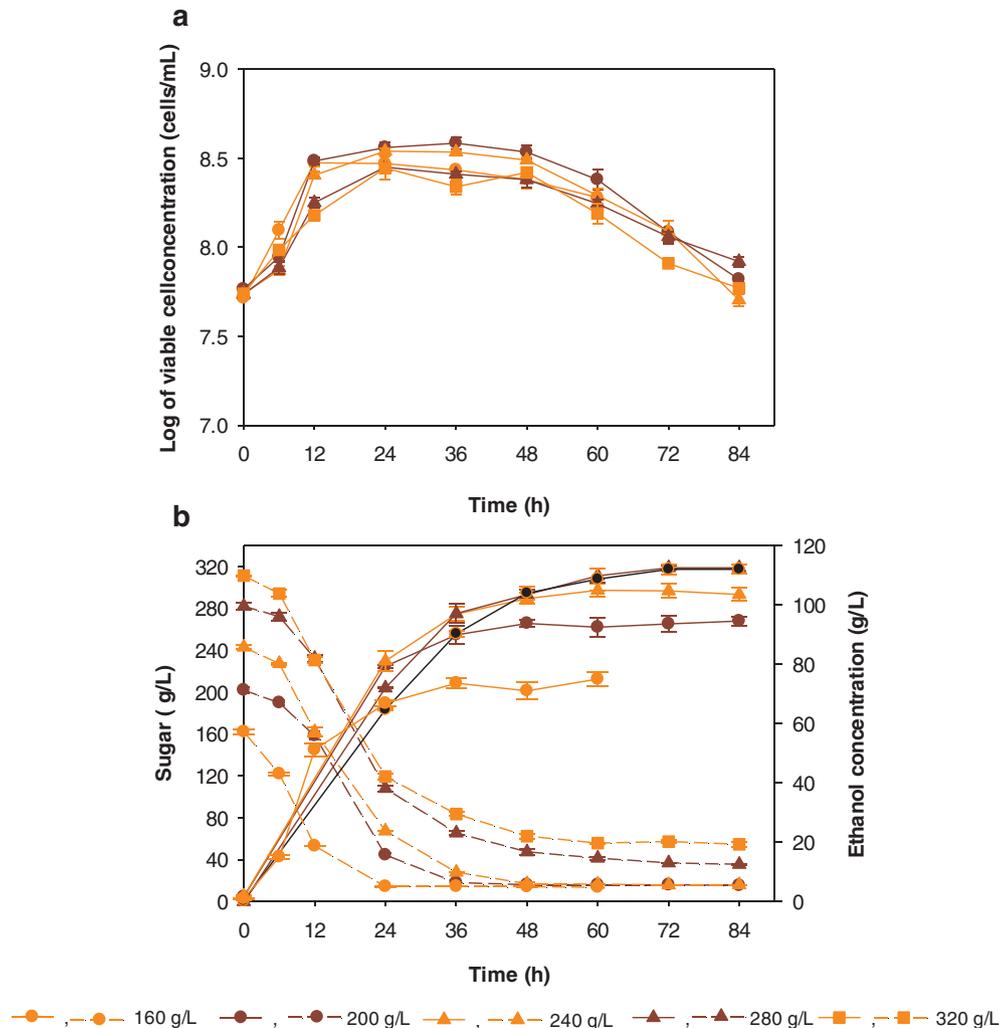
Fig. 4. Batch culture profiles of viable cells (a), sugar (b: dashed lines), and ethanol (b: solid lines) during ethanol fermentation from SSJ containing 160–320 g/L of sugar and 9 g/L of yeast extract.
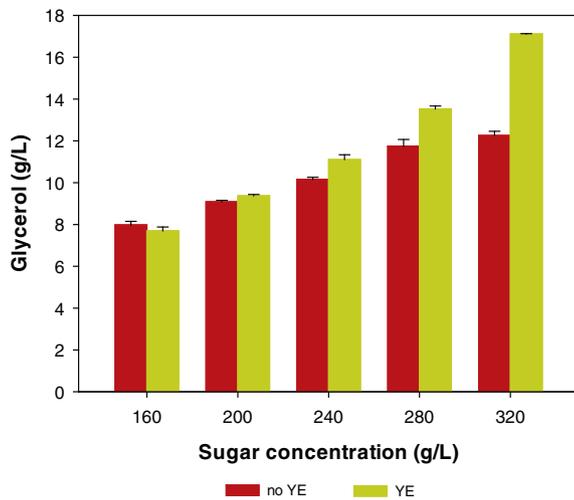
Fig. 5. Glycerol production in batch ethanol fermentation from SSJ containing 160–320 g/L of sugar with and without yeast extract (YE) supplementation.

during the first 24 h. It seemed that NP01 showed better ethanol tolerance at 15% ethanol. It could survive for 6 h with ~36% survival rate, whereas ATCC 4132 could survive for only 4 h at this ethanol concentration, with only ~8% survival rate. However, neither strain could survive after 30 min of exposure to 18% ethanol.

The effects of ethanol concentration on the specific growth rates (μ) of *S. cerevisiae* NP01 and ATCC 4132 are shown in Fig. 2. With no ethanol, the μ of NP01 and ATCC 4132 were similar (0.166–0.168/h). At 6% of ethanol concentration, the μ of NP01 and ATCC 4132 were lower (0.153 and 0.116/h, respectively). When the ethanol concentrations were further increased, μ decreased sharply. The inhibition of yeast growth at 9–12% of ethanol was almost complete. Similar results were observed by Zhang et al. [18], who found that the end product (ethanol) was shown to be the primary factor inhibiting yeast growth and fermentation activity because the yeast completely stopped growing and fermenting when the exogenous ethanol concentration exceeded 70 g/L (~9%, v/v).

Ethanol tolerance of yeast depends on not only the yeast strain used but also the composition of the growth medium. In the current study, higher ethanol tolerance of the two yeast strains may be obtained if they were cultured in an enriched medium. This was supported by Kumar et al. [19], who reported that *S. cerevisiae* could tolerate up to 15% ethanol for 48 h in a yeast extract–peptone–glucose medium. In this experiment, SSJ containing 100 g/L of sugar was used to mimic real conditions during ethanol fermentation from SSJ. According to the current experiment, NP01 could grow and tolerate ethanol better than ATCC 4132. Therefore, NP01 was selected for use in the subsequent experiments.

3.2. Batch ethanol fermentation

The changes of viable yeast cell count, sugar and ethanol concentrations during batch fermentation from the EP media without nutrient supplementations under NG, HG, and VHG conditions are shown in Fig. 3. The viable cell concentration increased during the first 12 h and remained constant in the experiments with initial sugar concentrations of 160–240 g/L. At higher initial sugar concentrations (280–320 g/L), the viable cell counts decreased after 72 h, which might have been due to osmotic and ethanol stress [4]. The residual sugar increased with increasing initial sugar concentration. The sugar consumption (S_C) was about 90% when the initial sugar concentrations were 160 and 200 g/L (Table 1). The sugar consumption and ethanol productivity (Q_E) decreased with increasing initial sugar concentration, indicating that high substrate concentration might lower the yeast fermentation capacity. The highest ethanol concentration was obtained with an initial sugar of 200 g/L. However, the sugar was not completely consumed at all concentrations, implying that essential nutrients might be insufficient (Table 1). Therefore, yeast extract was used to supplement the EP media and thereby improve sugar consumption and ethanol production.

When SSJ was supplemented with 9 g/L of yeast extract (Fig. 4), the viable cell counts at all conditions increased during the first 24 h, except with 160 g/L of initial sugar. These values dramatically decreased after 48 h. It was found that fermentation of SSJ with nutrient supplementation gave higher viable cell count and ethanol concentration. This suggested that yeast extract could promote cell growth, which in turn resulted in enhanced ethanol production. However, the viable cell counts under nutrient supplementation decreased more severely during the later stage of the fermentation compared to those with no supplementation, which might have been due to ethanol toxicity to the yeast cells (Fig. 3a and Fig. 4a).
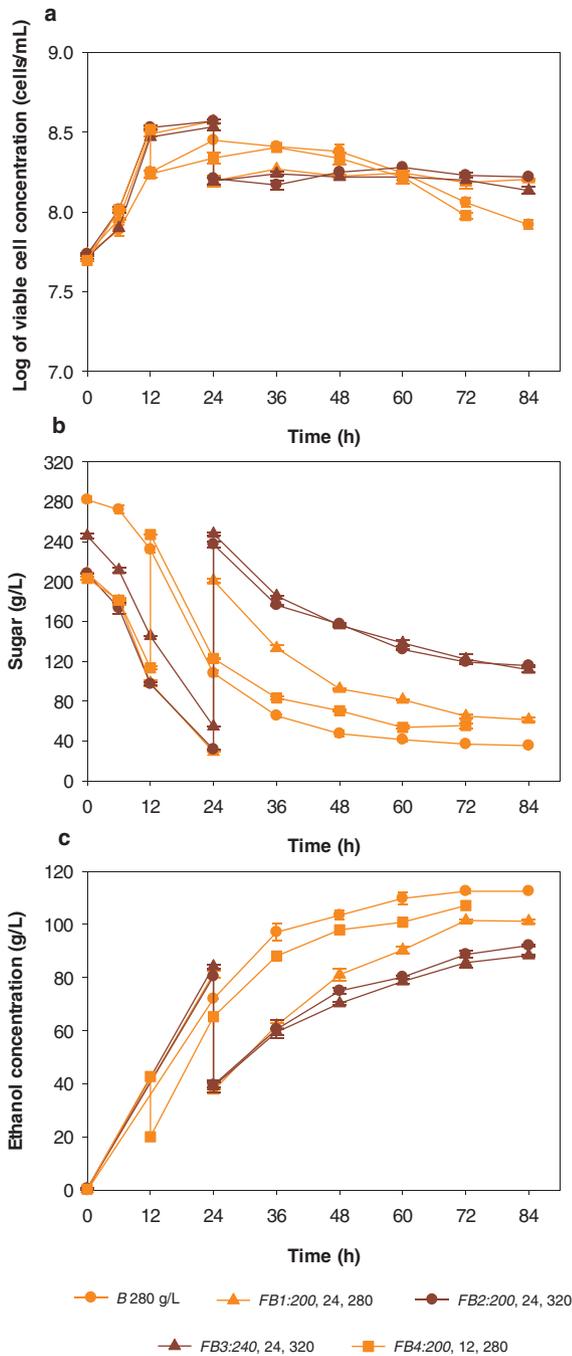
FAN was used in this study to monitor the utilization of nitrogen during the fermentation process. FAN is a collective term that refers to individual amino acids and small peptides of up to 3 units, which have been found essential for yeast growth [20]. Adequate provision of FAN resulted in higher rates of sugar uptake and consequently higher ethanol concentrations [21,22]. The availability and consumption of FAN in this study are given in Table 1. The initial FAN concentrations in the media were slightly different because of the varying amounts of concentrated SSJ juice used to prepare the EP media (data not shown). In the media without yeast extract supplementation, the initial values ranged from 183.0 to 220.8 g/L. The ability of the yeast to consume FAN was found to decrease with increasing initial sugar concentration from 81.0 to 64.7%, when the initial sugar concentration was increased from 160 to 240 g/L. Comparing with the sugar consumption (S_C, %), a correlation between S_C and FAN consumption was observed. However, this correlation was not observed under the HG and VHG conditions with 240–320 g/L of initial sugar. Even so, the percentage of S_C decreased with increasing initial sugar concentration. FAN utilization was similar, ranging from 63.6 to 65.3%. When the juices were supplemented with 9 g/L of yeast extract, the initial FAN concentrations were in the range of 516.6–560.3 mg/L (9 g/L yeast extract contained 334–339 mg/L FAN). The utilization of FAN in the

Table 2
Four regimes used in fed-batch fermentations by stepwise feeding with an initial working volume of 50%.

Regime*	Initial sugar concentration (g/L)	Feeding time (h)	Sugar concentration in feeding medium (g/L)	Sugar concentration in the broth after feeding (g/L)	Summation of sugar concentration (g/L)
1 (*FB1:200*, 24, 280)	200	24	356	200	280
2 (*FB2:200*, 24, 320)	200	24	434	240	320
3 (*FB3:240*, 24, 320)	240	24	413	240	320
4 (*FB4:200*, 12, 280)	200	12	356	240	280

* *FB1:200*, 24, 280 = fed-batch fermentation: initial sugar, 200 g/L; feeding time, 24 h; all sugar, 280 g/L, *FB2:200*, 24, 320 = fed-batch fermentation: initial sugar, 200 g/L; feeding time, 24 h; all sugar, 320 g/L, *FB3:240*, 24, 320 = fed-batch fermentation: initial sugar, 240 g/L; feeding time, 24 h; all sugar, 320 g/L, and *FB4:200*, 12, 280 = fed-batch fermentation: initial sugar, 200 g/L; feeding time, 12 h; all sugar, 280 g/L.

Fig. 6. Profiles of viable cell counts (a), sugar (b), and ethanol (c) under fed-batch fermentation by stepwise feeding of SSJ (280 and 320 g/L of all sugar) at feeding times of 24 and 12 h; B = batch system and FB = fed-batch system.

supplemented media was approximately double that in the media without yeast extract. It was found to slightly decrease from 59.0 to 53.4% when the concentration of the initial sugar was increased from 160 to 320 g/L. The presence of yeast extract, *i.e.* FAN, in the media resulted in higher sugar consumption by up to 17.4% with the same initial sugar concentration (Table 1). This was considered the main reason for the enhanced yeast growth and ethanol production during a shorter fermentation time.

Table 1 summarizes the important fermentation parameters in ethanol production from SSJ with and without yeast extract supplementation. With yeast extract supplementation, the S_C values were higher, particularly at higher initial sugar concentrations, than those with no nutrient supplementation (Table 1). At initial sugar concentrations of 200–240 g/L with yeast extract supplementation, the S_C increased to 93%, indicating that yeast extract may help alleviate osmotic stress due to a high sugar concentration resulting in higher Q_E. However, substrate inhibition still markedly occurred at initial sugar concentrations of 280–320 g/L resulting in only 82–87% S_C. With yeast extract supplementation, the S_C, P_E and Q_E values markedly increased at all initial sugar concentrations. The highest ethanol production efficiency was obtained at an initial sugar concentration of 280 g/L. The P_E, Q_E, and $Y_{E/S}$ values were 112.5 g/L, 1.56 g/L·h, and 0.46 g/g, respectively, at 72 h. At an initial sugar concentration of 240 g/L or lower, yeast extract markedly promoted both P_E and Q_E, whereas at higher initial sugar concentrations (280–320 g/L), nutrient supplement promoted P_E but the rate of ethanol production or Q_E was reduced. This might have been due to substrate inhibition under VHG conditions.

In the process of ethanol fermentation by *S. cerevisiae*, the main by-product is glycerol. It is a metabolite that regulates osmotic pressure produced by high concentration of sugar and ethanol in the fermentation process [23,24]. Fig. 5 shows glycerol production from the EP media with and without yeast extract. The glycerol concentration increased with increasing sugar concentration. At 160 and 200 g/L of sugar, glycerol production levels were similar regardless of the presence of yeast extract, indicating that the stresses under both conditions were similar. At higher initial sugar concentrations, glycerol concentrations under yeast extract supplementation were significantly higher than those without nutrient supplementation. This might have been due to high osmotic stress coupled with ethanol stress on yeast cells at high sugar concentrations. The highest glycerol concentration (P_G, 17.1 g/L) was detected in the broth containing the highest initial sugar and ethanol concentrations (SSJ containing 320 g/L of sugar and 9 g/L of yeast extract).

From the batch ethanol fermentation, SSJ containing 280 g/L of sugar and 9 g/L yeast extract gave relatively high P_E (112.5 g/L). However, the residual sugar was ~37 g/L (~86.9% S_C) with a Q_E of only 1.56 g/L·h. Therefore, to improve sugar consumption and ethanol production efficiency, the fed-batch fermentation was further investigated.

Table 3
Fermentation parameters of fed-batch ethanol fermentation using a stepwise feeding from SSJ under VHG conditions (280 and 320 g/L of all sugar) at feeding times of 24 and 12 h.

Regime	Fermentation parameter***					
	S_C (%)	P_E (g/L)	Q_E (g/L·h)	$Y_{E/S}$ (g/g)	P_G (g/L)	t (h)
B280*	86.9 ± 0.2[d]	112.5 ± 0.7[e]	1.56 ± 0.01[c]	0.46 ± 0.00[c]	13.9 ± 0.0[e]	72
FB1:200, 24, 280**	77.0 ± 0.7[b]	101.5 ± 0.0[c]	1.41 ± 0.00[c]	0.47 ± 0.00[c]	8.9 ± 0.2[b]	72
FB2:200, 24,320**	62.8 ± 0.5[a]	88.7 ± 0.0[b]	1.23 ± 0.00[b]	0.44 ± 0.00[b]	8.8 ± 0.1[a]	72
FB3:240, 24, 320**	62.7 ± 1.8[a]	85.6 ± 1.9[a]	1.19 ± 0.03[a]	0.42 ± 0.00[a]	9.6 ± 0.0[c]	72
FB4:200, 12, 280**	80.7 ± 0.9[c]	107.1 ± 0.0[d]	1.49 ± 0.03[d]	0.46 ± 0.02[c]	11.4 ± 0.0[d]	72

The experiments were performed in triplicate and the results were expressed as mean ± SD.
[a, b, c, d,] and [e]: means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at the level of 0.05.
  * $B280$ = batch fermentation at 280 g/L of sugar with 9 g/L of yeast extract supplementation.
 ** See Table 2.
*** S_C = sugar consumption, P_E = ethanol concentration, Q_E = ethanol productivity, $Y_{E/S}$ = ethanol yield, P_G = glycerol concentration and t = fermentation time.

3.3. Sugar consumption rate under NG and HG conditions

In fed-batch fermentations, the initial sugar concentration at the level of NG or HG condition was used to prevent substrate inhibition. Feeding of the substrate was initiated when most of the substrates had been consumed and the yeast growth was still in the exponential phase [25]. Before studying fed-batch fermentation, the sugar consumption rates under NG and HG conditions were calculated. The sugar concentration in SSJ containing 160–240 g/L of initial sugar and 9 g/L yeast extract (NG and HG conditions) decreased sharply during the first 24 h (Fig. 4b). The sugar consumption rate during 24 h of batch fermentations with an initial sugar concentration of 160 g/L was the lowest (6.16 g/L·h), whereas these values with 200 and 240 g/L of initial sugar were similar at 7.22 and 7.32 g/L·h, respectively. Therefore, initial sugar concentrations of 200 and 240 g/L were used in the fed-batch fermentations.

3.4. Fed-batch ethanol fermentation

In this research, two feeding regimes were studied:

3.4.1. Stepwise feeding

SSJ media containing 200 and 240 g/L of sugar and 9 g/L of yeast extract were used as EP media in fed-batch fermentations, employing 50% of the initial working volume [8]. According to Fig. 4b, the remaining 50% of the medium was fed at 12 and 24 h during which time the yeast cells were still active. Four regimes were conducted, and the overall sugar concentrations in the EP media were in VHG conditions at 280 and 320 g/L as shown in Table 2.

The viable cell counts continued to increase until fresh medium was fed to the flask at either 12 or 24 h (Fig. 6a). The cell concentration decreased after feeding fresh medium and then slightly increased. However, the maximum cell number after the feeding did not reach the maximum values that were obtained before feeding. The viable cell counts were relatively constant, except in Regime 4 (*FB4*). At 48 h, the viable cell count at feeding time at 12 h was higher than that at 24 h. In comparison to the control (batch system), the viable cell count of the fed-batch system at feeding time of 12 h and the control were similar until 72 h.

Changes in sugar and ethanol concentrations in the EP media under various fed-batch fermentations were different (Fig. 6b and c). The sugar and feeding time affected the P_E, Q_E, and $Y_{E/S}$ (Table 3). At a feeding time of 24 h, the S_C in *FB1* was higher than that in *FB2* and *FB3*, resulting in a higher P_E. At feeding time of 12 h (*FB4*), the S_C and P_E were higher than those at feeding time of 24 h. In *FB1* and *FB4* (overall sugar concentration of 280 g/L), the feeding time at 12 h (*FB4*) gave higher values of ethanol production, with the P_E and Q_E of 107.1 g/L and 1.49 g/L·h, respectively (Table 3).

However, the S_C and P_E of *FB4* were lower than those of the control (batch system) (Table 3). Glycerol concentrations at a feeding time of 24 h (8.8 to 9.6 g/L) were lower than that at a feeding time of 12 h (11.4 g/L). This might have been due to lower ethanol concentrations at feeding time of 24 h. Glycerol concentrations under all fed-batch conditions were lower than those under batch fermentation (13.9 g/L) (Table 3). This, again, might have been due to the lower stresses of high sugar and ethanol concentrations [26].

The results showed that the fed-batch fermentation with 1:1 stepwise feeding at feeding times of 12 and 24 h could not improve ethanol production efficiency from SSJ compared to that in the batch fermentation. To improve fed-batch ethanol production, continuous feeding was studied at a feeding time of 12 h.

3.4.2. Continuous feeding

According to the stepwise feeding fed-batch fermentation, *FB4* (initial sugar, 200 g/L; feeding time, 12 h; overall sugar concentration, 280 g/L) gave the highest S_C, P_E, and Q_E values (Table 3). Therefore, the conditions used in *FB4* were applied in continuous feeding.

The fed-batch fermentation by continuous feeding was performed in a 2-L fermenter. It was started by filling 50% of working volume of the fermenter with SSJ containing 200 g/L of initial sugar and 9 g/L of yeast extract. As discussed in Section 3.3, the sugar consumption rate at the initial sugar of 200 g/L was 7.22 g/L·h. Therefore in the fed-batch fermentation after 12 h, fresh medium (360 g/L of sugar) was fed continuously at 1X (27 mL/h, 10 g sugar/h) and 2X (54 mL/h, 20 g sugar/h). The results showed that the viable cell counts under these regimes were higher than those of the control during the first
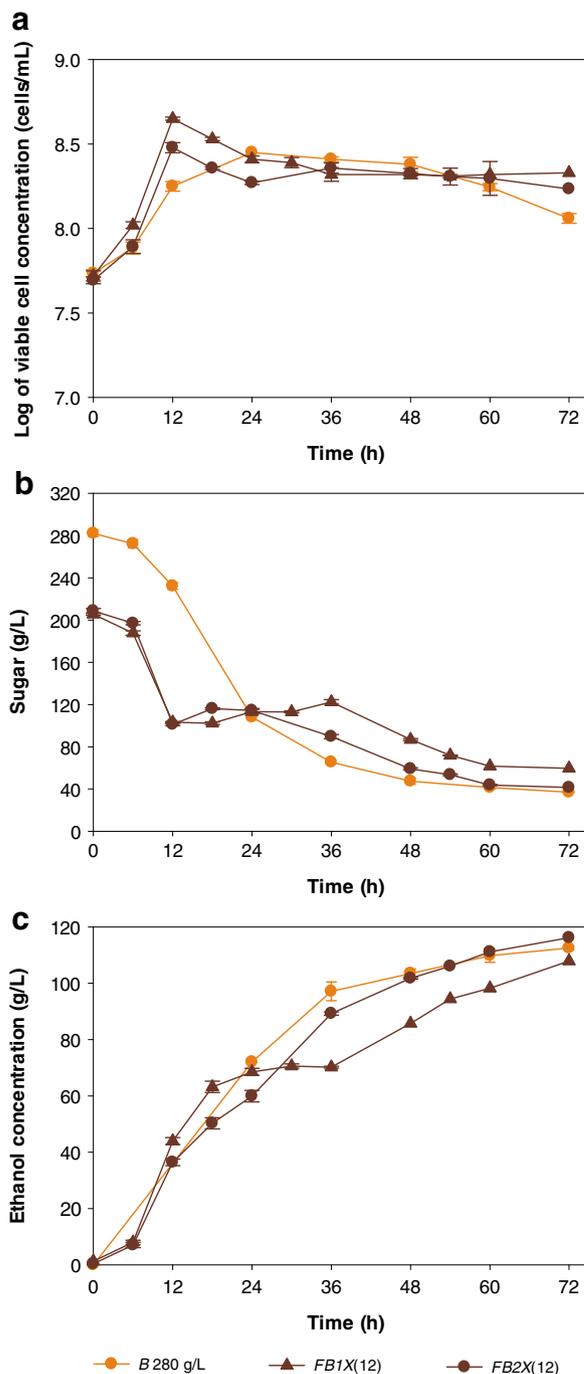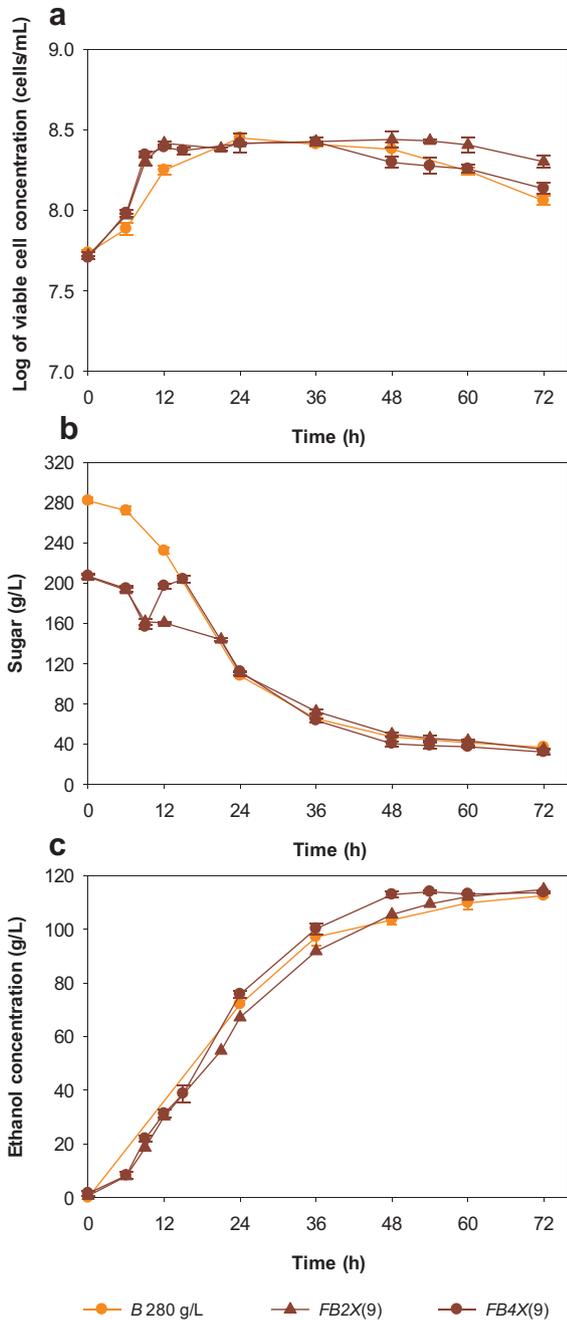

Fig. 7. Profiles of viable cell counts (a), sugar (b), and ethanol (c) under fed-batch fermentation by continuous feeding of SSJ (280 g/L of all sugar) at a feeding time of 12 h and feeding rate of 1X (10 g sugar/h) and 2X (20 g sugar/h); B = batch system and FB = fed-batch system.

Fig. 8. Profiles of viable cell counts (a), sugar (b), and ethanol (c) under fed-batch fermentation by continuous feeding of SSJ (280 g/L of all sugar) at a feeding time of 9 h and feeding rate of 2X (20 g sugar/h) and 4X (40 g sugar/h); B = batch system and FB = fed-batch system.

12 h, which might have been due to lower osmotic stress. However, after 24, the viable cell counts under all conditions were similar (Fig. 7a). After 24 h, the S_C and P_E of the fermentation at feeding time of 12 h and the feeding rate 2X [$FB2X(12)$] were higher than those of 1X [$FB1X(12)$] (Fig. 7b and c). However, these values at feeding time of 12 h were similar to the batch control. Therefore, the feeding was started earlier, at 9 h, and the feeding rates of 2X and 4X (108 mL/h, 40 g sugar/h) were further investigated to improve ethanol production (Fig. 8). The results showed that at a feeding time of 9 h, the feeding rate of 4X gave better sugar consumption and ethanol production rate than 2X (Table 4).

In the fed-batch fermentation with continuous feeding, feeding time and feeding rate affected P_E and Q_E (Table 4). The best conditions for ethanol production were to start feeding at 9 h at a rate of 40 g sugar/h. Under these conditions, the P_E, Q_E, and $Y_{E/S}$ values were 112.9 g/L, 2.35 g/L·h, and 0.47 g/g, respectively, at 48 h. Comparison of ethanol production between batch and fed-batch fermentations revealed that the P_E and $Y_{E/S}$ values in the fed-batch fermentation at 9 h and feeding rate of 40 g sugar/h were not different from those of the batch system, but the Q_E of the latter was higher because the fermentation time was shortened from 72 to 48 h. Moreover, the glycerol concentration decreased from 13.9 to 12.3 g/L compared to that in the batch control (Table 4), indicating that stresses under the fed-batch fermentation were less.

In the fed-batch process, the fermentation was initiated with a sugar concentration in the range of HG conditions (initial sugar concentration of 200 and 240 g/L). Then, the feed medium containing high sugar concentration was fed to attain overall sugar concentrations in the range of VHG conditions. Therefore, this process can avoid substrate inhibition of cell growth. In the current study, the fed-batch fermentation with continuous feeding improved ethanol productivity by ~51%. To further improve sugar consumption and ethanol production efficiency, aeration may be supplied [27] and/or some essential trace elements or osmoprotectant could be added to the EP medium [3,12]. Moreover, increasing the initial cell concentration may also improve ethanol productivity [28].

4. Conclusions

S. cerevisiae NP01 and ATCC 4132 could tolerate up to 12% (v/v) ethanol without loss of cell viability. At 15% ethanol, NP01 showed higher ethanol tolerance than ATCC 4132. In batch ethanol fermentations from SSJ, yeast extract supplementation promoted yeast growth, leading to an increase in ethanol production and reduced fermentation time, especially under HG and VHG fermentations. In fed-batch fermentations with continuous feeding, apart from nitrogen supplementation, feeding time and feeding rate were the key parameters to improve ethanol production efficiency under VHG conditions. In this study, continuous feeding starting at 9 h with a feeding rate of 40 g sugar/h gave the highest ethanol production efficiency.

Table 4
Fermentation parameters of fed-batch ethanol fermentation under a VHG condition (280 g/L of all sugar) with continuous feeding (starting at 9 and 12 h at different feeding rates).

Condition*	Fermentation parameter**					
	S_C (%)	P_E (g/L)	Q_E (g/L·h)	$Y_{E/S}$ (g/g)	P_G (g/L)	t (h)
B280	86.9 ± 0.2[e]	112.5 ± 0.7[d]	1.56 ± 0.01[a]	0.46 ± 0.00[b]	13.9 ± 0.0[e]	72
FB1X(12)	77.8 ± 0.8[a]	98.2 ± 1.1[a]	1.64 ± 0.01[b]	0.45 ± 0.00[a]	10.9 ± 0.3[b]	60
FB2X(12)	81.0 ± 1.9[b]	111.1 ± 1.3[b]	1.85 ± 0.00[c]	0.47 ± 0.00[c]	8.9 ± 0.1[a]	60
FB2X(9)	84.7 ± 1.4[c]	112.1 ± 0.7[c]	1.87 ± 0.01[d]	0.47 ± 0.00[c]	11.8 ± 0.2[c]	60
FB4X(9)	85.6 ± 1.2[d]	112.9 ± 0.1[e]	2.35 ± 0.00[e]	0.47 ± 0.01[c]	12.3 ± 0.4[d]	48

a, b, c, d, and e: values with the same letter within the same column are not significantly different using Duncan's multiple range test at 0.05 level of significance.
* B280 = batch fermentation at 280 g/L of sugar with 9 g/L of yeast extract supplementation, FB1X(12) = fed-batch fermentation at feeding time of 12 h and feeding rate of 10 g sugar/h, FB2X(12) = fed-batch fermentation at feeding time of 12 h and feeding rate of 20 g sugar/h, FB2X(9) = fed-batch fermentation at feeding time of 9 h and feeding rate of 20 g sugar/h, and FB4X(9) = fed-batch fermentation at feeding time of 9 h and feeding rate of 40 g sugar/h.
** See Table 3.

Conflict of interest

The authors declare no conflict of interest.

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