Electronic Journal of Biotechnology 46 (2020) 55–64

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research Article

Enhancement of ethanol production efficiency in repeated-batch fermentation from sweet sorghum stem juice: Effect of initial sugar, nitrogen and aeration

Benjaporn Sriputorn [a], Pattana Laopaiboon [b,c], Niphaphat Phukoetphim [b], Nilubol Polsokchuak [b], Khanittha Butkun [b], Lakkana Laopaiboon [b,d,*]

[a] Graduate School, Khon Kaen University, Khon Kaen 40002, Thailand
[b] Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand
[c] Center for Alternative Energy Research and Development, Khon Kaen University, Khon Kaen 40002, Thailand
[d] Fermentation Research Center for Value-Added Agricultural Products, Khon Kaen University, Khon Kaen 40002, Thailand

ARTICLE INFO

Article history:
Received 15 November 2019
Accepted 3 June 2020
Available online 8 June 2020

Keywords:
Aeration
Bio ethanol
Ethanol concentration
Ethanol fermentation
Ethanol productivity
High gravity fermentation
Normal gravity fermentation
Repeated-batch fermentation
Saccharomyces cerevisiae
Sugar consumption
Sweet sorghum
Yeast growth

ABSTRACT

Background: Ethanol concentration (P_E), ethanol productivity (Q_P) and sugar consumption (*SC*) are important values in industrial ethanol production. In this study, initial sugar and nitrogen (urea) concentrations in sweet sorghum stem juice (SSJ) were optimized for high P_E (≥10%, *v*/*v*), Q_P, (≥2.5 g/L·h) and *SC* (≥90%) by *Saccharomyces cerevisiae* SSJKKU01. Then, repeated-batch fermentations under normal gravity (NG) and high gravity (HG) conditions were studied.

Results: The initial sugar at 208 g/L and urea at 2.75 g/L were the optimum values to meet the criteria. At the initial yeast cell concentration of ~1×10^8 cells/mL, the P_E, Q_P and *SC* were 97.06 g/L, 3.24 g/L·h and 95.43%, respectively. Repeated-batch fermentations showed that the ethanol production efficiency of eight successive cycles with and without aeration were not significantly different when the initial sugar of cycles 2 to 8 was under NG conditions (~140 g/L). Positive effects of aeration were observed when the initial sugar from cycle 2 was under HG conditions (180–200 g/L). The P_E and Q_P under no aeration were consecutively lower from cycle 1 to cycle 6. Additionally, aeration affected ergosterol formation in yeast cell membrane at high ethanol concentrations, whereas trehalose content under all conditions was not different.

Conclusion: Initial sugar, sufficient nitrogen and appropriated aeration are necessary for promoting yeast growth and ethanol fermentation. The SSJ was successfully used as an ethanol production medium for a high level of ethanol production. Aeration was not essential for repeated-batch fermentation under NG conditions, but it was beneficial under HG conditions.

How to cite: Sriputorn B, Laopaiboon P, Phukoetphim N, et al. Enhancement of ethanol production efficiency in repeated-batch fermentation from sweet sorghum stem juice: Effect of initial sugar, nitrogen and aeration. Electron J Biotechnol 2020;46. https://doi.org/10.1016/j.ejbt.2020.06.001

1. Introduction

Bioethanol is of interest as an alternative fuel to mitigate the increasing demand for fossil fuels and to reduce greenhouse gas emissions. Ethanol can be produced from biomass-based fermentation, which is less toxic and easy to integrate with transport fuel, i.e., E10, E20 or E85 in Thailand. Yeast is commonly selected for ethanol fermentation of several agricultural feedstocks, i.e., sugar, starch and lignocellulosic materials [1,2,3].

Sweet sorghum, a C4 crop, is a promising bioenergy crop because it can be used as feedstock for ethanol fermentation from soluble sugar-based (stalk), starch-based (grain) and lignocellosic (bagasse) materials [4,5]. In this study, we are interested in the juice from its stalks because it consists of directly fermentable sugars and many trace elements essential for microbial growth [6]. In China, a pilot plant for ethanol production from sweet sorghum stem juice (SSJ) has been developed because it is a potential substitute for conventional bioethanol plant [7].

* Corresponding author.
E-mail address: lakcha@kku.ac.th (L. Laopaiboon).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

Additionally, it was reported that SSJ was mixed with sugarcane juice to increase the sugar concentration in a medium for butanol production [8].

Batch fermentation is traditionally used since it is simple to do as the fermentation medium, and the microorganisms (yeast) are added at the beginning of the process. However, it requires many upstream processes, and the yeasts are affected by product inhibition, slowing their growth [9]. In repeated-batch fermentation, the medium is withdrawn at specified time intervals and a residual part of the medium is used as an inoculum for the next cycle. This process offers many benefits over batch fermentation, not only the reuse of microbial cells for subsequent batches, but also less time is required for the operation and increased productivity [6,10,11].

It is established that the yeast, *Saccharomyces cerevisiae,* is highly capable of producing ethanol under anaerobic conditions, converting 1 g of glucose to 0.511 g of ethanol with the release of carbon dioxide. Therefore, a high concentration of a carbon source like sugar in the ethanol production medium will raise the ethanol concentration as well. Apart from a carbon source, a nitrogen source is necessary for promoting yeast growth with increased ethanol tolerance and ethanol production efficiency [6,12]. Additionally, the initial cell concentration in ethanol production medium was also reported as one of the main factors affecting ethanol production efficiency [13,14,15,16]. Typically, the initial sugar concentrations used in ethanol fermentation are at one of three levels. Normally in the ethanol industry, a fermentation medium has an initial sugar concentration that is less than 180 g/L. This range is called the "normal gravity" or NG condition. High gravity or HG condition means the fermentation used initial sugar concentrations ranging from ~180 to 240 g/L. The "very high gravity" or VHG condition contains initial sugar concentrations of at least 250 g/L [17,18,19]. High initial sugar concentrations can cause greater osmotic pressure, resulting in a stuck fermentation. However, under suitable environmental and nutritional conditions, yeast can produce and tolerate high ethanol concentrations under high initial sugar concentrations [6,20].

The oxygen level is one of the main factors affecting yeast growth. It is required for yeast propagation to generate the components of yeast cells and make them survive during the ethanol fermentation [21]. Ergosterol is an important component in yeast plasma membranes because it plays a critical role in ethanol tolerance in terms of membrane fluidity [22]. Apart from plasma membrane composition, a storage carbohydrate in the cytosol, trehalose, is an important component to protect cells from inactivation and denaturation by stress conditions, such as high levels of temperature, initial sugar or ethanol [23]. Therefore, monitoring changes of oxygen at low levels is necessary during the fermentation. A typical instrument for oxygen measurement, the dissolved oxygen (DO) electrode, may not have sufficient sensitivity to follow the changes of oxygen levels during fermentation under facultative to nearly anaerobic conditions [24]. Oxidation–reduction potential, or ORP, is a real-time process parameter that can be conveniently monitored at very low levels of oxygen in the fermentation medium [25,26]. Normally, a momentary redox neutral process occurs when converting glucose to ethanol by yeast. NADH is produced from pre-pyruvate metabolism and serves as the electron donor. Oxygen serves as electron acceptor resulting from the oxidation–reduction reaction. They are the primary compounds that change the redox potential of intracellular metabolism during ethanol fermentation [27,28].

Our previous work achieved two sets of optimum conditions. The first optimized high ethanol concentration (P_E) and the second maximized ethanol productivity (Q_P) [16]. However, in industrial ethanol production, effective sugar utilization must also be considered in addition to attaining high P_E or Q_P values. Therefore, our previous work could not be entirely applied in industrial practice. The goal of the current study was to improve ethanol production efficiency from sweet sorghum stem juice (SSJ) by *S. cerevisiae* SSJKKU01 while optimizing all three of these parameters. The concentrations of the initial sugar and nitrogen supplement (urea) were first optimized for high ethanol concentration, ethanol productivity and sugar consumption. These values are important in industrial ethanol production [6,18,29]. Then the effects of initial cell concentration and ORP control during batch ethanol fermentation were studied. Finally, repeated-batch fermentations under NG and HG conditions with and without aeration were performed. The effects of aeration on yeast cell components (trehalose and ergosterol) in repeated-batch fermentations were also investigated.

2. Materials and methods

2.1. Raw material and ethanol production medium preparation

Sweet sorghum stalks (*cv*. KKU40) obtained from the Faculty of Agriculture, Khon Kaen University, Thailand were harvested. The juice was extracted from its stalks using sugarcane a juice extractor. The juice contained ~17°Brix of total soluble solids. It was concentrated to ~65°Brix and stored at −20°C until use.

Diluting concentrated juice with an appropriate amount of distilled water was done to make an ethanol production medium or SSJ medium. A nitrogen source, urea, was added to the SSJ medium (as indicated in Table 1) before the medium was sterilized at 110°C for 28 min [6].

2.2. Microorganism

S. cerevisiae SSJKKU01 was isolated from the juice of sweet sorghum stalks. It was added into a 250-mL Erlenmeyer flask containing 150 mL of yeast extract malt extract (YM) medium [16]. The flask was shaken at 150 rpm in an incubator at 32°C for 18 h. After that, the yeast was transferred into SSJ containing 100 g/L of sugar and incubated at 32°C. After 15 h, the yeast was used as the inoculum for ethanol fermentation.

2.3. Experiments

All fermentations were conducted at 32°C with an agitation rate of 200 rpm. Three primary experiments were carried out, and samples were periodically collected for analyses during the fermentations.

2.3.1. Optimization of initial sugar and urea concentrations in batch fermentation and verification experiments

Response surface methodology (RSM) based on central composite design (CCD) was used to evaluate three responses from two factors (the independent variables, i.e., initial sugar, X_1 and urea, X_2 concentrations) with three levels. The three responses (dependent variables) were ethanol concentration (P_E), ethanol productivity (Q_P) and sugar consumption (*SC*). Experimental design was done using Design-Expert 7 software (trial version, STAT-EASE Inc., USA), and a total of 13 experimental runs are shown in Table 1. The ethanol

Table 1
Thirteen experimental runs and responses (P_E, Q_P and *SC*) of CCD used for RSM.

Run	Factor 1: X_1 (g/L)	Factor 2: X_2 (g/L)	Response 1: P_E (g/L)	Response 2: Q_P (g/L·h)	Response 3: *SC* (%)
1	250	3.07	110.38	2.30	93.37
2	170	3.07	78.02	2.60	90.56
3	210	0.29	96.49	1.32	89.57
4	210	1.92	90.51	2.51	93.18
5	210	3.55	95.57	2.66	93.85
6	267	1.92	113.58	1.89	87.80
7	210	1.92	91.34	2.54	93.43
8	153	1.92	71.44	2.98	92.92
9	170	0.77	80.30	2.23	94.06
10	210	1.92	90.51	2.40	93.53
11	210	1.92	91.42	2.54	93.30
12	210	1.92	90.95	2.53	93.36
13	250	0.77	105.33	1.76	86.91

Factor 1 = sugar concentration (X_1), Factor 2 = urea concentration (X_2), Response 1 = ethanol concentration (P_E), Response 2 = ethanol productivity (Q_P) and Response 3 = sugar consumption (*SC*). All experiments were done in triplicate.

fermentations from the sterile SSJ medium containing various sugar and urea concentrations (Table 1) were carried out under a batch mode in 500-mL Erlenmeyer air-locked flasks, at an initial cell concentration of ~5×10^7 cells/mL. The response variables were used to correlate the level of the factors for the maximum responses ($P_E \geq 10\%$, v/v, $Q_P \geq 2.5$ g/L·h and $SC \geq 90\%$). These are acceptable values for industrial scale production.

In order to confirm the reliability of the results, verification experiments were performed under the optimum conditions of the two factors in the air-locked flask and a 2-L fermenter at an agitation rate of 200 rpm.

2.3.2. Effect of initial cell concentration and ORP control during batch fermentation

To improve the ethanol production efficiency, the initial cell concentration was increased to ~1×10^8 cells/mL. A fermentation under the control condition (SSJ medium containing the initial sugar concentration at the optimum level with no urea) was also done.

For the ORP control experiment, the inoculum was transferred into 1-L of the optimized SSJ medium (from Section 2.3.1) in a 2-L fermenter. An ORP probe was used to maintain the ORP value at a set point by adjusting aeration. ORP set points were − 150, −100 mV and without ORP control [25]. When the ORP values were lower than the set point, the sterile air was supplied to the fermenter at a flow rate of 0.5 vvm through a 0.2-μm sterile membrane filter (modified from Khongsay et al. [30]).

2.3.3. Repeated-batch fermentation: effect of aeration and sugar concentration

Repeated-batch fermentations were performed under the optimum conditions (Section 2.3.1 and Section 2.3.2) in a 2-L fermenter with a 1-L working volume and agitation rate of 200 rpm. The fermentation was carried out until the residual sugar concentration in the medium was ~10% of the initial value. Then, the agitation was stopped to let the cells flocculate under a static condition for 30 min. Then 75% of the fermentation medium was withdrawn, and the same volume of the fresh sterile SSJ medium was immediately replaced using a peristaltic pump [31]. After that, agitation was started and aeration was supplied at 2.0 vvm for 4 h at the beginning of each cycle (modified from Khongsay et al. [32]). The repeated-batch fermentation without aeration served as a control treatment.

The repeated-batch fermentation was executed using two different feeding media. One medium contained 208 g/L of sugar while the other contained 240 g/L of sugar. These media are referred to as repeated-batch 1 (RB1) and repeated-batch 2 (RB2) media, respectively. Urea at the optimum value (from Section 2.3.1) was also added to both feeding media. At least eight successive cycles were performed or until the fermentation efficiency was lower than 25% of the first cycle.

2.4. Analytical methods

A haemacytometer with methylene blue staining was used to determine the viable yeast cell numbers in the fermentation medium. The pH and total soluble solids values of the fermentation medium were determined using a pH meter and hand-held refractometer, respectively [33]. To measure sugar and ethanol concentrations, a sample of the fermentation medium was centrifuged at 12,000 rpm for 10 min, and the supernatant was filtered through a 0.45-μm nylon membrane before analysis. The sugar in the fermented medium was analyzed using HPLC with a refractive index detector. The stationary and mobile phases were a carbohydrate analysis column (WAT044355, 250 mm × 4.6 mm ID) and 75% acetonitrile/25% water solution, respectively. The flow rate was controlled at 0.8 mL/min. Column temperature was controlled at 35°C (modified from Sirisantimethakom et al. [34]). The ethanol concentration was analyzed via GC with a flame ionization detector, and propanol was used as an internal standard. The stationary phase was polyethylene glycol packed column (PEG-20 M). The column and injection temperatures were controlled at 150 and 180°C, respectively. The temperature of the detector was 250°C. Nitrogen gas was used as a carrier [6]. The Q_P value and percentage of SC were calculated as follows:

$$Q_P\,(\text{g/L}\cdot\text{h}) = P_E \text{ produced (g/L)/fermentation time (h)}$$

$$SC\,(\%) = [\text{sugar utilized (g/L)/initial sugar concentration (g/L)}] \times 100$$

Glycerol, the main by-product during ethanol fermentation, was determined by HPLC with the refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 mm × 7.8 mm ID) and 5 mM H_2SO_4, respectively. The flow rate was controlled at 0.6 mL/min. The column temperature was held constant at 40°C [34].

At the end of fermentation, yeast cells were harvested by centrifugation at 8,000 rpm for 10 min at 4°C. The resulting yeast cell pellet was used to analyze the trehalose and ergosterol contents. The trehalose content in the yeast cells was measured by adding 3 mL of distilled water into 2 g (wet weight) of a yeast cell pellet before boiling. Next, a mixture was made of 1 mL of 15% (w/v) of a potassium ferrocyanide solution, 1 mL of 23% (w/v) of a zinc acetate solution and 5 mL of 5-mM sulfuric acid. Then it was added to the yeast cell pellet suspension (modified from Pereira et al. [35]). The mixture was centrifuged at 12,000 rpm for 10 min and filtered through a 0.45-μm nylon membrane. The supernatant containing trehalose was then analyzed using HPLC with refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 mm × 7.8 mm ID) and 5-Mm H_2SO_4 flowing at 0.7 mL/min, respectively. The column temperature was controlled at 60°C (modified from Liu et al. [36]). Ergosterol was extracted from 2 g of yeast cell pellet and then analyzed according to Inoue et al. [37]. The dry weight of the yeast cell pellet was also determined.

3. Results and discussion

3.1. Optimization of initial sugar and urea concentrations with verification of results

In this study, the optimum initial sugar and urea concentrations for ethanol fermentation must meet pre-determined criteria as discussed in Section 2.3.1. Table 1 shows the experimental data of the 13 runs. These values were used to predict the optimum initial sugar and urea concentrations. Based on the experimental data, second order polynomial models showing the relationship between the independent variables (initial sugar concentration and urea concentration) and dependent variables (P_E, Q_P and SC) were developed as follows:

$$P_E = 52.7844 + 0.1339X_1 - 15.0335X_2 + 0.0398X_1X_2 + 0.0004{X_1}^2 + 1.7710{X_2}^2, R^2 = 0.9974 \quad (1)$$

$$Q_P = 2.2166 - 0.0006X_1 + 0.8498X_2 + 0.0009X_1X_2 - 0.00002{X_1}^2 - 0.1924{X_2}^2, R^2 = 0.9094 \quad (2)$$

$$SC = 78.7026 + 0.2412X_1 - 8.1302X_2 + 0.0541X_1X_2 - 0.0009{X_1}^2 - 0.5879{X_2}^2, R^2 = 0.9665 \quad (3)$$

where X_1 = initial sugar concentration (g/L) and X_2 = urea concentration (g/L).

The resulting P_E, Q_P and *SC* values were significant at the $p < 0.05$ level. Equation 1, Equation 2 and Equation 3 show that the initial sugar concentration alone (X_1) had positive effects on P_E and *SC*, but the opposite effect on Q_P. Alternatively, urea concentration alone (X_2) had positive effect on Q_P, but the opposite effect on P_E and *SC*. However, the interaction of initial sugar and urea concentrations (X_1X_2) acted positively on all ethanol fermentation efficiency (P_E, Q_P and *SC*) values.

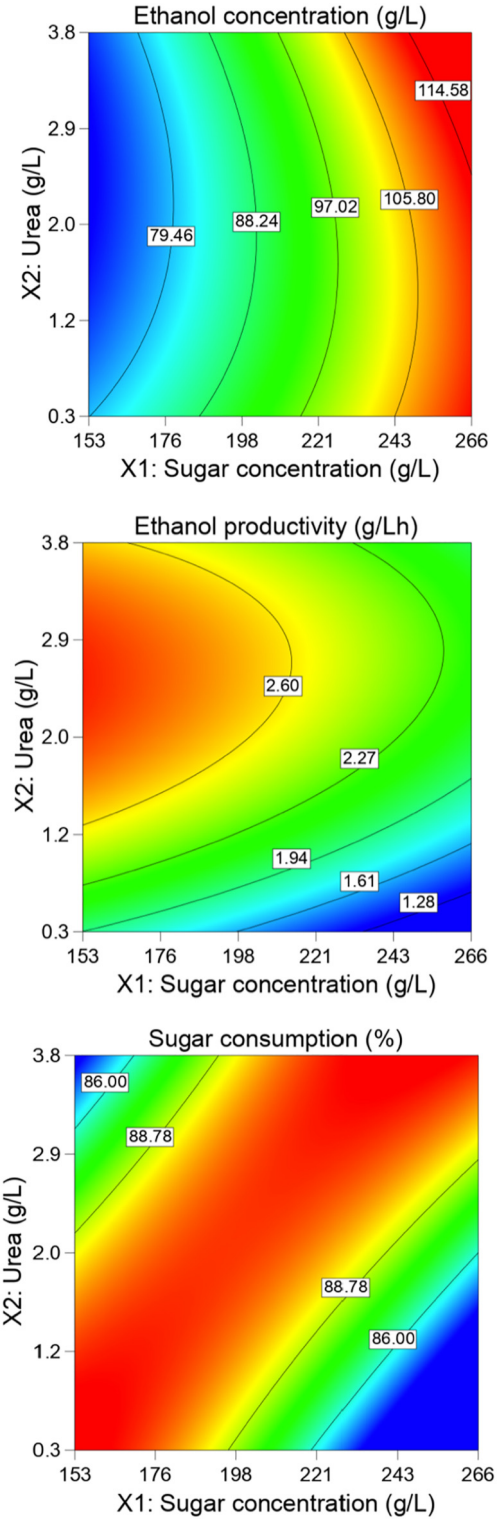

Fig. 1. Response surface plot of interaction on P_E, Q_P and *SC* values between sugar concentration (X_1) and urea concentration (X_2). The red region shows the highest response values, whereas the blue region shows the lowest the response values.

Response surface plot interactions of P_E, Q_P and *SC* (Fig. 1) indicate that the optimum conditions for obtaining the maximum P_E, Q_P and *SC* were an initial sugar concentration at 208 g/L and urea at 2.75 g/L. Under this condition, the predicted P_E, Q_P and *SC* values were 94.61 g/L, 2.70 g/L·h and 93.75%, respectively. When the fermentation under the optimum condition was carried out in the air-locked flask and the 2-L fermenter, it was found that the experimental values of P_E, Q_P and *SC* in both reactors were not different. The P_E (95.62 g/L), Q_P (2.73 g/L·h) and *SC* (93.15%) in the 2-L fermenter were close to the predicted values. The results obtained indicated high reliability of the model in predicting the experimental values.

3.2. Effects of the initial cell concentration, ORP control and urea supplementation during batch fermentation

The initial yeast cell concentration is also one of the primary factors affecting P_E, Q_P and *SC* values [14,15,16]. To improve these values, the initial cell concentration in this study was doubled to ~1×10^8 cells/mL. Table 2 shows the results of ethanol fermentation under the optimum initial sugar and urea concentrations at various conditions. The P_E values at an initial cell concentration of ~1×10^8 cells/mL (97.06 g/L) were not significantly different from that at initial cell concentration of ~5×10^7 cells/mL. However, the ethanol production rate or Q_P value at higher initial cell concentrations was markedly higher than at lower cell numbers because the fermentation time was decreased from 35 to 30 h. Under this condition, 95.43% *SC* was obtained. Therefore, the higher initial cell concentration, ~1×10^8 cells/mL, was used for ethanol fermentation in the next experiment. The results also showed that urea supplementation into SSJ at 2.75 g/L (optimum values) significantly promoted ethanol production efficiency, and especially, the rates of sugar consumption and ethanol production. In the control condition (208 g/L of initial sugar with no urea), the fermentation time was markedly longer, 60 h, and the P_E was 89.99 g/L with Q_P and *SC* values of only 1.50 g/L·h and 86.06%, respectively (Table 2).

Controlling ORP is necessary for a HG fermentation process to avoid the negative effects of high glucose levels, which result in the buildup of ethanol that adversely affects yeast causing growth to stop [25]. The ORP profile controlled at −100 mV and no ORP control are compared in Fig. 2. With no ORP control, the oxygen in the fermentation medium was rapidly depleted due to yeast growth, and the negative value of ORP appeared at the lowest ORP value of approximately −100 mV at 6 h (thus ORP control at −150 mV was not carried out). Subsequently, those values increased until the end of the fermentation process. Under ORP control at −100 mV, the ORP profile was similar to that with no control of this parameter in the first 6 h. When the measured ORP reached its set point, sterile air was fed into the fermenter until around 29 h of fermentation time. After that, the ORP values were gradually increased, which may have resulted from low cell activity and ethanol buildup.

Changes of the primary fermentation parameters with and without ORP control were very close (Fig. 3). The similarity of P_E values (96.12 and 97.06 g/L) was evident with and without ORP control. Approximately 5% of the residual sugar remained at the end of fermentation under both conditions. Similar fermentation results indicate that ORP control was not necessary under this condition. Analogous results were found by previous researchers [25] who reported that ORP control had no benefit to a fermentation using 200 g/L of glucose, but ORP control at −150 mV improved the fermentation efficiency using 250 and 300 g/L of glucose. It was reported that ORP was auto-regulated to only −138 mV in the case of 203 g/L of glucose with 98.51 g/L of P_E obtained [38], which was very close to this study. For this reason, ORP control was not applied in the next experiment.

Table 2
Ethanol fermentation of sweet sorghum stem juice containing the optimum initial sugar and urea concentrations at an initial cell concentration of ~1 × 10^8 cells/mL under various conditions.

Condition	P_E (g/L)	Q_P (g/L·h)	SC (%)	$Y_{P/S}$	t (h)
No ORP control (~5 × 10^7 cells/mL)	95.62 ± 0.05[a]	2.73 ± 0.15[b]	93.15 ± 0.21[b]	0.47 ± 0.00[c]	35
No ORP control	97.06 ± 0.02[a]	3.24 ± 0.00[a]	95.43 ± 0.44[a]	0.50 ± 0.00[a]	30
ORP at −100 mV	96.12 ± 0.21[a]	3.20 ± 0.01[a]	95.06 ± 0.96[a]	0.48 ± 0.00[b]	30
Control conditions*	89.99 ± 0.56[b]	1.50 ± 0.01[c]	86.06 ± 0.36[c]	0.48 ± 0.01[b,c]	60

* Control condition = SSJ medium containing 208 g/L of initial sugar with no urea. All experiments were done in triplicate. [a,b,c] 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.

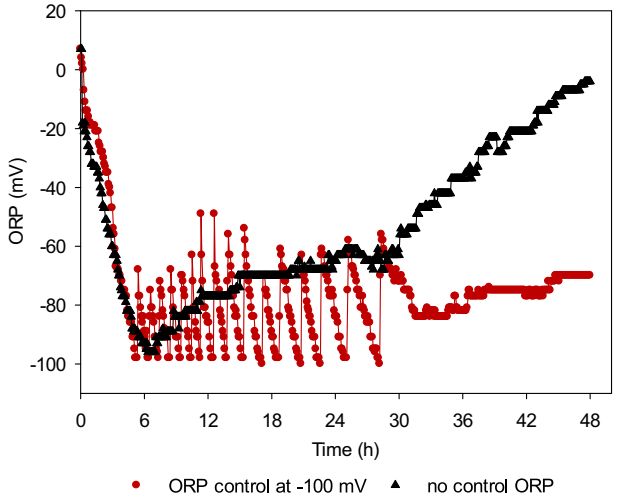

Fig. 2. ORP profiles during batch ethanol fermentation with ORP control at −100 mV and no control ORP.

3.3. Repeated-batch fermentation with aeration supply

3.3.1. RB1 condition: feeding medium containing 208 g/L of sugar

The repeated-batch fermentation was performed for eight successive cycles, and an initial aeration (2.0 vvm for 4 h) was supplied in every cycle. In the first cycle, the changes of log viable cell number, residual sugar and ethanol concentration (Fig. 4a–c) were similar to those found in batch fermentation (Fig. 3). The initial cell concentrations in the eight successive cycles were quite similar ranging from 6.0 to 9.3 × 10^7 cells/mL, and they were in the range of 1.37 to 2.51 × 10^8 cells/mL at the end of each cycle (Fig. 4a). In the first cycle, 75% of the fermented medium was withdrawn from the fermentation vessel, and a feeding medium containing 208 g/L of sugar was added to replace the removed medium. The initial sugar concentration of next cycle was reduced to levels ranging from 123.39 to 140.92 g/L of sugar (Fig. 4b). The residual sugar levels were 3.02 to 10.00 g/L, whereas the P_E values of each cycle ranged from 92.28 to 97.72 g/L. The average P_E of the eight successive cycles was 94.36 g/L (Fig. 4c), and the total amount of ethanol (P_E*) was 590.62 g in 231 h of total fermentation time (t*) in a 6-L fermentation (Table 3). The fermentation time of the first cycle (30 h) was the same as that of the batch fermentation. However, in some of the subsequent cycles, a shorter fermentation time (27 h) was attained, which might have been due to lower initial sugar concentration in the later cycles.

Eight successive cycles of repeated-batch fermentation with no aeration were also carried out as a control treatment (Fig. 4d–f). The initial cells numbers continuously decreased from the first to the eighth cycle. The lowest initial cell concentration was 3.4 × 10^7 cells/mL at the eighth cycle (Fig. 4d). The initial sugar concentrations in cycles 2 to 8 were similar at 133.36 to 143.40 g/L (Fig. 4e). Even though the viable cells in each cycle with no aeration were lower than those under aeration, the average P_E, P_E* and t* values under this condition were similar to those subjected to aeration (Table 3). Additionally, the main by-product or glycerol concentrations (P_G) with and without aeration were quite similar with values of 12.01 and 11.47 g/L, respectively.

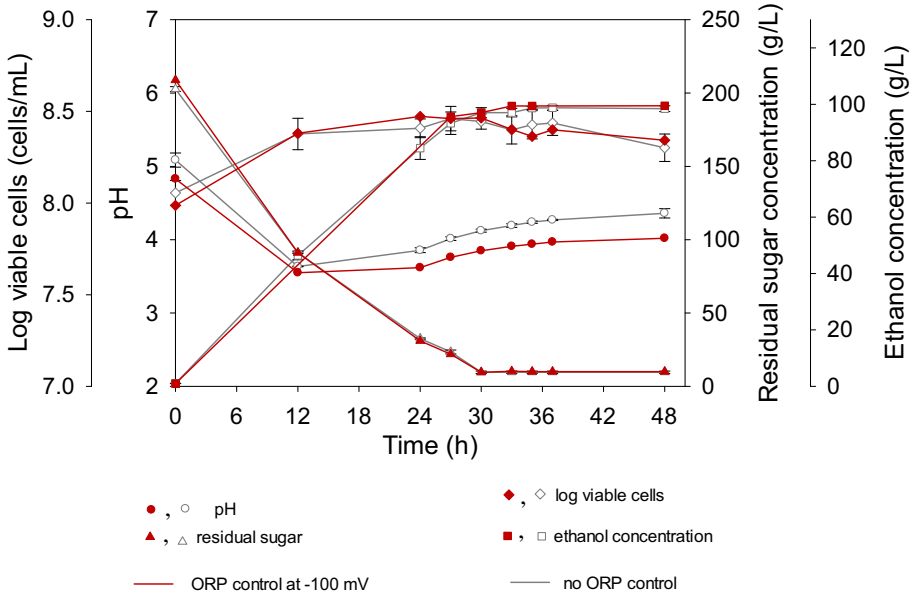

Fig. 3. Ethanol fermentation from sweet sorghum stem juice containing 208 g/L of sugar and 2.75 g/L of urea with and without ORP control conditions.

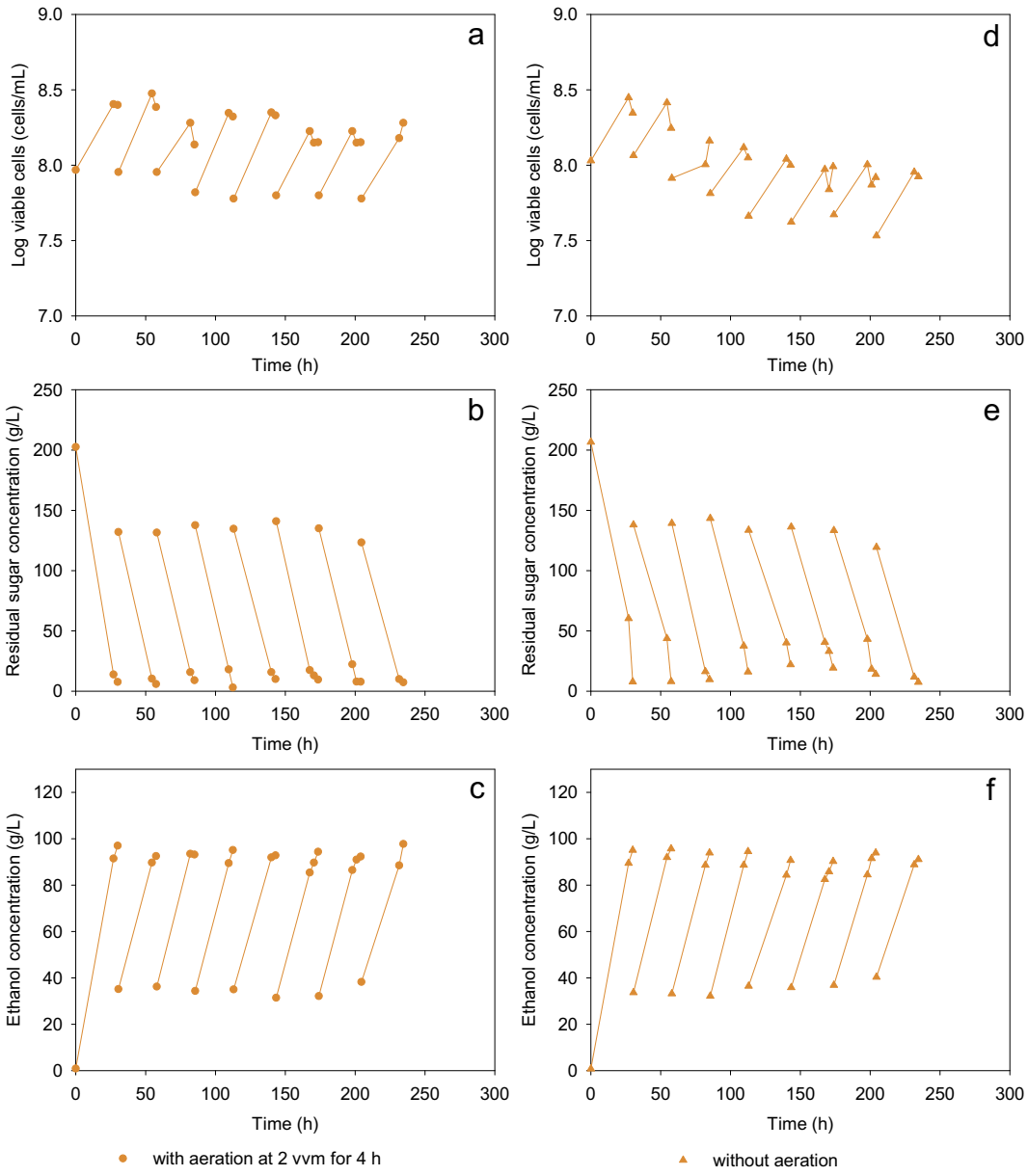

Fig. 4. Profiles of viable cells (a,d), residual sugar (b,e) and ethanol concentration (c,f) during repeated-batch ethanol fermentation from sweet sorghum stem juice in an RB1 condition with and without aeration at 2 vvm for 4 h at the beginning of each cycle.

Table 3
Ethanol fermentation parameters of repeated-batch fermentation under various conditions.

Condition		Average values for eight successive cycles								
		P_E	Q_P	$Y_{P/S}$	SC	P_G	t	P_E*	t*	Q_P*
RB1	Aeration	94.36 ± 1.95[b]	2.21 ± 0.39[a]	0.47 ± 0.02[a]	93.27 ± 3.91[a]	12.01 ± 0.31[b]	27–30	590.62	231	2.56
	No aeration	93.14 ± 1.99[b]	2.14 ± 0.45[a]	0.47 ± 0.01[a]	88.30 ± 6.96[a,b]	11.47 ± 0.43[b]	27–30	581.63	231	2.52
RB2	Aeration	105.16 ± 3.70[a]	2.16 ± 0.41[a]	0.43 ± 0.03[a]	87.49 ± 3.65[a,b]	13.15 ± 0.30[a]	30–36	657.20	279	2.36
	No aeration									
	- Cycle 1	94.63	3.12	0.50	93.08	11.71	30	–	–	–
	- Cycle 2	98.68	2.00	0.48	74.83	13.19	33	–	–	–
	- Cycle 3	93.89	1.41	0.40	76.85	13.41	44	–	–	–
	- Cycle 4	88.27	1.29	0.35	78.79	13.54	44	–	–	–
	- Cycle 5	80.40	1.17	0.30	78.60	13.92	44	–	–	–
	- Cycle 6	80.40	1.17	0.30	78.60	13.92	44	–	–	–
	Average	89.38 ± 7.03[b]	1.69 ± 0.70[a]	0.39 ± 0.08[a]	80.12 ± 5.96[b]	13.28 ± 0.82[a]	30–44	442.41	239	1.85

RB1 refers to repeated-batch fermentation using feeding medium containing 208 g/L of sugar, and RB2 refers to repeated-batch fermentation using feeding medium containing 240 g/L of sugar. P_E = ethanol concentration (g/L), Q_P = ethanol productivity (g/L·h), $Y_{P/S}$ = ethanol yield , SC = sugar consumption (%), P_G = glycerol concentration (g/L), t = fermentation time (h), P_E* = total amount of ethanol (g), t* = total fermentation time (h) and Q_P* = total ethanol productivity (g/h).
[a,b]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.

3.3.2. RB2 condition: feeding medium containing 240 g/L of sugar

To control the initial sugar concentration of each cycle so that it remained at similar levels, SSJ medium containing 240 g/L of sugar was used as the feeding medium in cycles 2 to 8. The fermentation was performed for eight successive cycles under aeration. At the end of each cycle, the viable cell concentrations were still high, about 2.01 to 2.46 × 10^8 cells/mL (Fig. 5a). The initial sugar concentrations of each cycle ranged from 179.60 to 217.00 g/L (Fig. 5b). The residual sugar levels ranged from 13.14 to 37.37 g/L. The average P_E of eight cycles was 105.16 g/L (Fig. 5c and Table 3), whereas the total P_E* was 657.20 g for 279 h of fermentation with a total volume of 6 L (Table 3). In cycles 2 to 8, the fermentation time was slightly extended from 30 to 36 h. Increasing the initial sugar concentrations caused longer fermentation times, up to 36 h, compared to using RB1 (t = 27 h). However, the ethanol production rates or Q_P (2.16 g/L·h) were not significantly different compared to RB1 (2.14 to 2.21 g/L·h) (Table 3). Regarding glycerol production, the P_G value of cycle 1 was the lowest (11.54 g/L). It increased in cycles 2 to 8 ranging from 12.76 to 13.49 g/L. Higher P_G values in later cycles might have been due to ethanol stress (105.16 g/L). This was supported by Appiah-Nkansah et al. [39], Phukoetphim et al. [40] and Wang et al. [41], who reported that the glycerol accumulation could be attributed to high osmotic stresses caused by high initial sugar and ethanol concentrations during the fermentation process.

With no aeration, the fermentation efficiencies continuously decreased in the later cycles. Consequently, this fermentation was conducted for only six cycles. The results showed that the viable cells numbers continuously decreased and only about 1.15 × 10^8 cells/mL remained at the end of cycle 6 (Fig. 5d). The initial sugar concentrations in cycles 2 to 6 ranged from 182.10 to 220.19 g/L, and the residual sugar ranged from 43.07 to 47.13 g/L (Fig. 5e), which was markedly higher than those grown under aeration. The P_E declined from the first (94.63 g/L) to the sixth cycle (80.40 g/L) (Fig. 5f and Table 3), and the average value was about 89.38 g/L with a P_E* value of 442.41 g after 239 h of fermentation with a working volume of 4.5 L (Table 3). Although the fermentation time in the first cycle was equal

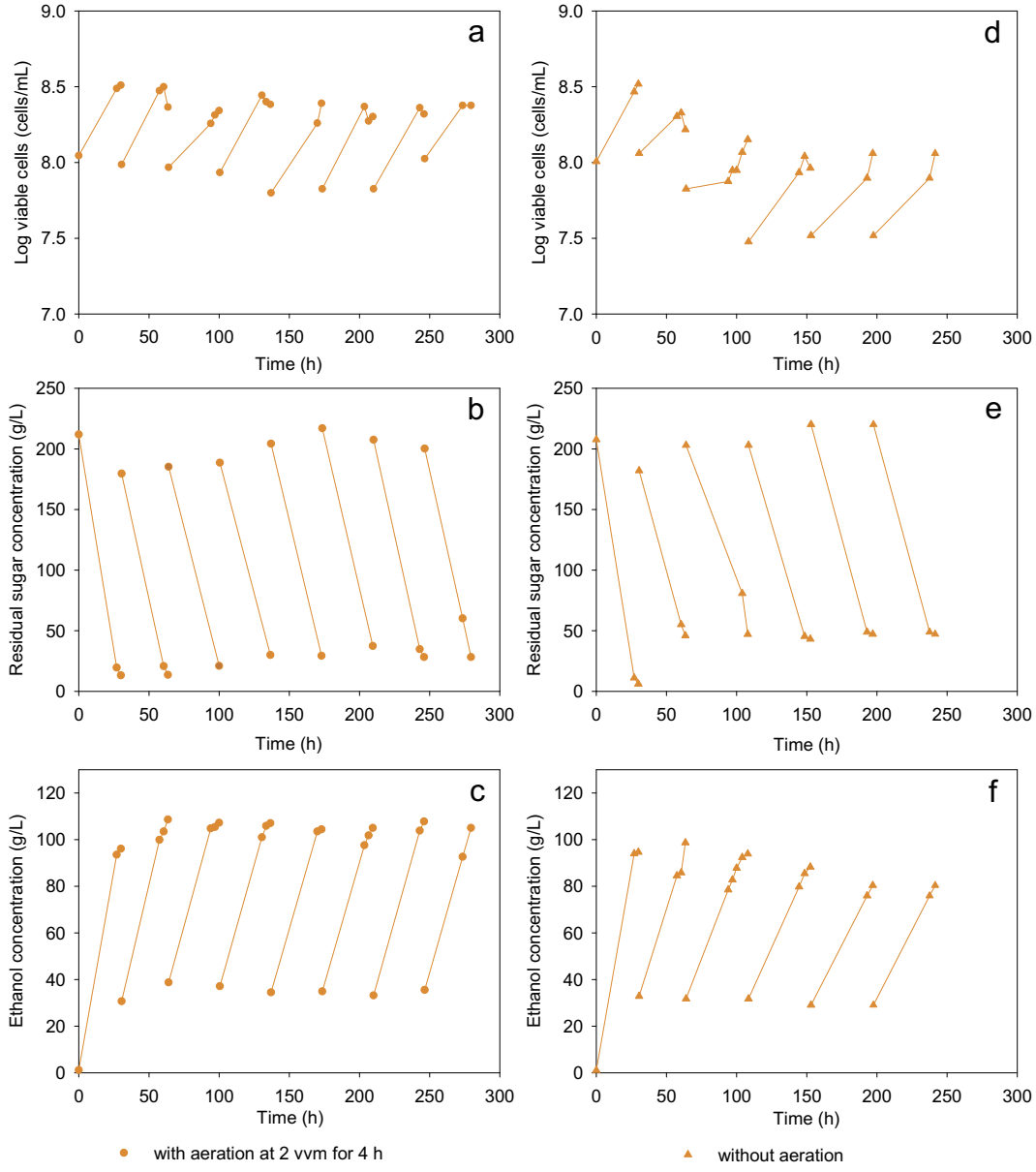

Fig. 5. Profiles of viable cell numbers (a,d), residual sugar (b,e) and ethanol concentration (c,f) during repeated-batch ethanol fermentation from sweet sorghum stem juice in an RB2 condition with and without aeration at 2 vvm for 4 h at the beginning of each cycle.

to that of batch fermentation, the fermentation time of the later cycles increased to 44 h in cycle 6. This might have been due to stress caused by high initial sugar levels. Additionally, high ethanol concentrations may have been toxic to yeast cells. Together, they may have resulted in decreased fermentation efficiencies and numbers of viable cells. These stresses with no aeration also affected the P_G levels, which were higher in cycles 2 to 6 (13.19 to 13.92 g/L) compared to that of cycle 1 (11.71 g/L). This is supported by Aili and Xun [42] and Pagliardini et al. [43] who reported that apart from high initial sugar and ethanol concentrations, glycerol also accumulated under an anaerobic condition. In cycle 6, the P_E was only 80.40 g/L, corresponding to Q_P of 1.17 g/L·h. These values were 15.04 and 62.50% lower than those of cycle 1, respectively. Therefore, the repeated-batch with no aeration was operated for only six successive cycles.

3.4. Comparison of repeated-batch fermentation under NG and HG conditions

The fermentation results using RB1 with and without aeration were similar (Table 3). This might have been due to the relatively low sugar concentrations at NG conditions with initial sugar concentrations of 120 to 143 g/L in cycles 2 to 8. Under these conditions, *S. cerevisiae* SSJKKU01 could quickly grow and produce ethanol. Additionally, the results indicate that no stress of sugar and ethanol occurred under the NG condition. Using RB1 with and without aeration, the total amount of ethanol for eight successive cycles (P_E*) was 581.63 to 590.62 g (total fermentation time of 231 h) with a satisfactory production rate (Q_P*) of 2.52 to 2.56 g/h. The ethanol fermentation efficiencies under both conditions were not different indicating that aeration under NG conditions was not necessary.

Different results were observed using RB2 media. The P_E of all eight cycles under aeration were similar, but the P_E value was decreased by 14 g/L from cycle 1 (94.63 g/L) to 6 (80.40 g/L) with no aeration (Table 3). This resulted in decreasing ethanol fermentation efficiency in terms of Q_P and $Y_{P/S}$ (Table 3). The P_E (105.16 vs. 89.38 g/L) and Q_P (2.16 vs. 1.69 g/L·h) under both conditions were significantly different. The longer fermentation time (44 vs. 30 h) with no aeration might have been due to the higher initial sugar concentration in the medium (180 to 220 g/L) which is a HG condition. High ethanol content using RB2 (105.16 g/L) can stress yeast cells, but these stresses were alleviated under low aeration. Aeration contributed to biomass formation and increased cell viability [44]. Furthermore, the sugar consumption with aeration was about 7% higher than without aeration. This shows that appropriate aeration can enhance fermentation efficiency. It was found that the P_G values using the RB2 medium (13.15 to 13.28 g/L) were slightly higher than those using RB1 (11.47 to 12.31 g/L). This indicates more stress due to high ethanol content and/or insufficient aeration under RB2 conditions [42,43].

The RB2 medium with aeration gave a higher P_E* value (657.20 g) than RB1 (581.63 to 590.62 g). However fermentation time of eight successive cycles using RB2 was 48 h longer than RB1, resulting in a slightly lower Q_P* value (2.36 g/h) than when using RB1 (2.52 to 2.56 g/L). The P_E* (442.41 g) and Q_P* (1.85 g/h) values with RB2 medium and no aeration were the lowest since the P_E value was lowest, and only six successive cycles were operated. These results indicate that aeration is not required for repeated batch ethanol fermentation under NG conditions, but it is essential for repeated batch fermentation under HG conditions.

3.5. Effects of aeration on yeast cell composition in repeated-batch fermentation

The composition of yeast cells is important for their tolerance to ethanol and osmotic pressure. Trehalose, a non-reducing sugar, is beneficial for yeast because it functions as an osmoprotectant [45,46].

Table 4
Ergosterol and trehalose concentrations in S. cerevisiae SSJKKU01 at the end of repeated-batch fermentation.

Condition		Concentration (mg/g DCW)	
		Trehalose	Ergosterol
RB1	Aeration	88.21 ± 0.54[a]	13.66 ± 0.43[a]
	No aeration	91.46 ± 2.93[a]	14.09 ± 0.05[a]
RB2	Aeration	91.77 ± 5.00[a]	11.30 ± 0.72[b]
	No aeration	95.64 ± 4.10[a]	8.53 ± 0.14[c]

[a,b,c.] 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.

In this study, the trehalose contents of yeast cells were not different with and without aeration (Table 4), indicating that aeration did not significantly affect trehalose content. The P_E under those conditions (92.58 to 105.16 g/L) did not adversely affect the highly ethanol tolerant strain, *S. cerevisiae* SSJKKU01 . Ergosterol responds to ethanol toxicity by increasing membrane fluidity to facilitate ethanol transport out of the yeast cells [47,48]. Mannazzu et al. [49] reported that yeast cells were unable to synthesize ergosterol under anaerobic conditions. In the current study, the ergosterol contents using RB1 medium with and without aeration conditions were similar. Under these conditions, the average P_E was about 94 g/L (Table 4). Conversely, ergosterol content using RB2 with aeration (P_E 105.16 g/L) was significantly higher than with no aeration (P_E 80.40 g/L). This indicated that the yeast cells were stressed using RB2 with aeration owing to the high P_E (105.16 g/L). Therefore, greater synthesis of ergosterol promoted cell survival during the fermentation. The trehalose and egosterol levels observed in the current study were in range of those reported by Xue et al. [50], who studied the changes of trehalose and ergosterol in flocculating yeast. They found that the trehalose and ergosterol contents were 76.50 to 109.20 and 5.64 to 8.67 g/L, respectively, under ethanol fermentation using 300 g/L of glucose by a self-flocculating yeast SPSC01.

4. Conclusions

Sweet sorghum stem juice containing 208 g/L of initial sugar and 2.75 g/L of urea was successfully used as an ethanol production medium for a high level of ethanol production. ORP control during the fermentation did not enhance the fermentation efficiency, but increasing initial cell concentration could improve ethanol productivity. A low aeration rate (2.0 vvm for 4 h) was not necessary for repeated-batch fermentation under a NG condition, but it was beneficial under HG for the health and productivity of the yeast culture. Repeated-batch fermentation showed enhanced ethanol productivity over that of batch fermentation because inoculum preparation was not required. Finally, short aeration did not promote trehalose accumulation in yeast cells during repeated-batch fermentation, but it affected the ergosterol content in yeast cells at a high ethanol concentration (105 g/L).

Financial support

This research was financially supported by the Thailand Research Fund (TRF) through the Royal Golden Jubilee (RGJ) Ph.D. Program (Grant Number PHD0178/2558), the Post-doctoral Program (Grant Number 59153) from Research Affairs and Graduate School, Khon Kaen University (KKU), Thailand.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank Associate Prof. Dr. Prasit Jaisil, Faculty of Agriculture, KKU, Thailand for providing the sweet sorghum stem juice.

References

[1] Li Z, Wang D, Shi YC. Effects of nitrogen source on ethanol production in very high gravity fermentation of corn starch. J Taiwan Inst Chem Eng. 2017;70:229–35. https://doi.org/10.1016/j.jtice.2016.10.055.

[2] Wattana S. Bioenergy development in Thailand: challenges and strategies. Energy Procedia. 2014;52:506–15. https://doi.org/10.1016/j.egypro.2014.07.104.

[3] Zabed H, Sahu JN, Suely A, et al. Bioethanol production from renewable sources: current perspectives and technological progress. Renew Sustain Energy Rev. 2017;71:475–501. https://doi.org/10.1016/j.rser.2016.12.076.

[4] Nuanpeng S, Laopaiboon L, Srinophakun P, et al. Ethanol production from sweet sorghum juice under very high gravity conditions: batch, repeated-batch and scale up fermentation. Electron J Biotechnol. 2011;14:1–12. https://doi.org/10.2225/vol14-issue1-fulltext-2.

[5] Appiah-Nkansah NB, Li J, Rooney W, et al. A review of sweet sorghum as a viable renewable bioenergy crop and its techno-economic analysis. Renew Energy. 2019;143:1121–32. https://doi.org/10.1016/j.renene.2019.05.066.

[6] Laopaiboon L, Nuanpeng S, Srinophakun P, et al. Ethanol production from sweet sorghum juice using very high gravity technology: effects of carbon and nitrogen supplementations. Bioresour Technol. 2009;100:4176–82. https://doi.org/10.1016/j.biortech.2009.03.046 PMid: 19375908.

[7] Zhang C, Wen H, Zheng J, et al. A combination of evaporation and chemical preservation for long-term storage of fresh sweet sorghum juice and subsequent bioethanol production. J Food Process Preserv. 2018;42:1–9. https://doi.org/10.1111/jfpp.13825.

[8] Rochón E, Ferrari MD, Lareo C. Integrated ABE fermentation-gas stripping process for enhanced butanol production from sugarcane-sweet sorghum juices. Biomass Bioenergy. 2017;98:153–60. https://doi.org/10.1016/j.biombioe.2017.01.011.

[9] Najafpour G, Younesi H, Ku Ismail KS. Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. Bioresour Technol. 2004;92:251–60. https://doi.org/10.1016/j.biortech.2003.09.009 PMid: 14766158.

[10] Ganjali Dashti M, Abdeshahian P, Wan Yusoff WM, et al. Repeated Batch Fermentation Biotechnology for the Biosynthesis of Lipid and Gamma-Linolenic Acid by *Cunninghamella bainieri* 2A1. Biomed Res Int. 2014;2014. https://doi.org/10.1155/2014/831783 PMid: 25147817.

[11] Walker Graeme M. Yeast Physiology and Biotechnology. Chichester, West Sussex PO19 IUD, England; 1998 [362 p].

[12] Bafrncová P, Šmogrovičová D, Sláviková I, et al. Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*. Biotechnol Lett. 1999;21:337–41. https://doi.org/10.1023/A:1005436816047.

[13] Breisha GZ. Production of 16% ethanol from 35% sucrose. Biomass Bioenergy. 2010;34:1243–9. https://doi.org/10.1016/j.biombioe.2010.03.017.

[14] Laopaiboon L, Thanonkeo P, Jaisil P, et al. Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by *Saccharomyces cerevisiae*. World J Microbiol Biotechnol. 2007;23:1497–501. https://doi.org/10.1007/s11274-007-9383-x.

[15] Sridee W, Laopaiboon L, Jaisil P, et al. The use of dried spent yeast as a low-cost nitrogen supplement in ethanol fermentation from sweet sorghum juice under very high gravity conditions. Electron J Biotechnol. 2011;14:1–15. https://doi.org/10.2225/vol14-issue6-fulltext-5.

[16] Sriputorn B, Laopaiboon P, Laopaiboon L. Increase in ethanol production efficiency from sweet sorghum juice by *Saccharomyces cerevisiae*. Chiang Mai J Sci. 2017;44:59–71.

[17] Bai FW, Anderson WA, Moo-Young M. Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv. 2008;26:89–105. https://doi.org/10.1016/j.biotechadv.2007.09.002 PMid: 17964107.

[18] Deesuth O, Laopaiboon P, Klanrit P, et al. Improvement of ethanol production from sweet sorghum juice under high gravity and very high gravity conditions: effects of nutrient supplementation and aeration. Ind Crops Prod. 2015;74:95–102. https://doi.org/10.1016/j.indcrop.2015.04.068.

[19] Puligundla P, Smogrovicova D, Obulam VSR, et al. Very high gravity (VHG) ethanolic brewing and fermentation: a research update. J Ind Microbiol Biotechnol. 2011;38:1133–44. https://doi.org/10.1007/s10295-011-0999-3 PMid: 21695540.

[20] Yue G, Yu J, Zhang X, et al. The influence of nitrogen sources on ethanol production by yeast from concentrated sweet sorghum juice. Biomass Bioenergy. 2012;39:48–52. https://doi.org/10.1016/j.biombioe.2010.08.041.

[21] Liu YK, Lien PM. Bioethanol production from potato starch by a novel vertical mass-flow type bioreactor with a co-cultured-cell strategy. J Taiwan Inst Chem Eng. 2016;62:162–8. https://doi.org/10.1016/j.jtice.2016.01.027.

[22] Saini P, Beniwal A, Kokkiligadda A, et al. Response and tolerance of yeast to changing environmental stress during ethanol fermentation. Process Biochem. 2018;72:1–12. https://doi.org/10.1016/j.procbio.2018.07.001.

[23] Wang M, Chen Y, Xia X, et al. Energy efficiency and environmental performance of bioethanol production from sweet sorghum stem based on life cycle analysis. Bioresour Technol. 2014;163:74–81. https://doi.org/10.1016/j.biortech.2014.04.014 PMid: 24787319.

[24] Liu CG, Hao XM, Lin YH, et al. Redox potential driven aeration during very-high-gravity ethanol fermentation by using flocculating yeast. Sci Rep. 2016;6:1–6. https://doi.org/10.1038/srep25763 PMid: 27161047.

[25] Lin YH, Chien WS, Duan KJ. Correlations between reduction-oxidation potential profiles and growth patterns of Saccharomyces cerevisiae during very-high-gravity fermentation. Process Biochem. 2010;45:765–70. https://doi.org/10.1016/j.procbio.2010.01.018.

[26] Liu CG, Qin JC, Lin YH. Fermentation and Redox Potential. In: Jozala A, editor. Fermentation Processes. IntechOpen; 2017. p. 23–42. https://doi.org/10.5772/64640.

[27] Gao J, Yuan W, Li Y, et al. Application of redox potential control to improve ethanol productivity from inulin by consolidated bioprocessing. Process Biochem. 2016;51:1544–51. https://doi.org/10.1016/j.procbio.2016.07.011.

[28] Liu CG, Xue C, Lin YH, et al. Redox potential control and applications in microaerobic and anaerobic fermentations. Biotechnol Adv. 2013;31:257–65. https://doi.org/10.1016/j.biotechadv.2012.11.005 PMid: 23178703.

[29] Liang L, Zhang YP, Zhang L, et al. Study of sugarcane pieces as yeast supports for ethanol production from sugarcane juice and molasses. J Ind Microbiol Biotechnol. 2008;35:1605–13. https://doi.org/10.1007/s10295-008-0404-z PMid: 18685877.

[30] Khongsay N, Lin YH, Laopaiboon P, et al. Improvement of very-high-gravity ethanol fermentation from sweet sorghum juice by controlling fermentation redox potential. J Taiwan Inst Chem Eng. 2014;45:302–7. https://doi.org/10.1016/j.jtice.2013.07.011.

[31] Ariyajarearnwong P, Laopaiboon L, Laopaiboon P, et al. Repeated-batch ethanol fermentation from sweet sorghum juice by free cells of Saccharomyces NP 01. Afr J Biotechnol. 2011;10:13909–18. https://doi.org/10.5897/AJB11.1285.

[32] Khongsay N, Laopaiboon L, Jaisil P, et al. Optimization of agitation and aeration for very high gravity ethanol fermentation from sweet sorghum juice by *Saccharomyces cerevisiae* using an orthogonal array design. Energies. 2012;5:561–76. https://doi.org/10.3390/en5030561.

[33] Zoecklein BW, Fugelsang KC, Gump BH, et al. Wine analysis and production. Philadelphia, United States: Aspen Publishers Inc., U.S.; 1995 639 p. https://doi.org/10.1007/978-1-4757-6978-4.

[34] Sirisantimethakom L, Laopaiboon L, Danvirutai P, et al. Volatile compounds of a traditional Thai rice wine. Biotechnology. 2008;7:505–13. https://doi.org/10.3923/biotech.2008.505.513.

[35] Pereira FB, Guimarães PMR, Teixeira JA, et al. Optimization of low-cost medium for very high gravity ethanol fermentations by *Saccharomyces cerevisiae* using statistical experimental designs. Bioresour Technol. 2010;101:7856–63. https://doi.org/10.1016/j.biortech.2010.04.082 PMid: 20627715.

[36] Liu H-J, Liu D-H, Zhong J-J. Interesting physiological response of the osmophilic yeast *Candida krusei* to heat shock. Enzyme Microb Technol. 2005;36:409–16. https://doi.org/10.1016/j.enzmictec.2004.04.024.

[37] Inoue T, Iefuji H, Fujii T, et al. Cloning and Characterization of a Gene Complementing the Mutation of an Ethanol-sensitive Mutant of Sake Yeast. Biosci Biotechnol Biochem. 2000;64:229–36. https://doi.org/10.1271/bbb.64.229 PMid: 10737174.

[38] Liu CG, Lin YH, Bai FW. Development of redox potential-controlled schemes for very-high-gravity ethanol fermentation. J Biotechnol. 2011;153:42–7. https://doi.org/10.1016/j.jbiotec.2011.03.007 PMid: 21419814.

[39] Appiah-Nkansah NB, Zhang K, Rooney W, et al. Ethanol production from mixtures of sweet sorghum juice and sorghum starch using very high gravity fermentation with urea supplementation. Ind Crops Prod. 2018;111:247–53. https://doi.org/10.1016/j.indcrop.2017.10.028.

[40] Phukoetphim N, Salakkam A, Laopaiboon P, et al. Improvement of ethanol production from sweet sorghum juice under batch and fed-batch fermentations: effects of sugar levels, nitrogen supplementation, and feeding regimes. Electron J Biotechnol. 2017;26:84–92. https://doi.org/10.1016/j.ejbt.2017.01.005.

[41] Wang L, Zhao XQ, Xue C, et al. Impact of osmotic stress and ethanol inhibition in yeast cells on process oscillation associated with continuous very-high-gravity ethanol fermentation. Biotechnol Biofuels. 2013;6:1–10. https://doi.org/10.1186/1754-6834-6-133 PMid: 24041271.

[42] Aili Z, Xun C. Improve ethanol yield through minimizing glycerol yield in ethanol fermentation of *Saccharomyces cerevisiae*. Chin J Chem Eng. 2008;16:620–5. https://doi.org/10.1016/S1004-9541(08)60130-5.

[43] Pagliardini J, Alfenore S, Bideaux C, et al. The metabolic costs of improving ethanol yield by reducing glycerol formation capacity under anaerobic conditions in *Saccharomyces cerevisiae*. Microb Cell Fact. 2013;12:1–14. https://doi.org/10.1186/1475-2859-12-29 PMid: 23537043.

[44] Liu Y, Qi T, Shen N, et al. Improvement of ethanol concentration and yield by initial aeration and agitation culture in very high gravity fermentation. Chinese J Appl Environ Biol. 2009;15:563–7.

[45] Auesukaree C. Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. J Biosci Bioeng. 2017;124:133–42. https://doi.org/10.1016/j.jbiosc.2017.03.009 PMid: 28427825.

[46] Elbein AD, Pan YT, Pastuszak I, et al. New insights on trehalose: a multifunctional molecule. Glycobiology. 2003;13:17–27. https://doi.org/10.1093/glycob/cwg047 PMid: 12626396.

[47] Dong SJ, Yi CF, Li H. Changes of *Saccharomyces cerevisiae* cell membrane components and promotion to ethanol tolerance during the bioethanol fermentation. Int J Biochem Cell Biol. 2015;69:196–203. https://doi.org/10.1016/j.biocel.2015.10.025 PMid: 26515124.

[48] Vanegas JM, Contreras MF, Faller R, et al. Role of unsaturated lipid and ergosterol in ethanol tolerance of model yeast biomembranes. Biophys J. 2012;102:507–16. https://doi.org/10.1016/j.bpj.2011.12.038 PMid: 22325273.

[49] Mannazzu I, Angelozzi D, Belviso S, et al. Behaviour of *Saccharomyces cerevisiae* wine strains during adaptation to unfavourable conditions of fermentation on synthetic medium: cell lipid composition, membrane integrity, viability and fermentative

activity. Int J Food Microbiol. 2008;121:84–91. https://doi.org/10.1016/j.ijfoodmicro.2007.11.003 PMid: 18055051.

[50] Xue C, Zhao XQ, Bai FW. Effect of the size of yeast flocs and zinc supplementation on continuous ethanol fermentation performance and metabolic flux distribution under very high concentration conditions. Biotechnol Bioeng. 2010;105:935–44. https://doi.org/10.1002/bit.22610 PMid: 19953674.