

Article

Repeated-Batch Ethanol Production from Sweet Sorghum Juice by *Saccharomyces cerevisiae* Immobilized on Sweet Sorghum Stalks

Pongthep Ariyajaroenwong¹, Pattana Laopaiboon^{2,3}, Prasit Jaisil⁴ and Lakkana Laopaiboon^{2,3,*}

¹ Graduate School, Khon Kaen University, Khon Kaen 40002, Thailand; E-Mail: piccolosth@yahoo.com

² Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand; E-Mail: patlao@kku.ac.th

³ Fermentation Research Center for Value Added Agricultural Products, Khon Kaen University, Khon Kaen 40002, Thailand

⁴ Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand; E-Mail: pjaisil@gmail.com

* Authors to whom correspondence should be addressed; E-Mail: lakcha@kku.ac.th; Tel.: +66-4336-2121; Fax: +66-4336-2121.

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Abstract: Sweet sorghum stalks were used as a low cost carrier for immobilization of *Saccharomyces cerevisiae* NP 01 to produce ethanol from sweet sorghum juice. The effects on ethanol production of carrier size ($6 \times 6 \times 6$ to $20 \times 20 \times 20$ mm³) and initial cell concentrations (5×10^7 to 2×10^8 cells mL⁻¹) for cell immobilization were investigated. The ethanol production medium was the juice containing 230 g L⁻¹ of total sugar without nutrient supplementation. The fermentations were carried out under static conditions in 500-mL air-locked Erlenmeyer flasks at 30 °C. The results showed that the optimum size of sorghum stalk pieces for repeated-batch ethanol production was $6 \times 6 \times 6$ mm³, while the optimum initial cell concentration for the immobilization was 1.0×10^8 cells mL⁻¹. The immobilized yeast under these conditions could be used for at least eight successive batches without any losses of ethanol production efficiencies. The average ethanol concentration, productivity and yield of the eight successive batches were 99.28 ± 3.53 g L⁻¹, 1.36 ± 0.05 g L⁻¹ h⁻¹ and 0.47 ± 0.03 g g⁻¹, respectively.

Keywords: ethanol fermentation; immobilization; repeated-batch; sweet sorghum; *Saccharomyces cerevisiae*

1. Introduction

Currently, bioethanol has emerged as one of the most viable options in the area of non-conventional sources of energy [1]. The use of fuel ethanol can reduce the toxic exhaust emissions and greenhouse gases from vehicles [2]. Apart from the raw materials *i.e.*, sugarcane, molasses, corn and cassava that are widely used for industrial ethanol production, one of the prime sources of raw material being investigated for ethanol is sweet sorghum. Sweet sorghum [*Sorghum bicolor* (L.) Moench] is a high biomass- and sugar-yielding crop. The juice from its stalks contains a large amount of fermentable sugar (sucrose, glucose and fructose) and many essential trace elements suitable for microbial growth and ethanol production [3,4]. It can be cultivated at nearly all temperatures and tropical climate areas with the growing period of 120–150 days [5]. In Thailand, the growing period of sweet sorghum cv. KKKU 40 is only 90–100 days [6], therefore it can be planted for 3 cycles/year with an average yield of 30 tons/hectare/round or 90 tons/hectare/year, while sugarcane can be cultivated for only one round/year or 63 tons/hectare/year [7].

In this research, repeated-batch fermentation was used in the conversion process of sugars to ethanol because this process has several advantages compared to conventional batch fermentation such as no new inoculum is required for each batch and long-term productivity [8,9]. In addition, no time is wasted for cleaning and reesterilization, and the operational control is easier than that of a continuous mode. In the repeated-batch process using free cells of *Saccharomyces cerevisiae*, the portion of the fermented broth is withdrawn at time intervals, and the residual part of the broth is used as an inoculum for the next batch. However, this method causes a reduction in yeast cell concentration, resulting in lower ethanol production in the subsequent batches [10,11]. To avoid this phenomenon, the repeated-batch process using immobilized yeast cells is proposed. The use of immobilization systems can minimize the production costs, because this system offers several advantages over the free cell fermentation operation *i.e.*, higher yeast cell concentration, higher fermentation rate, easier cell recycle and lesser product inhibition [12–14].

The most widely used immobilization method is cell entrapment on Ca-alginate [15–17]. The main drawback of using Ca-alginate as a carrier for the immobilization is the instability of the carrier against phosphates and the disruption of gel particles due to CO₂ evolution during fermentation [18,19]. In addition, the price of alginate is expensive, which is a disadvantage for its use for industrial purposes. Sweet sorghum is a natural lignocellulosic material which has several advantages. Its stalk is much more porous than other lignocellulosic materials (e.g., straw and wood chips). In addition, there are lots of pores among the sorghum cells, which make the sorghum integrative, and will make the transmission of substrates and products among the sorghum cells more easy [18]. Other low-cost carriers used for yeast immobilization for bioethanol production are sugarcane bagasse and corncobs [19,20]. In this study, apart from using the juice squeezed from sweet sorghum stalk as a raw material for ethanol production, the fresh stalk itself is also employed as a natural and low-cost carrier

for cell immobilization to avoid the drawbacks of Ca-alginate. To our knowledge, this is the first report of the use of fresh sweet sorghum as the carrier for cell immobilization to produce ethanol from its juice.

The aims of this research were to study the ability of sweet sorghum stalk pieces as the low-cost carrier for the immobilization of *S. cerevisiae* cells for ethanol fermentation from sweet sorghum juice and to investigate their stability in repeated-batch ethanol fermentation from the juice. The effects of carrier sizes and initial cell concentrations for cell immobilization on ethanol production were also investigated.

2. Experimental Section

2.1. Microorganism and Inoculum Preparation

S. cerevisiae NP 01, an osmotolerant yeast, was isolated from Loog-pang (Chinese yeast cake for Thai rice wine making) from Nakorn Phanom province, Thailand. It was inoculated into a 250-mL Erlenmeyer flask containing 150 mL of yeast extract malt extract (YM) medium. The medium contained (g L⁻¹) yeast extract 3, malt extract 3, peptone 5 and glucose 10. The flask was incubated on rotating shaker at 150 rpm, 30 °C for 15 h. The yeast was transferred into a 500-mL Erlenmeyer flask containing 350 mL of YM medium containing 20 g L⁻¹ of glucose to give the initial cell concentration of approximately 5 × 10⁶ cells mL⁻¹. The flasks were further incubated under the conditions as previously mentioned. After 15 h, the cells were harvested and used as an inoculum for cell immobilization.

2.2. Raw Material

Sweet sorghum juice extracted from its stalks (cv. KKU 40) was obtained from Division of Agronomy, Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Thailand. To avoid storage problem and bacterial contamination, the juice containing total soluble solids of 18 °Brix (% by weight) was concentrated to 75 °Brix and stored at 4 °C until use.

2.3. Ethanol Production Medium

The concentrated juice was diluted with distilled water to the total sugar concentration of 230 g L⁻¹ and used as an ethanol production (EP) medium [19,21]. In all experiments, the EP medium was transferred into a 500-mL air-locked Erlenmeyer flask with a final working volume of 350 mL before autoclaving at 110 °C for 28 min [3].

2.4. Cell Immobilization on Carrier

The sweet sorghum cv. KKU 40 at 100–110 days old was harvested. The diameter of the sorghum stalk from top to bottom ranged from 5 to 30 mm. The skin of sorghum stalk was removed, and it was cut into three different particle sizes (approximately 6 × 6 × 6, 12 × 12 × 12 and 20 × 20 × 20 mm³) by a sharp knife. The stalk pieces (52.5 g corresponding to approximately 30% of the working volume) were autoclaved at 121 °C for 15 min before mixing with 100 mL fresh YM medium containing active yeast cell concentration of 1 × 10⁸ cells mL⁻¹. The immobilization was carried out at 30 °C under static

condition. After 24 h, the sorghum stalks were washed with sterile EP medium before being used as an inoculum (immobilized cells) for ethanol production.

2.5. Optimum Initial Cell Concentration for Cell Immobilization

Sorghum stalk pieces at the optimum size (from Section 2.4) were prepared and they were sterilized as previously mentioned. The sterile stalks were added into 100 mL of fresh YM medium containing different active yeast cell concentrations (0.5×10^8 , 1×10^8 and 2×10^8 cells mL⁻¹). The immobilization was performed as previously described.

2.6. Fermentation Processes

2.6.1. Batch Fermentation System

The immobilized yeast cells were inoculated into sterile EP medium in the 500-mL flask. The fermentation was operated at 30 °C under static conditions. The samples were collected at 12-h intervals for analysis.

2.6.2. Repeated-Batch Fermentation System

The repeated-batch fermentation was first carried out in batch mode. After 72 h, all fermented broth was drained and only the carriers with immobilized cells were retained in the flask. Then, the same amount of the sterile EP medium was immediately replaced to initiate the next cycle. Eight successive cycles were performed. During each cycle, the fermented broth was collected for analysis as performed in the batch process.

2.7. Analytical Methods

The viable cell numbers in the fermentation broth were determined by direct counting method using haemocytometer with methylene blue staining technique [22]. Ten grams of sorghum stalk pieces containing immobilized cells were blended with 90 mL of 0.85% NaCl solution [23]. The suspension was serially diluted and cell concentration in the suspension was determined as described above. The dry weight of the sorghum stalk was measured. The viable cells in the carrier (cells g⁻¹ dry weight of sorghum stalk) were then calculated.

The fermentation broth was centrifuged at 13,000 rpm for 10 min. The supernatant was determined for total and reducing sugar by a phenol-sulfuric acid method [24] and dinitrosalicylic acid (DNS) method [25], respectively. Ethanol concentration was analyzed by gas chromatography (Shimadzu GC-14B, Japan, solid phase: polyethylene glycol (PEG-20M), carrier gas: nitrogen, 150 °C isothermal packed column, injection temperature 180 °C, flame ionization detector temperature 250 °C; GC Solution analysis Version 2.30) and 2-propanol was used as an internal standard [3,21]. The ethanol yield ($Y_{p/s}$, g g⁻¹) was calculated as the actual ethanol produced and expressed as g ethanol per g sugar utilized (g g⁻¹). The volumetric ethanol productivity (Q_p , g L⁻¹ h⁻¹) was calculated by the actual ethanol concentration produced divided by the fermentation time giving the highest ethanol concentration for batch and repeated-batch fermentations.

2.8. Statistical Analysis

All the experiments were performed in triplicate, and the results were expressed as mean \pm SD. Statistical analysis was carried out using SPSS 15.0 for Windows.

3. Results and Discussion

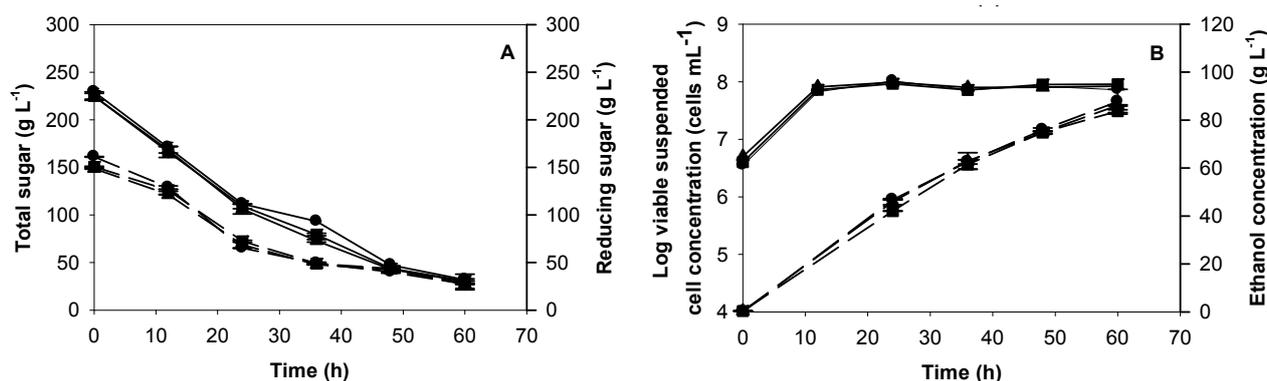
3.1. Effects of the Carrier Sizes for Yeast Cell Immobilization on Ethanol Production from Sweet Sorghum Juice

After 24 h of the cell immobilization process in sorghum stalk pieces of different sizes, the cells immobilized on the stalks were enumerated. The cell concentrations immobilized on $6 \times 6 \times 6$, $12 \times 12 \times 12$ and $20 \times 20 \times 20$ mm³ sorghum stalks before use as the inoculum for ethanol production were $(7.84 \pm 0.11) \times 10^9$, $(1.15 \pm 0.26) \times 10^{10}$ and $(1.41 \pm 0.14) \times 10^{10}$ cells g⁻¹ dry weight of sorghum, respectively. The results showed that the larger pieces contained slightly higher cell concentrations than the smaller ones. The cell numbers in the $20 \times 20 \times 20$ and $12 \times 12 \times 12$ mm³ sorghums were 0.25 and 0.17 log scale higher than that of the $6 \times 6 \times 6$ mm³ sorghum. This finding was supported by the results of Yu *et al.* [18] who reported that if the total mass of the carrier was equal, the large pieces had more intact stalk cells than the small ones, and more yeast cells could be immobilized on every unit. The cells of the large sorghum pieces were large, long and porous. The cell retention was due to the action of capillary forces during immobilization process, which pulled the cells closer to the surface and through the channels where they could be entrapped or attached, and multiplied [26].

The changes of total sugar, reducing sugar, viable suspended cell and ethanol concentration in the broth during the batch fermentation from the sweet sorghum juice by the yeast immobilized on different sizes of sorghum stalks are shown in Figure 1. The initial pH of the broth was 4.6 and it decreased to about 4.0 in 24 h and remained constant throughout the experiment (data not shown). At the $6 \times 6 \times 6$ mm³ sorghum, the initial total sugar and reducing sugar in the juice were 229.27 ± 3.62 and 161.00 ± 0.41 g L⁻¹, respectively. However, the sugars were not completely consumed at the end of the fermentation. The total sugar and reducing sugar remaining in the fermented broth were 31.89 ± 0.07 and 27.05 ± 0.24 g L⁻¹, respectively. This might be due to nutrient deficiency and/or the inhibition by high ethanol concentration in the broth at the end of the fermentation. Extension of the fermentation time longer than 60 h might further decrease only small amount of the remaining sugars because only 13–15 g L⁻¹ of the sugars were utilized from 48 h to 60 h (Figure 1). Similar values of these two sugars at the end of the fermentation indicated that all sucrose containing in the sweet sorghum juice was converted to glucose and fructose during the fermentation. The initial freely suspended cell concentrations in the broth was 3.5×10^6 cells mL⁻¹, then it was sharply increased to 7.3×10^7 cells mL⁻¹ in 12 h and remained constant throughout the experiment. The ethanol concentration was produced continuously until the end of the experiment with the value of 87.66 ± 1.41 g L⁻¹. The changes of sugars, cell and ethanol for $12 \times 12 \times 12$ mm³ and $20 \times 20 \times 20$ mm³ sorghums were similar to those of $6 \times 6 \times 6$ mm³ counterparts (Figure 1). Regarding the visual observation of the carriers before and after ethanol fermentation, it was found that physical characteristics of the $6 \times 6 \times 6$ and $12 \times 12 \times 12$ mm³ sorghums were not changed. On the other hand, some of the $20 \times 20 \times 20$ sorghum stalks were slightly macerated in the middle. Like other grasses

such as sugarcane bagasse, the pith of the sweet sorghum stalk constructed by several fibrous bundles meshed together in spongy tissue [27]. The diameter of the raw stalk cv. KKU 40 varied from 5 to 30 mm. Therefore, when the $20 \times 20 \times 20 \text{ mm}^3$ sorghums are prepared, it is inevitable to avoid the sponge area. This may cause the $20 \times 20 \times 20 \text{ mm}^3$ sorghum easier to be disrupted if they are reused.

Figure 1. Batch culture profiles of ethanol production from sweet sorghum juice by *S. cerevisiae* NP 01 immobilized on $6 \times 6 \times 6$ (●), $12 \times 12 \times 12$ (▲) and $20 \times 20 \times 20$ (■) mm^3 sorghum stalks. (A) Total sugar (solid lines) and reducing sugar (dash lines); (B) log viable suspended cell concentration (solid lines) and ethanol concentration (dash lines).



Liang *et al.* [26] studied ethanol production from sugarcane juice and molasses by immobilized yeast on sugarcane pieces. They reported that the immobilization occurred by physical adsorption due to electrostatic forces between the cell membrane and the carrier. In this study, the viable cells immobilized on the carrier at different sizes before use as the inoculum for ethanol production slightly increased when the carrier size increased; however the leaked cells that migrated into the fermentation broth of the three different carrier sizes at the beginning of the fermentation were similar (3.5×10^6 to 5×10^6 cells mL^{-1}). The suspended cell concentration in the broth was relatively high implying that sugar conversion occurred by both freely suspended cells and immobilized cells in the system. Table 1 summarizes the important parameters (P , Q_p and $Y_{p/s}$) of ethanol fermentation by the yeast immobilized on different sizes of sorghum stalk. The results showed that the sizes of sorghum stalk used as the carrier did not significantly affect $Y_{p/s}$, while the P and Q_p values of the $6 \times 6 \times 6$ and $12 \times 12 \times 12 \text{ mm}^3$ sorghums were approximately 2 to 5% higher than those of the largest sorghum. Similar results were observed by Yu *et al.* [18] who studied ethanol production using immobilized yeast on sorghum stalk from synthetic medium containing the total sugar of 200 g L^{-1} . They found that the ethanol productivity was decreased when the carrier size (5, 10 and 15-mm) increased. As the carrier size increased, the mass transfer in the inner sorghum stalk would become more difficult, and finally influence the fermentation productivity. However, it seemed that the mass transfer in the $6 \times 6 \times 6$ and $12 \times 12 \times 12 \text{ mm}^3$ sorghums in our study might not be restricted as the ethanol production efficiencies of these two carrier sizes were not different. It was reported that pretreatment of the carriers by cellulase addition could increase specific surface areas of the carriers for cell attachment [20]. However, this method was not applied in our study because it would increase the cost of the carrier pretreatment for ethanol production. In addition, the largest size was not suitable for the repeated-batch fermentation because of the easily disrupted sponge area as previously described. Considering the

external surface area of sorghum stalk, the $6 \times 6 \times 6 \text{ mm}^3$ sorghum had more surface area than the $12 \times 12 \times 12 \text{ mm}^3$ sorghum if biomass of sorghum was equal. Therefore, the $6 \times 6 \times 6 \text{ mm}^3$ sorghum stalk was selected to be used in the following experiments.

Table 1. Fermentation parameters at 60 h of batch ethanol production from sweet sorghum juice by immobilized yeast on different sizes of sorghum stalk.

Sizes of sorghum stalk (mm^3)	Parameters (mean \pm SD)		
	P (g L^{-1})	Q_p ($\text{g L}^{-1} \text{ h}^{-1}$)	$Y_{p/s}$ (g g^{-1})
$6 \times 6 \times 6$	87.66 ± 1.41^a	1.46 ± 0.02^a	0.43 ± 0.00^a
$12 \times 12 \times 12$	86.09 ± 0.29^a	1.43 ± 0.01^a	0.44 ± 0.01^a
$20 \times 20 \times 20$	83.69 ± 0.58^b	1.39 ± 0.01^b	0.43 ± 0.00^a

P , ethanol concentration; Q_p , ethanol productivity and $Y_{p/s}$ ethanol yield. ^{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.

3.2. Effects of Initial Cell Concentration for Cell Immobilization on Sorghum Stalk

High yeast cell concentration had been widely used for immobilization in many researches. The cell concentration of $2 \times 10^8 \text{ cells mL}^{-1}$ was used as the initial cell concentration for yeast immobilization on calcium alginate, corncob and sorghum bagasses, while the $3.8 \times 10^8 \text{ cells mL}^{-1}$ was used for yeast immobilization on sugarcane pieces [18,19,27]. The use of high cell concentration for immobilization causes an increase in the operation cost in an industrial scale. Therefore, in this experiment, the effects of initial cell concentration for immobilization on ethanol production were investigated. The sterile $6 \times 6 \times 6 \text{ mm}^3$ sorghum stalks were transferred into 100 mL YM broth containing active *S. cerevisiae* NP 01 at the concentrations of 0.5×10^8 , 1×10^8 and $2 \times 10^8 \text{ cells mL}^{-1}$. After 24 h, the cell concentrations immobilized in the stalks were enumerated and the cell concentrations immobilized on the stalks at the initial cell concentrations of 0.5×10^8 , 1×10^8 and $2 \times 10^8 \text{ cells mL}^{-1}$ were found to be $(6.72 \pm 0.01) \times 10^9$, $(7.95 \pm 0.11) \times 10^9$ and $(1.03 \pm 0.09) \times 10^{10} \text{ cells g}^{-1}$ dry weight of sorghum, respectively. The results showed that when the initial cell concentration for immobilization was increased, the cell numbers in the carrier increased slightly.

The time profiles of total and reducing sugars, viable suspended cells and ethanol concentration in the broth during the batch ethanol fermentation by the immobilized yeast prepared under the different initial cell concentrations for immobilization are compared in Figure 2. The changes of total sugar, reducing sugar and cell concentrations were similar among the different immobilization conditions, while the ethanol concentrations were similar in the first 24 h. After that, the ethanol concentration increased with increasing in the initial cell concentration for immobilization. However, after 48 h, the initial cell concentrations at 1.0×10^8 and $2.0 \times 10^8 \text{ cells mL}^{-1}$ gave the same ethanol concentrations.

Figure 2. Batch culture profiles of ethanol production from sweet sorghum juice by *S. cerevisiae* NP 01 immobilized on $6 \times 6 \times 6$ mm³ sorghum stalks. The immobilized yeast cells were prepared at different initial cell concentrations in YM medium: (●) = 0.5×10^8 , (▲) = 1.0×10^8 and (■) = 2.0×10^8 cells mL⁻¹. (A) total sugar (solid lines) and reducing sugar (dash lines); (B) log viable suspended cell concentration (solid lines) and ethanol concentration (dash lines).

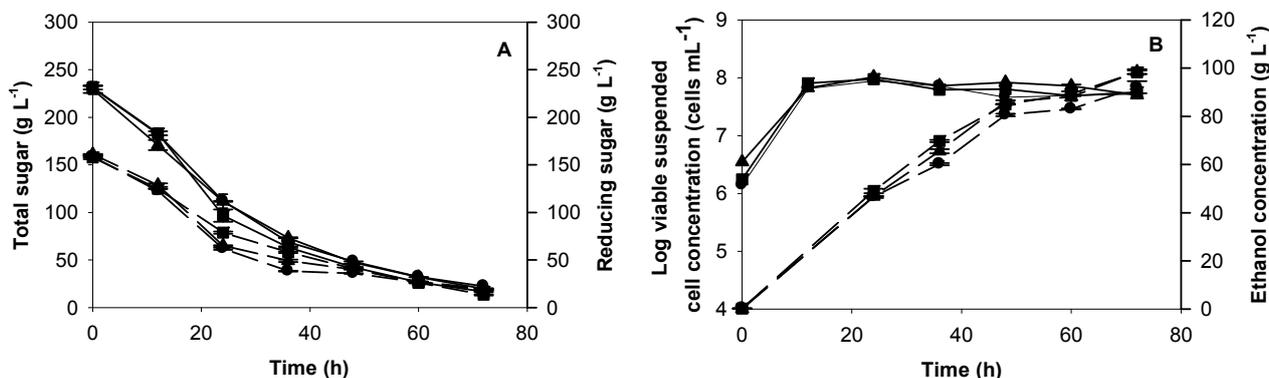


Table 2 summarizes the important parameters of ethanol fermentation under the different initial cell concentrations for immobilization.

Table 2. Fermentation parameters at 72 h of batch ethanol production from sweet sorghum juice by immobilized yeast. The immobilized yeast cells were prepared at different initial cell concentrations in YM medium.

Initial cell concentration for immobilization (cells mL ⁻¹)	Parameters (mean ± SD)		
	P (g L ⁻¹)	Q_p (g L ⁻¹ h ⁻¹)	$Y_{p/s}$ (g g ⁻¹)
0.5×10^8	92.08 ± 2.50 ^b	1.28 ± 0.04 ^b	0.44 ± 0.00 ^b
1.0×10^8	98.48 ± 1.03 ^a	1.37 ± 0.01 ^a	0.47 ± 0.01 ^a
2.0×10^8	98.27 ± 0.73 ^a	1.36 ± 0.01 ^a	0.46 ± 0.00 ^a

* P , ethanol concentration; Q_p , ethanol productivity and $Y_{p/s}$ ethanol yield. ^{a,b} Means having the same letter in the same column are not significantly different at $P < 0.05$.

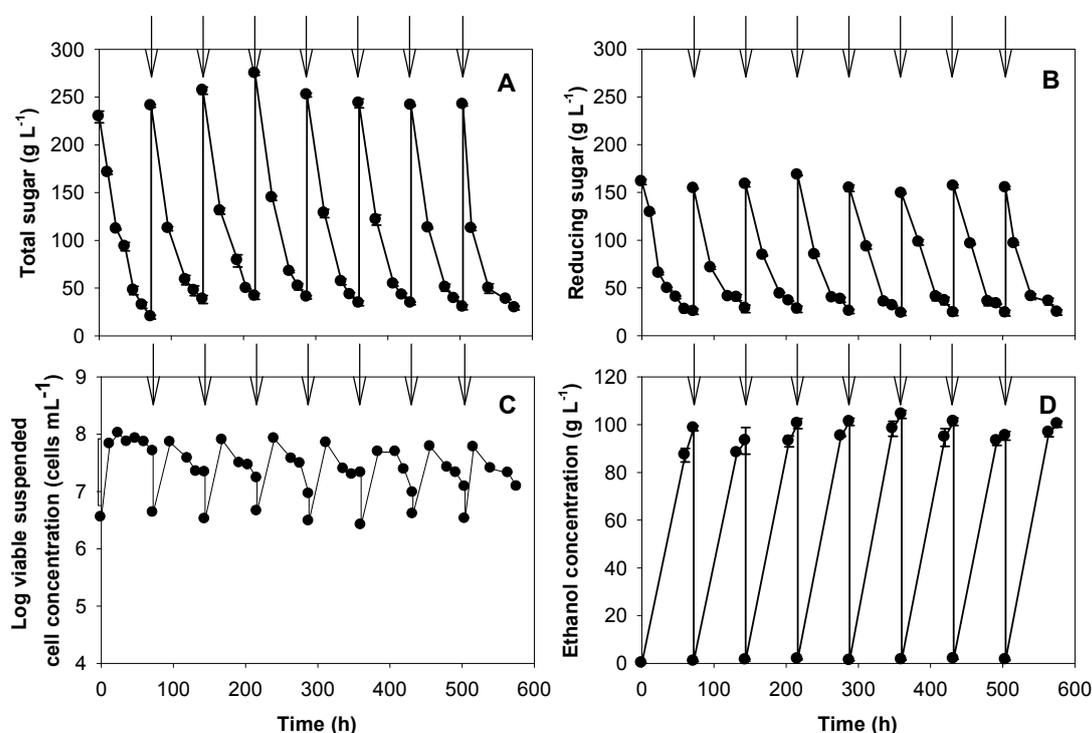
The results showed that the initial cell concentration for immobilization up to 1.0×10^8 cells mL⁻¹ had significant effects on the main fermentation parameters. Higher ethanol production efficiencies were obtained at the initial cell concentration of 1.0×10^8 and 2.0×10^8 cells mL⁻¹. The P , Q_p and $Y_{p/s}$ values at these two initial cell concentrations were not significantly different. To reduce the cost of inoculum preparation; therefore, the initial cell concentration of 1.0×10^8 cells mL⁻¹ was selected as the optimum initial cell concentration for immobilization in the subsequent experiments.

3.3. Repeated-Batch Ethanol Fermentation by Immobilized Yeast on Sorghum Stalk

The repeated-batch ethanol fermentation from the sweet sorghum juice by the immobilized yeast was carried out. Changes of all parameters measured during the fermentation are illustrated in Figure 3, and the main fermentation parameters are shown in Table 3. In batch 1, the sugar was almost utilized in 72 h with the ethanol concentration of 98.48 g L⁻¹. In batches 2 to 8, the residual sugar

concentrations were approximately 21 to 41 g L⁻¹, while the ethanol concentrations at 72 h (93.24 to 104.26 g L⁻¹) were similar to that of batch 1. The Q_p and $Y_{p/s}$ values of batches 1 to 8 were also similar ranging from 1.28 to 1.43 g L⁻¹ h⁻¹ and 0.45 to 0.48 g g⁻¹, respectively. The initial cell concentrations in the broth of batches 2 to 8 were $(2.56$ to $4.50) \times 10^6$ cells mL⁻¹ indicating that the immobilized cells in each cycle were able to grow and leaked or migrated into the fermenting broth. The sorghum stalks containing yeast cells seemed to act as the source of inoculum for ethanol production. The cell number in the broth increased to almost one log scale at the end of each cycle implying that the sweet sorghum juice contained some essential nutrients for yeast growth.

Figure 3. Repeated-batch culture profiles of ethanol production from sweet sorghum juice by *S. cerevisiae* NP 01 immobilized on 6 × 6 × 6 mm³ sorghum stalks. (A) total sugar; (B) reducing sugar; (C) viable suspended cell concentration; and (D) ethanol concentration. The arrows indicate the start time of each cycle.



At the end of batch 8, the sorghum stalks were collected and blended to determine the viable cell concentration in the carrier. The total viable immobilized cells were $(2.54$ to $3.92) \times 10^9$ cells g⁻¹ dry weight of sorghum stalk. Similar values were observed by de Vasconcelos *et al.* [28] who found that in continuous ethanol fermentation, the concentration of immobilized cells on sugarcane stalk after 60 days operation was approximately 10⁹ cells g⁻¹ of dry stalk. Higher cell density was reported by Liang *et al.* [26], who studied repeated-batch ethanol production using immobilized *S. cerevisiae* AS2.1190 on sugarcane pieces from sugarcane juice and molasses containing total sugar concentration of 176 and 154 g L⁻¹, respectively. They found that at the end of batch 10, the viable immobilized cells were 1.5 × 10¹¹ cells g⁻¹ of the carrier. The higher viable cells in the carrier compared with our results might be due to the differences in the type of carrier and the initial sugar concentration used. In our study, the initial sugar concentration was 230 g L⁻¹ which might inhibit yeast growth to some

extent [29,30]. Only eight cycles were conducted in this study due to our limited supplies of sweet sorghum juice. However, relatively high cell concentrations were maintained in the fermentation broth and in the carriers after eight successive cycles of the repeated-batch fermentation. Also, the ethanol production efficiencies (P , Q_p and $Y_{p/s}$) did not decrease, implying that the immobilized cells could be reused several times in the following batches.

Table 3. Comparison of the fermentation parameters of ethanol production from the sweet sorghum juice by the immobilized cell and free cell systems in the repeated-batch fermentation.

Batch number	Parameters (mean \pm SD)				Cell concentration in the broth (cells mL ⁻¹)		Reference
	P (g L ⁻¹)	Q_p (g L ⁻¹ h ⁻¹)	$Y_{p/s}$ (g g ⁻¹)	t (h)	Initial	Final	
1	98.48 \pm 1.03 ^{bc}	1.37 \pm 0.01 ^a	0.47 \pm 0.01 ^{ab}	72	3.50 \times 10 ⁶	7.20 \times 10 ⁷	This study
2	93.24 \pm 5.57 ^d	1.28 \pm 0.08 ^b	0.46 \pm 0.04 ^{ab}	72	4.25 \times 10 ⁶	2.15 \times 10 ⁷	
3	100.39 \pm 2.10 ^{ab}	1.38 \pm 0.04 ^a	0.46 \pm 0.01 ^{ab}	72	3.25 \times 10 ⁶	1.70 \times 10 ⁷	
4	101.15 \pm 1.49 ^{ab}	1.38 \pm 0.02 ^a	0.46 \pm 0.01 ^{ab}	72	4.50 \times 10 ⁶	0.90 \times 10 ⁷	
5	104.26 \pm 1.66 ^a	1.43 \pm 0.02 ^a	0.47 \pm 0.00 ^{ab}	72	3.00 \times 10 ⁶	2.10 \times 10 ⁷	
6	101.25 \pm 1.54 ^{ab}	1.39 \pm 0.02 ^a	0.48 \pm 0.02 ^a	72	2.56 \times 10 ⁶	0.95 \times 10 ⁷	
7	95.30 \pm 1.78 ^{cd}	1.30 \pm 0.03 ^b	0.45 \pm 0.01 ^b	72	4.00 \times 10 ⁶	1.20 \times 10 ⁷	
8	100.21 \pm 1.27 ^{ab}	1.37 \pm 0.01 ^a	0.46 \pm 0.00 ^{ab}	72	3.25 \times 10 ⁶	1.20 \times 10 ⁷	
Average	99.28 \pm 3.53	1.36 \pm 0.05	0.47 \pm 0.03	576 *	3.54 \times 10 ⁶	2.18 \times 10 ⁷	This study
Average free cells	95.97 \pm 7.95	1.30 \pm 0.41	0.50 \pm 0.04	468 *	4.91 \times 10 ⁷	1.36 \times 10 ⁸	[11]

P , ethanol concentration; Q_p , ethanol productivity; $Y_{p/s}$ ethanol yield and t , fermentation time. ^{a, b, ab, bc, cd and d} Means having the same letter in the same column are not significantly different at $P < 0.05$. * Total fermentation time.

The parameters of repeated-batch ethanol fermentation by the immobilized yeast cells were compared with those of free cells as reported by Ariyajarearnwong *et al.* [11] (Table 3). It was found that the fermentation time in batch 1 (48 h) and batches 2 to 8 (60 h each) of the free cell system was 24 and 12 h shorter than that of the immobilization system, respectively; however, the average P and $Y_{p/s}$ values of both systems in eight successive batches were not significantly different. In the immobilization system, the suspended cells in the broth of each batch increased with the average cell production of 1.83×10^7 cells mL⁻¹. When the total ethanol production rate (g ethanol day⁻¹) was calculated, this value of the immobilization system (11.52 g ethanol day⁻¹) was higher than that of the free cell system (10.32 g ethanol day⁻¹). The reason was that all fermented broth was harvested in the immobilized cell system, while only 75% of the working volume was drained in the free cell system. Therefore, this could be accounted as one of the advantages of using the immobilized cell system over the free cell system under repeated-batch fermentation. In addition, it was found that the physical observation of the carriers containing the immobilized cells before and after eight successive cycles of ethanol fermentation were not different (Figure 4), indicating that the sorghum stalk was suitable to be used as the carrier for cell immobilization.

Figure 4. Comparison of sorghum stalk pieces before (A) and after (B) the ethanol fermentation for the eight successive batches.



Sweet sorghum was also used as the carrier for cell immobilization in repeated-batch ethanol fermentation [18]. A comparison of using sweet sorghum as the carrier between Yu *et al.* [18] and our study is shown in Table 4. The advantages of using sorghum stalk in our study over Yu *et al.* [18] are the lack of carrier pretreatment (only size reduction) and lower initial cell concentrations for the immobilization. In our study, the fresh sorghum stalk was used directly for cell immobilization, while the stalk was dried before immobilization in Yu *et al.* [18], resulting in higher cell concentration after immobilization in the dried sorghum stalk. Rapid sugar consumption was observed in Yu's work with the fermentation time of 16 h, resulting in high ethanol productivity. This might be due to very high proportion between carrier and fermentation broth at the ratio of 1:1. Even though the cell concentration in the carrier after immobilization of our study was 3 folds lower than that of Yu *et al.* [18], ethanol concentrations of the two studies were comparable. In addition, the total ethanol production rate in terms of g ethanol produced day⁻¹ in our study was 58% higher than that previously reported by Yu *et al.* [18]. When the ethanol production efficiencies in the repeated-batch fermentation by *S. cerevisiae* NP 01 immobilized on the sorghum stalks were compared with the values obtained from the immobilization on corncobs [19]. It was found that the yeast immobilized on the sorghum stalks gave significantly higher ethanol concentration than those ($90.75 \pm 3.05 \text{ g L}^{-1}$) on the corncobs.

Table 4. Comparison of repeated-batch ethanol fermentation by yeast cells immobilized on sweet sorghum stalk.

Operating conditions	Yu <i>et al.</i> [18]	This study
Reactor and working volume	Column reactor with working volume of 50 mL	500-mL air-locked Erlenmeyer flasks with working volume of 350 mL
Yeast strain	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> NP 01
Fermentation medium	Synthetic medium containing 100 g L ⁻¹ of glucose and 100 g L ⁻¹ of sucrose	Sweet sorghum juice containing 230 g L ⁻¹ of total sugar without nutrient supplementation
Carrier for cell immobilization	dried sweet sorghum (10 × 10 × 10 mm ³)	Fresh sweet sorghum (6 × 6 × 6 mm ³)

Table 4. Cont.

Operating conditions	Yu <i>et al.</i> [18]	This study
Initial cell concentrations for the immobilization (cells mL ⁻¹)	2.0×10^8	1.0×10^8
Cell concentration after immobilization (g DCW g ⁻¹ DW of carrier)	0.6	0.2
Carrier (g) : fermentation medium (mL)	1:1 (50 g : 50 mL)	1:7 (52.5 g : 350 mL)
Total batches (cycles)	21	8
Fermentation time of each batch (h)	16	72
Total fermentation time (days)	14	24
Average ethanol concentration (g L ⁻¹)	96 (batches 1–13), 100 (batches 14–21)	99 (8 batches)
Ethanol yield (g g ⁻¹)	0.48	0.47
Total ethanol production rate (g ethanol day ⁻¹)	7.31	11.52

4. Conclusions

Sweet sorghum stalks, a low cost carrier, were found to be suitable as a support material for yeast cell immobilization. Sizes of the stalk pieces ($6 \times 6 \times 6$ to $20 \times 20 \times 20$ mm³) did not affect ethanol production efficiency. However, the $20 \times 20 \times 20$ mm³ sorghum stalk pieces might be more easily degraded and/or broken down when it was reused in repeated-batch fermentations because it contained the sponge area. The initial cell concentration in the fresh YM medium at 1×10^8 cells mL⁻¹ was optimum for using in cell immobilization process on the sorghum stalk. The immobilized yeast cells on the $6 \times 6 \times 6$ mm³ sorghum stalk could be reused at least eight successive batches without any losses of ethanol production efficiencies. A slice of sorghum stalk without skin removal will be further studied because the skin may help maintain the structure of the carrier when it is used in repeated-batch fermentation. This will also reduce the cost and time of the carrier preparation and will be more practical for ethanol production in industrial scales.

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