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Batch and Continuous Ethanol Fermentation by *Saccharomyces cerevisiae* NP 01 Using Cell Recycling Technique

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Abstract

The aims of this research were to compare batch ethanol production efficiency of *Saccharomyces cerevisiae* NP 01 between using sweet sorghum juice (230 g/l of total sugar) and three synthetic media (230 g/l of sucrose) as substrates and to study the effects of nitrogen supplementation on the ethanol fermentation from the juice. Continuous ethanol fermentation with and without cell recycling system were also studied. In the batch fermentation at 30°C, the results showed that nitrogen supplementation obviously improved cell growth, sugar consumption and ethanol production. The juice supplemented with 6 g/l of yeast extract gave the highest ethanol concentration (*P*, 111.57 g/l) and ethanol productivity (Q_P, 2.32 g/l.h). For the synthetic media, the modified Melzoch's medium containing 6 g/l of yeast extract gave the highest *P* (109.71 g/l) and Q_P (1.31 g/l.h). When the continuous ethanol fermentation without cell recycling system using the modified Melzoch's medium was operated at the dilution rate of 0.02 h^{-1}, it was found that the aeration rate of 0.2 vvm promoted yeast growth but it did not improve sugar consumption and ethanol production. At the steady state, the cell and residual sugar concentrations were 1.76×10^8 cells/ml and 107.03 g/l, respectively corresponding to *P* and Q_P of 45.51 g/l and 0.90 g/l.h, respectively. When the continuous fermentation with cell recycling system was performed at the recycled ratio of 2, almost sugar was utilized. At the steady state, the cell and residual sugar concentrations were 2.86×10^8 cells/ml and 16.59 g/l, respectively corresponding to *P* and Q_P of 75.43 g/l and 1.49 g/l.h, respectively.

Keywords: Ethanol, cell recycling system, continuous fermentation, sweet sorghum, *Saccharomyces*

1. Introduction

Nowadays, the research effort on ethanol production is looking for new and effective nutritional sources and progressive fermentation techniques which can achieve both high substrate consumption and high productivity. There are three main fermentation processes i.e. batch, fed-batch and continuous systems. In general, the continuous fermentation gives higher yield and better efficiency than other fermentation processes by providing both high product concentration and high productivity (1). An important key consideration of process improvement is an appropriate level of cell concentration during the fermentation process since it affects product concentration, productivity as well as sugar consumption rate (2). Moreover, a large quantity of cells with high activity can

successfully repress bacterial contamination (3). Although, the continuous fermentation process has more advantages over other fermentation techniques, some of cell proportion in an effluent is withdrawn from the cultivation system. Consequently, recycling of the cells back to the continuous fermentation system will increase cell concentration during the fermentation process. Therefore, cell recycling technique is an important process for improvement of continuous ethanol fermentation by involving the cell circulation in the process resulting in a high biomass concentration.

In Thailand, the main raw materials for ethanol production are sugarcane molasses and cassava. Regarding to the energy policy, Thai government has planned to enlarge ethanol production from 3,000,000 to 9,000,000 litres/day within 15 years (by 2008 to 2022) (4). Therefore, it is possible that Thailand may face a shortage of these materials for ethanol production.

Sweet sorghum, a rapid growing plant, can be easily cultivated in dried and warmer areas. The juice from its stalk contains high fermentable sugar (5). Therefore, it has been a particular interest as a substrate for ethanol production. In addition, other nutrients especially nitrogen source are required to improve yeast growth and ethanol production (6).

This research aims to compare the batch ethanol production of *Saccharomyces cerevisiae* NP 01 from sweet sorghum juice, a high potential raw material for ethanol production and synthetic media under different nutrient supplementations and to preliminarily investigate the continuous ethanol fermentation from a synthetic medium with and without cell recycling system.

2. Materials and methods

2.1 Microorganism and inoculum preparation

S. cerevisiae NP 01 was kindly donated by Asst. Prof. Dr. Paiboon Danvirutai from Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand. This strain was isolated from Loog-Pang (dried starter for rice wine production) from Nakorn Pranom province, Thailand. *S. cerevisiae* NP 01 was grown in yeast extract malt extract (YM) broth containing 10 g/l of glucose on a rotary shaker of 200 rpm, at 30 ^{o}C for 18 h. To increase cell concentration, the 18 h pre-culture was transferred into YM broth containing 150 g/l of glucose to obtain the initial cell concentration approximately $5x10^{6}$ cells/ml. The culture was grown under the same conditions for 18 h before use as the inoculum for ethanol production.

2.2 Ethanol production medium

The ethanol production media were classified into 2 groups as follows:

1) Sweet sorghum juice cv. KKU40 (faculty of Agriculture, Khon Kaen University) containing 230 g/l of total sugar supplemented with 0, 3 and 6 g/l of yeast extract. The composition of the raw juice was reported by Laopaiboon *et al.* (5).

2) The three synthetic media containing 230 g/l of sucrose. The compositions of the media are shown in Table 1.

Table 1. Compositions of three synthetic media

Synthetic media	Compositions
SM1	Sucrose, 230 g/l; peptone, 5 g/l and yeast extract, 3 g/l (7)
SM2	Sucrose, 230 g/l and yeast extract, 6 g/l
SM3 (modified Melzoch's medium)	Sucrose, 230 g/l; yeast extract, 6 g/l; $ZnSO_4.7H_2O$, 1.4 mg/l; $MnSO_4.H_2O$, 3 mg/l; KH_2PO_4, 0.5 g/l; $MgSO_4.7H_2O$, 0.025 g/l; $(NH_4)_2SO_4$, 1 g/l and $CaCl_2.2H_2O$, 1 g/l (modified from 8)

2.3 Fermentation conditions

2.3.1 Batch ethanol fermentation

The ethanol production medium was transferred into a 2-litre fermenter with a final working volume of 1 litre and autoclaved at 110 °C for 28 min. The ethanol fermentation was carried in batch system by *S. cerevisiae* NP 01 at the initial cell concentration of 2.0 to $3.0x10^7$ cells/ml, 30 °C, and the agitation rate of 200 rpm. Aeration was provided at 2.5 vvm for the first 4 h of the fermentation (9).

2.3.2 Continuous fermentation without cell recycling system

The optimum synthetic medium (from the batch experiment) was used as substrate. The continuous fermentation was first carried out as in batch mode. When the total sugar in the broth remained about half of the initial value as found in the batch fermentation, the sterile medium was fed to the system at the dilution rate of 0.02 h^{-1} (7) without aeration.

2.3.3 Continuous fermentation with cell recycling system

The continuous fermentation with cell recycling system is shown in Figure 1. The ultrafiltration module for cell recycling was the hollow fibre with pore size of 0.65 μm (Pall Corporation Co. Ltd., Japan). The system was first carried out as in batch mode. When the total sugar in the fermented broth remained approximately half of the initial value, the sterile medium was fed to the system at the dilution rate of 0.02 h^{-1} (7) and aeration was continuously supplied at the rate of 0.2 vvm (10). According to Tang *et al.* (3), the recycled ratio (α) of this process was fixed at 2. This value corresponded to the flow rate of the feeding fresh medium and cell circulation into the fermenter at 20 and 40 ml h^{-1}, respectively.

2.4 Analytical methods

During the fermentations, the samples were withdrawn aseptically for analysis. The total sugar and viable cell concentration were measured by phenol sulfuric acid method (12) and direct counting method using haemacytometer with methylene blue staining technique (13), respectively. Ethanol concentration was assayed by gas chromatography (5).

3. Results and Discussion

3.1. Comparison of batch ethanol fermentation efficiency from sweet sorghum juice and synthetic media

Batch profiles of ethanol fermentation by *S. cerevisiae* NP 01 from the sweet sorghum juice supplemented with yeast extract at different concentrations

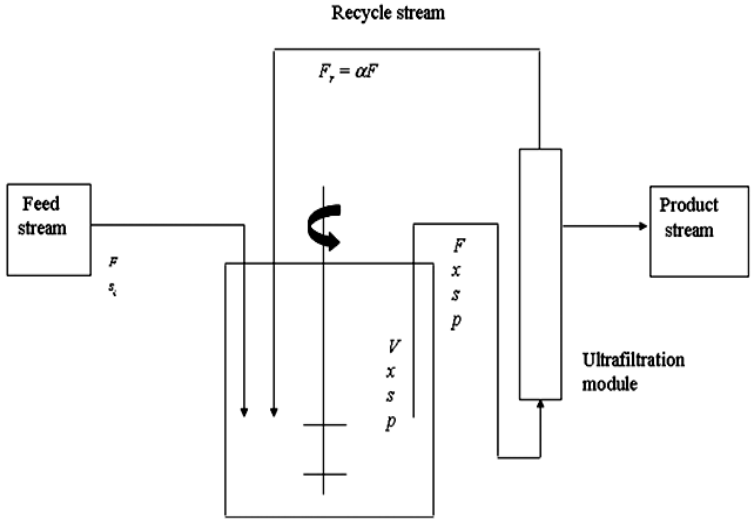

Figure 1. Schematic diagram of cell recycling system. F, flow rate; F_r, recycled flow rate; α, recycled ratio; V, working volume; x, cell concentration; s, substrate concentration; p, product concentration (modified from 11).

are shown in Figure 2. Changes of pH in all treatments were similar throughout the experiments. Under no nutrient supplementation, the viable yeast cell concentrations significantly increased in 12 h. After that they were relatively constant with the values of 2.13×10^8 cells/ml. At the end of the fermentation, the residual sugar and ethanol concentrations were 77.95 g/l and 70.16 g/l, respectively when using the sweet sorghum juice without nutrient supplementation (Table 2). These results might be due to nutritional insufficiency in the juice (14). Due to low ethanol concentration and low sugar consumption, the juice was supplemented with 3 and 6 g/l of yeast extract. The results showed that the juice supplemented with 6 g/l of yeast extract gave the highest sugar consumption of 96.95 % corresponding to ethanol concentration (P) of 111.57 g/l. Under this condition, the ethanol productivity (Q_P) and ethanol yield ($Y_{P/S}$) were 2.32 g/l.h and 0.50 g/g, respectively (Table 2). These results indicated that supplementation of yeast extract improved the sugar consumption and ethanol production of *S. cerevisiae* NP 01 during the fermentation. Higher yeast extract concentration was not further studied because it would increase the ethanol production cost and the sugar consumption was almost completely consumed at 6 g/l of yeast extract.

Several synthetic ethanol production media were successfully used for ethanol production. In this study, the three synthetic media (Table 1) were selected because the initial sugar concentrations were comparable to that of the sweet sorghum juice. Batch profiles of ethanol fermentation by *S. cerevisiae* NP 01 from the synthetic media are shown in Figure 3. The results showed that the highest sugar consumption (96.03 %) and P (109.71 g/l) were obtained when the SM3 medium or the modified Melzoch's medium was used as the substrate. Under this condition, Q_P and $Y_{P/S}$ were 1.31 g/l.h and 0.50 g/g, respectively (Table 2).

When the ethanol production efficiencies were compared between using the sweet sorghum juice supplemented with 6 g/l of yeast extract and the SM3 medium, it was found that P and $Y_{P/S}$ of the two media were similar, while Q_P of the SM3 medium was only 56 % of the other (Table 2). The results indicated that some trace elements in the sweet sorghum juice (absence in the SM3 medium) could promote ethanol production rate.

Table 2. The fermentation parameters of batch ethanol fermentation by *S. cerevisiae* NP 01 from different ethanol production (EP) media.

EP media	*RTS* (g/l)	S_C (%)	P (g/l)	Q_P (g/l.h)	$Y_{P/S}$ (g ethanol / g sugar utilized)	t (h)
SSJ (control)*	77.95 ± 0.74	66.11 ± 0.32	70.16 ± 0.57	0.97 ± 0.01	0.46 ± 0.01	72
SSJ (3 YE)*	29.74 ± 1.07	87.06 ± 0.47	91.48 ± 0.74	1.27 ± 0.01	0.46 ± 0.01	72
SSJ (6 YE)*	7.01 ± 0.95	96.95 ± 0.41	111.57 ± 0.45	2.32 ± 0.01	0.50 ± 0.00	48
SM1**	65.0 ± 0.62	72.22 ± 0.23	81.17 ± 0.47	0.97 ± 0.01	0.48 ± 0.00	84
SM2**	29.82 ± 1.41	87.15 ± 0.61	96.67 ± 0.23	1.15 ± 0.00	0.49 ± 0.00	84
SM3**	9.45 ± 1.02	96.03 ± 0.44	109.71 ± 0.35	1.31 ± 0.00	0.50 ± 0.00	84

*SSJ(control), sweet sorghum juice without yeast extract supplementation; SSJ (3YE), sweet sorghum juice supplemented with 3 g/l of yeast extract; SSJ (6YE), sweet sorghum juice supplemented with 6 g/l of yeast extract.

**See Table 1.

RTS, residual total sugar; S_c, sugar consumption; P, ethanol concentration; Q_P, ethanol productivity; $Y_{P/S}$, ethanol yield and t, fermentation time.

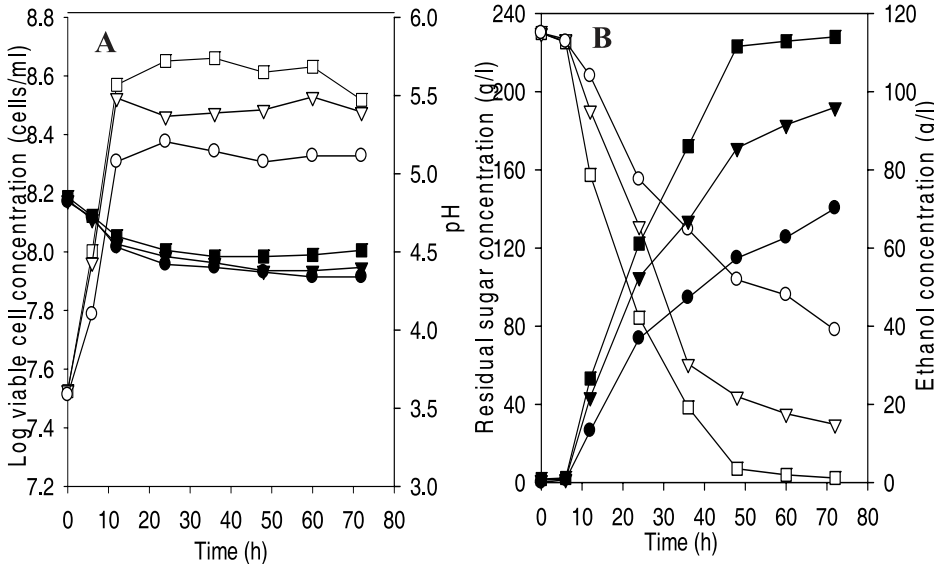

Figure 2. Batch profiles of ethanol fermentation from sweet sorghum juice containing 230 g/l of total sugar under different yeast extract supplementations. Control, (○,●); 3 g/l of yeast extract, (▽,▼) and 6 g/l of yeast extract, (□,■) (A) : Log viable cell concentration, (open symbol) and pH, (close symbol) (B) : residual sugar concentration, (open symbol) and ethanol concentration, (close symbol).

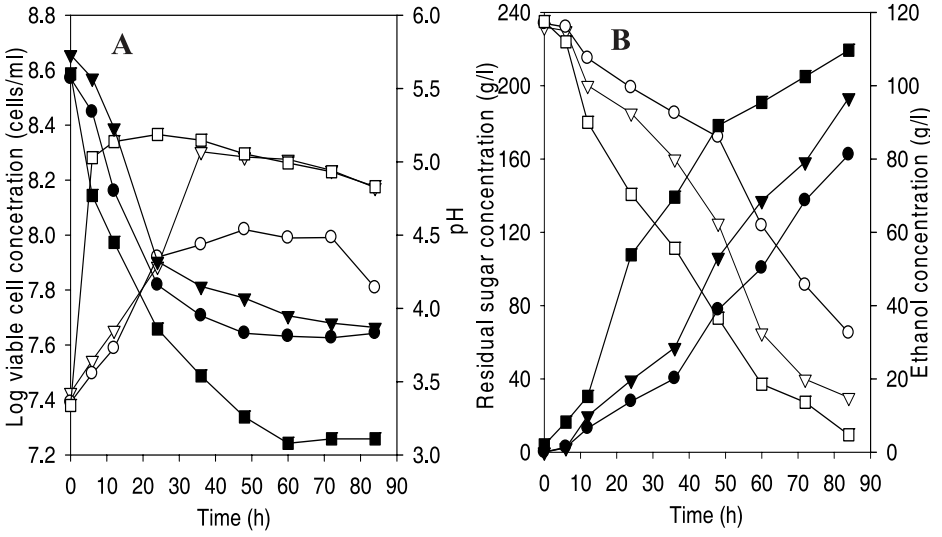

Figure 3. Batch profiles of ethanol fermentation from the synthetic media containing 230 g/l of sucrose under different nutrient supplementations. SM1, (○,●); SM2, (▽,▼) and SM3 (□,■) (A) : Log viable cell concentration, (open symbol) and pH, (close symbol) (B) : residual sugar concentration, (open symbol) and ethanol concentration, (close symbol).

The sweet sorghum juice extracted from its stalks contained some insoluble solids. This may cause membrane blocking in the hollow fibre of the recycling system in the next experiments. Therefore, the SM3 medium was selected to be the substrate for the continuous ethanol fermentation with and without cell recycling system.

3.2 Comparison of continuous ethanol fermentation efficiency by the process with and without cell recycling system

The profiles of continuous ethanol fermentation from SM3 medium without cell recycling system are shown in Figure 4. The process was initiated as in the batch fermentation with the aeration rate of 2.5 vvm for 4 h (9), the continuous fermentation was started at 18 h without aeration. The results showed that sugar consumption under steady state was only 52.34 % of the initial value corresponding to the average residual sugar concentration, P and Q_p of 112.01 g/l, 52.77 g/l and 1.04 g/l.h, respectively; while the viable cell concentration was considerably decreased from 2.21×10^8 cells/ml (at 18 h) to 5.3×10^7 cells/ml (Table 3). The results might be due to lack of oxygen for cell growth and maintenance (2). Therefore, the aeration had been continuously supplied at 0.2 vvm in order to raise the cell concentration since 169 h (Figure 4). The results showed that the viable cell concentration turned to be dramatically increased with the average value of 1.76×10^8 cells/ml under the steady state. However, the sugar consumption and ethanol production under the aeration condition were not significantly different from those under no aeration (Table 3). This might be due to non-appropriate level of aeration rate for ethanol production. This was supported by Lin *et al*. (2) who reported that levels of aeration supply during the fermentation significantly affected viable cell concentration, sugar consumption and ethanol production.

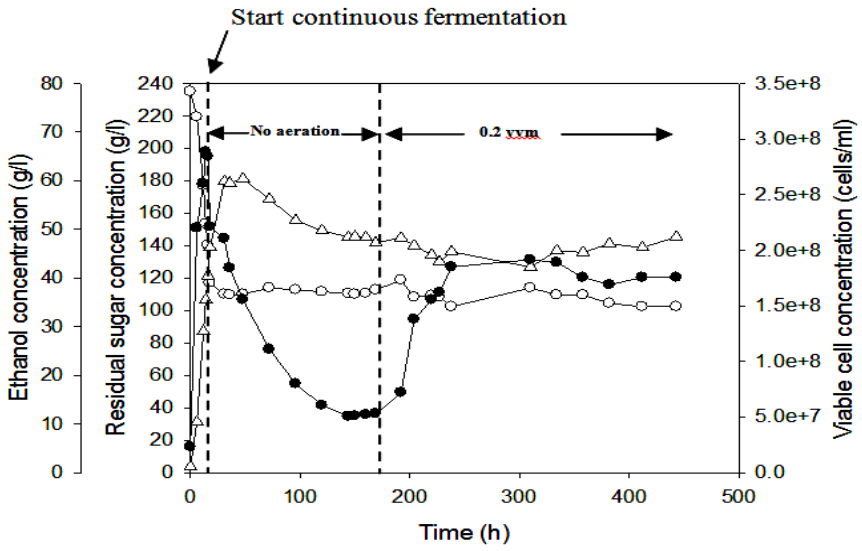

Figure 4. Profiles of ethanol production from SM3 medium in continuous fermentation without cell recycling system. Residual sugar concentration, (○); viable cell concentration, (●) and ethanol concentration, (△).

Table 3. Fermentation parameters of continuous ethanol fermentation with and without cell recycling system under steady state conditions.

Systems	Aeration (vvm)	X (cells/ml)	RTS (g/l)	S_C (%)	P (g/l)	Q_P (g/l.h)
without cell recycling	no	5.05 to 5.3×10^7	112.01 ± 1.21	52.34 ± 0.52	52.77 ± 1.29	1.04 ± 0.03
	0.2 vvm	1.69 to 1.76×10^8	107.03 ± 1.08	54.46 ± 0.50	45.51 ± 1.34	0.90 ± 0.02
with cell recycling (recycled ratio =2)	0.2 vvm	2.79 to 2.86×10^8	16.59 ± 0.65	92.85 ± 0.28	75.43 ± 0.22	1.49 ± 0.01

X, viable cell concentration; RTS, residual total sugar; S_c, sugar consumption; P, ethanol concentration and Q_P, ethanol productivity.

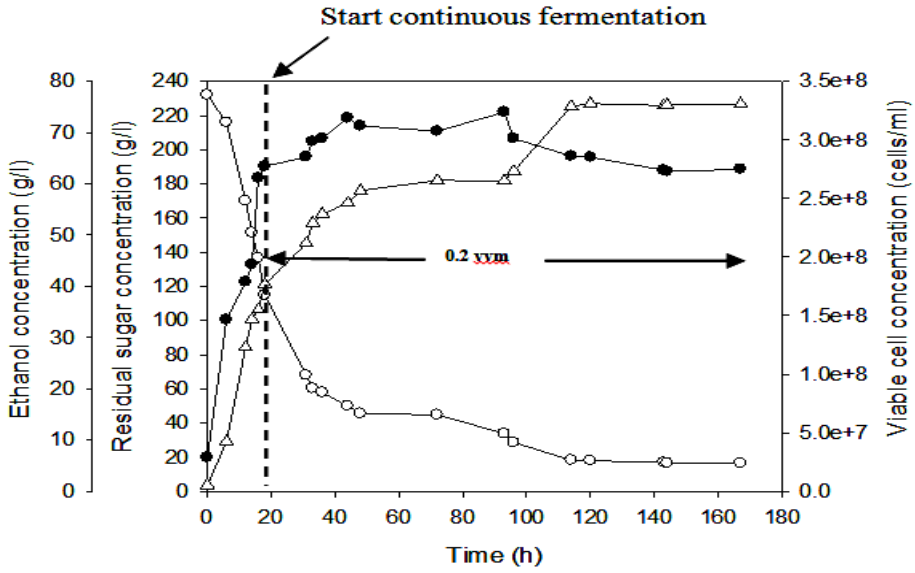

Figure 5. Profiles of ethanol production from SM3 medium in continuous fermentation with the cell recycled ratio of 2. Residual sugar concentration, (○); viable cell concentration, (●) and ethanol concentration, (△).

Regarding to the continuous ethanol fermentation with cell recycling system, the process was first carried out as that without cell recycling system (Figure 5). The aeration rate of 0.2 vvm was continuously supplied when the continuous fermentation was started at 18 h. At the recycled ratio of 2, the steady state was achieved in 114 h. The viable cell concentration was obviously increased with the average value of 2.86×10^8 cells/ml, while the residual sugar concentration was 16.59 g/l corresponding to sugar utilization of 92.85 %. The P and Q_P were increased to 75.43 g/l and 1.49 g/l.h, respectively (Table 3). The results obtained indicated that providing aeration coupling with cell recycling technique promoted both sugar consumption and ethanol production. In addition, the experiment also showed that the 0.65 μm hollow fibre and the recycled ratio of 2 were suitable for the preliminary study of the continuous fermentation with cell recycling system as there was no observation of membrane blocking of the hollow fibre.

Using microfiltration module for recycling the cells to the fermenter increased substantial numbers of the yeast cells during the fermentation. At the same level of aeration rate (0.2 vvm), the S_C, P and Q_P values of the process with cell recycling system were approximately 66 to 71 % higher than those of the process without cell recycling system. This indicated that the yeast numbers and cell recycling system markedly affected the continuous ethanol fermentation.

4. Conclusions

The sweet sorghum juice could be employed for ethanol fermentation under batch culture of *S. cerevisiae* NP 01. The supplementation of nitrogen source in the juice promoted both sugar consumption and ethanol production rates. The modified Melzoch's medium containing 6 g/l of yeast extract could be used to replace the sweet sorghum juice as the ethanol concentration and its yield of the two media were similar. In the continuous fermentation system at the aeration of 0.2 vvm, the viable cell concentration of the system with the cell recycled ratio of 2 was approximately 58 % higher than that without cell recycling system. Applying aeration coupling with cell recycling system for the continuous fermentation could improve sugar consumption, ethanol concentration as well as ethanol productivity.

5. Acknowledgements

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