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Development of High-Productivity Continuous Ethanol Production using PVA-Immobilized *Zymomonas mobilis* in an Immobilized-Cells Fermenter

Pengembangan Produktivitas dari Produksi Etanol Kontinyu Menggunakan *Zymomonas Mobilis* yang Di Immobilisasi PVA dalam Sel Fermenter Terimmobilisasi

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Abstract

Ethanol as one of renewable energy was being considered an excellent alternative clean-burning fuel to replace gasoline. Continuous ethanol fermentation systems had offered important economic advantages compared to traditional systems. Fermentation rates were significantly improved, especially when continuous fermentation was integrated with cell immobilization techniques to enrich the cells concentration in fermentor. Growing cells of *Zymomonas mobilis* immobilized in polyvinyl alcohol (PVA) gel beads were employed in an immobilized-cells fermentor for continuous ethanol fermentation from glucose. The glucose loading, dilution rate, and cells loading were varied in order to determine which best condition employed in obtaining both high ethanol production and low residual glucose with high dilution rate. In this study, 20 g/L, 100 g/L, 125 g/L and 150 g/L of glucose concentration and 20% (w/v), 40% (w/v) and 50% (w/v) of cells loading were employed with range of dilution rate at 0.25 to 1 h^{-1}. The most stable production was obtained for 25 days by employing 100 g/L of glucose loading. Meanwhile, the results also exhibited that 125 g/L of glucose loading as well as 40% (w/v) of cells loading yielded high ethanol concentration, high ethanol productivity, and acceptable residual glucose at 62.97 g/L, 15.74 g/L/h and 0.16 g/L, respectively. Furthermore, the dilution rate of 4 hour with 100 g/L and 40% (w/v) of glucose and cells loading was considered as the optimum condition with ethanol production, ethanol productivity and residual glucose obtained were 49.89 g/L, 12.47 g/L/h, and 2.04 g/L, respectively.

Keywords: ethanol, glucose fermentation, immobilized-cells fermenter, polyvinyl alcohol (PVA), *Zymomonas mobilis*

Abstrak

Sebagai salah satu energi terbarukan, etanol merupakan bahan bakar alternatif ramah lingkungan yang dapat digunakan sebagai pengganti bensin. Produksi etanol secara fermentasi menawarkan banyak keuntungan secara ekonomi dibandingkan produksi secara tradisional. Laju fermentasi meningkat secara signifikan, terutama setelah sistem diintegrasi dengan teknik sel immobilisasi untuk memperkaya konsentrasi sel dalam fermentor. Sel-sel *Zymomonas mobilis* yang diimobilisasi dalam polyvinyl alcohol (PVA) dimanfaatkan untuk proses produksi etanol dari fermentasi glukosa dalam fermentor dengan sel terimmobilisasi. Konsentrasi glukosa, laju dilusi dan konsentrasi sel divariasikan untuk menghitung kondisi terbaik untuk mendapatkan produksi etanol tertinggi serta residual glukosa yang rendah dengan laju dilusi yang tinggi. Pada penelitian ini, konsentrasi glukosa sebanyak , 20 g/L, 100 g/L, 125 g/L dan 150 g/L serta 20% (w/v), 40% (w/v) and 50% (w/v) sel digunakan dengan rentang laju dilusi pada 0,25 sampai 1 perjam. Produksi paling stabil dihasilkan selama 25 hari dengan menggunakan glukosa sebanyak 100 g/L. Sementara itu, hasil yang diperoleh juga menunjukkan bahwa penggunaan glukosa sebanyak 125 g/L dan konsentrasi sel 40% (w/v) menghasilkan kosentrasi dan produktivitas etanol yang tinggi serta residu glukosa yang bisa diterima yaitu 62.97 g/L, 15.74 g/L/h and 0.16 g/L berturut-turut. Selain itu, pada laju dilusi 4 jam dengan menggunakan konsentrasi glukosa dan sel sebanyak 100 g/L dan 40% (w/v) dianggap sebagai kondisi optimum proses dengan produk etanol, produktivitas dan residu glukosa yang dihasilkan yaitu berturut-turut sebanyak 49.89 g/L, 12.47 g/L/h, and 2.04 g/L.

Kata kunci: etanol, fermentasi glukosa, fermentor terimmobilisasi, polyvinyl alcohol (PVA), *Zymomonas mobilis*

1. Introduction

The ancient art alcohol fermentation has been documented for many years, and the well-known fermentation processes for converting sugar to alcohol by using yeast and bacteria can be found readily in past and current literature. In the past decades, microbial ethanol production had been focused and considered as an alternative fuel for future since fossil fuel was depleted. Several microorganisms, including *Clostridium sp.*, the well-known yeast ethanol producers, *Saccharomyces cere-visiae* and *Zymomonas mobilis* are suitable candidates to produce ethanol (Najafpour *et al*., 2004). *Zymomonas mobilis* has been extensively studied in ethanol fermentation. *Z. mobilis* metabolites sugar via Entner-Doudoroff (ED) pathway, which produces less ATP and less biomass. More carbon sources are thus channeled to ethanol, resulting in high ethanol yield, even higher than that found with the native ethanol fermenting yeast *Saccharomyces cerevisiae*. In addition, because of producing less ATP during ethanol fermentation, *Z. mobilis* maintains a higher glucose metabolic flux, normally three to five fold than that of *S. cerevisiae* (Bai *et al*., 2008).

Current batch processes for ethanol production from glucose using free-cell biocatalysts have a low volumetric productivity and require long fermentation times. The development of high-productivity processes and reactors can potentially reduce capital costs for commercial fuel ethanol production. Continuous culture fermentation provides advantages over batch fermentations including: optimized process conditions for maximal product productivity, long-term continuous productivity, higher volumetric productivity, reduced labor costs once steady state is reached, reduced vessel down time for cleaning, filling and sanitizing, and easier process control and operation then batch during steady state operation (Scott, dkk, 2013, Kelsall and Lyons, 1999, Sinclair and Cantero, 1990). Unfortunately, continuous fermentations are more susceptible to long-term bacteriological problems. For this reason, both manu-facturing processes are widely used worldwide (Bayrock and Ingledew, 2001). The use of continuous systems having high biocatalyst loading along with some form of biocatalyst retention mechanisms can improve ethanol productivities compared to traditional batch systems. These methods include cell recycle by filtration, sedimentation, entrapment in membranes or gels (Krishnan *et al*., 2000).

Microbial cells immobilization has several either advantages, disadvantages or overall prospects and problems of a technological development based on bound cell systems. Foremost among the advantages for using immobilized whole cells in continuous alcohol fermentation in higher product yields with a complementary reduction in cost. General operational stability is increased, as evidenced by continued reuse of microbial cells with a little amount of washout. Immobilized-cells technology has the disadvantage of the bound microorganisms being unable to carry out complex fermentation process involving coenzymes in multiple pathways (Alvira *et al*, 2010).

Immobilization also promises some advantages, such as stability, possible regeneration, no need for sterilization, and the potential development of a continuous process (Bokulich *et al*., 2013, Verbelen *et al*., 2006). Cell immobilization, with the feature of high biomass, was suitable for continuous and high sugar fermentation. It has been shown to be able to operate continuously over longer periods of time, and subsequently, it has higher productivities and lower operating costs (Jackson, 1994). The mechanisms of various immobilization methods and on a variety of materials have been widely reviewed (Branyik *et al*., 2005) However, because of difficulties in sampling, few researches have reported on the variation of immobilized cells during the whole process of repeated batch fermentations or on continuous fermentation (Ji *et al*., 2012).

The natural zeolite was used to immobilize yeast (Shindo *et al*., 2001). They investigated the capacity of immobilization and alcohol fermentation using a continuous fermentation system without analyzing the status of immobilized cells. Meanwhile, the orange peels also were utilized for immobilized yeast cells, and they employed for every several batches during repeated batch fermentations (Plessas *et al*., 2007). Currently, several natural polymers (i.e. agar, alginate, or chitosan) and synthetic polymers (i.e. polyvinyl alcohol, PVA) have been used as entrapment matrices. PVA has very good at mechanical strength, but often causes a reduction in cell viability because of high toxicity level, especially when it is synthesized by the boric acid method (Takei *et al*., 2011). Since during ethanol

fermentation, the microorganisms are usually under resting-cell conditions with very little cell growth, using of immobilized-cells has a particular advantage of being able to retain the cells in the systems during continuous operations. Therefore, although suspended-cell system is still dominant way for ethanol fermentation, there are still many reports describing the use of immobilized-cells (especially for immobilized *S. cerevisiae*) for bioethanol production (Bai *et al*., 2008, Kourkoutas *et al*., 2004, Verbelen *et al*., 2006). Nevertheless, much fewer studies about utilizing *Z. mobilis* cells for ethanol fermentation have been reported (Doelle *et al*., 1993). Even fewer efforts are prepared using PVA-immobilized *Z. mobilis* cells for bioethanol production. Therefore, in this study, the authors present the development of high-productivity continuous ethanol production using PVA-immobilized *Zymomonas mobilis* in an immobilized-cells fermentor.

2. Method

2.1. Materials

Zymomonas mobilis was employed as an ethanol-producing strain in this study. The culture medium contained the following composition (g/L): yeast extract, 10; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4$, 0.5; glucose, 20. *Z. mobilis* was cultured at 30°C for 12 h and the resulting *Z. mobilis* cells were immobilized in polyvinyl alcohol (PVA) gel beads, as described in the following section.

2.2. Immobilization of Cells

The broth containing *Z. mobilis* cells in 400 mL stock culture was centrifuged and washed by distilled water. The pellets were re-dissolved in 20 mL of NaCl 0.9% (w/v) solution and then mixed thoroughly with 80 mL of PVA 9% (w/v) solution. After proper mixing, the PVA gel beads were prepared by dropping PVA+cells solution into buffer solution. They were then washed using 4°C sterilized water after 2 h of agitation and stored in sterilized ice-water for overnight. Prior to use, the gel beads need to be re-cultivated using the original culture medium for 6-12 h to recover the cells activity.

2.3. Analytical Assays

Soluble metabolites (i.e. sugar, organic acid and ethanol) were determined by a high performance liquid chromatography (HPLC) system equipped with refractive index detection (RID, Waters 2414, USA).

3. Results and Discussion

3.1. Effect of Glucose Loading on Fermenter Stability

Continuous conversion of glucose 20 g/L and 100 g/L to ethanol by PVA-immobilized *Z. mobilis* is depicted in Figure 1(a) and 1(b), respectively. These initial experiments were carried out in an immobilized-cells fermenter. Immobilized *Z. mobilis* maintained its highest ethanol concentration with acceptable residual glucose for several days. In comparison between 20 g/L and 100 g/L of glucose initial loading with the same 20% (w/v) of cells loading employed, the 100 g/L of glucose loading maintained more stable of ethanol production until 25 days compared to 20 g/L of glucose loading. For 20 g/L of glucose loading, ethanol production was highest at first 3-7 days, with slightly decreased for every five days to 8.06 g/L after 24 days. This yield was still at high conversion, however. In contrast, very low level of residual glucose was found in the effluent about 0.21 g/L on average. Obviously, the results proved that at lower glucose initial loading, the cells grew on lack of substrate provided. This condition influenced ability of cells on ethanol production. Furthermore, this preliminary study exhibited that high glucose loading could convert to ethanol properly and maintained at stable production for long time. Therefore, based on the results mentioned above, an initial glucose concentration of 100 g/L with cells loading of 20% (w/v) was considered as the better conditions to maintain the stability of ethanol production and further could be applicable on industrial scale.

3.2. Effect of Cells Loading on Ethanol Production and Productivity

Fig. 2 represents the effect of various cells loading of 20% (w/v), 40% (w/v) and 50% (w/v) employed on ethanol concentration and its productivity. The optimum conditions for fermentation occurred at a 40% (w/v) of cells loading, which obtained the maximum ethanol concentration of 48.31 g/L and maximum productivity of 16.10 g/L/h. Originally, both at 20% (w/v) and 40% (w/v) of cells loading, the ethanol concentration achieved can be considered as the best condition.

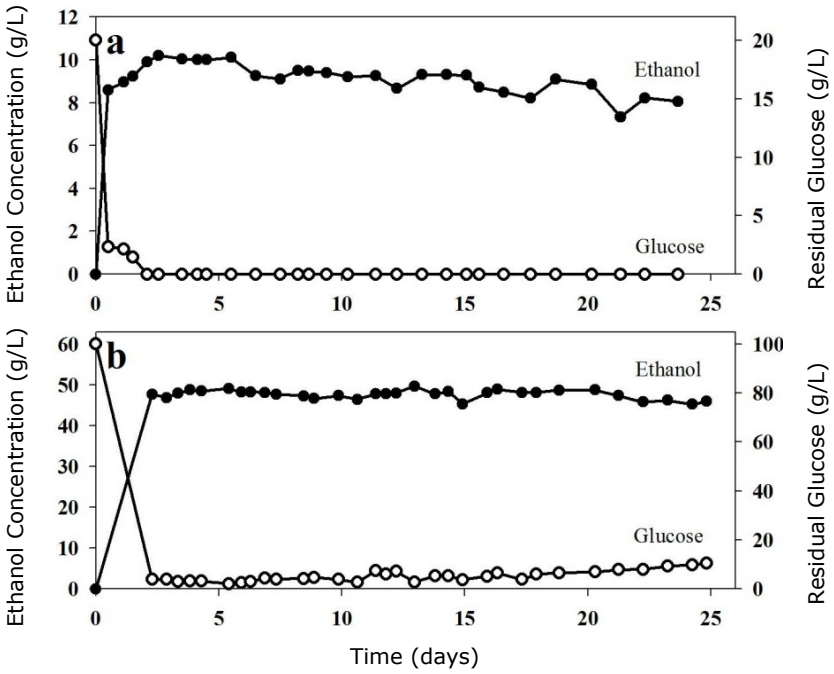

Fig. 1 Continuous production of ethanol from glucose (a) 20 g/L and (b) 100 g/L by PVA-immobilized *Zymomonas mobilis*

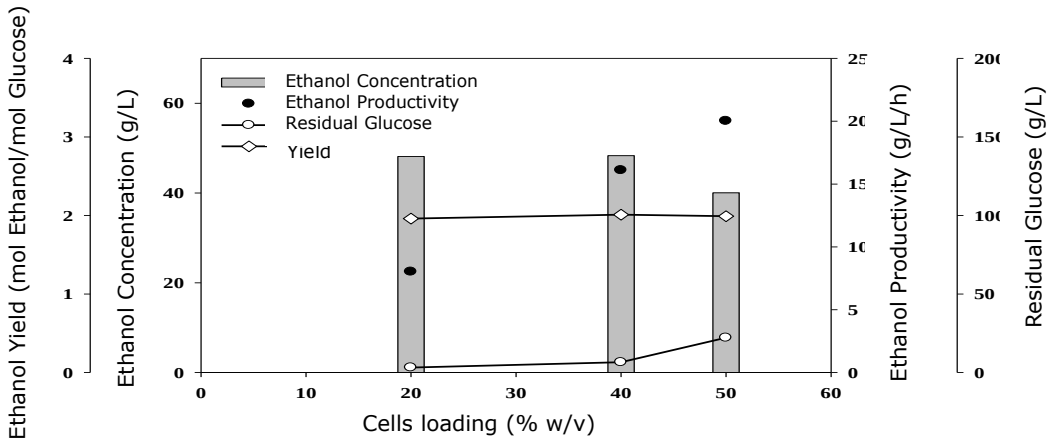

Fig. 2 Ethanol fermentation performance of PVA-immobilized *Zymomonas mobilis* ATCC 29191 cells based on the effect of cells loading (glucose loading = 100 g/L) on ethanol concentration and productivity

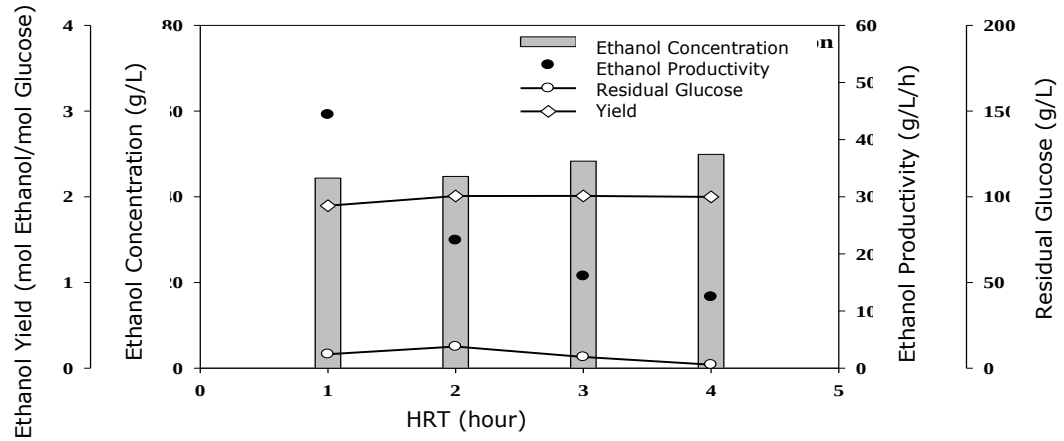

Fig. 3 Ethanol fermentation performance of PVA-immobilized *Zymomonas mobilis* ATCC 29191 cells based on the effect of HRT (glucose loading = 100 g/L, cells loading = 40% (w/v)) on ethanol concentration and productivity

However, the fermentation time required by 20% (w/v) of cells loading was longer than 40% (w/v) of cells loading, and eventually affected to their productivities. By deliberating these two factors, the cells loading of 40% (w/v) were selected as the optimum condition. Otherwise, the results also indicated that the higher cells loading of 50% (w/v) was unnecessary because of high residual sugar and low ethanol production.

3.3. Effect of Dilution Rate/HRT on Ethanol Production and Productivity

The productivity of ethanol was greatly influenced by dilution rate of substrate feeding or residence time (HRT) of substrate in a fermentor. According to previous work, the glucose loading of 100 g/L as well as cells loading of 40% (w/v) were tested in order to determine proper dilution rate/HRT in continuous fermentation system. By considering both remaining glucose in effluent and ethanol concentration obtained, the 4 hour of HRT (0.25 h^{-1} of dilution rate) was selected as the optimum condition as depicted in Fig. 3. The ethanol production, ethanol productivity and residual glucose obtained were 49.89 g/L, 12.47 g/L/h and 2.04 g/L, respectively. Since the higher glucose detected by employing 3 hour of HRT (6.55 g/L), this condition was not considered as the optimum value because of cost efficiency for substrate purchasing.

3.4 Ethanol Production Inhibition

The present work demonstrates that an ethanol concentration as high as 71.59 g/L can be achieved using single stage continuous immobilized-cells fermentor (Fig. 4). During this test, several of glucose initial loading (100 g/L, 125 g/L and 150 g/L) were employed to investigate maximum ethanol concentration achieved. Meanwhile, the residual glucose also was monitored to determine ethanol inhibition level.

For glucose initial loading of 100 g/L, 125 g/L and 150 g/L, the ethanol concentration obtained were 49.89 g/L, 62.97 g/L and 71.59 g/L, respectively. These three conditions were tested on optimum dilution rate from previous results in order to minimize residual glucose concentration in effluent. At high glucose initial loading (150 g/L), the remained glucose was at high concentration (8.68 g/L) because of ethanol inhibition on cells activity and eventually caused stuck or sluggish fermentation which significantly compromised ethanol yield. This phenomenon may be overcome by integrated separation system (i.e. *in-situ* membrane separation). Therefore, the optimum ethanol productivity achieved with completely glucose consumption was 15.74 g/L/h at 125 g/L of glucose loading.

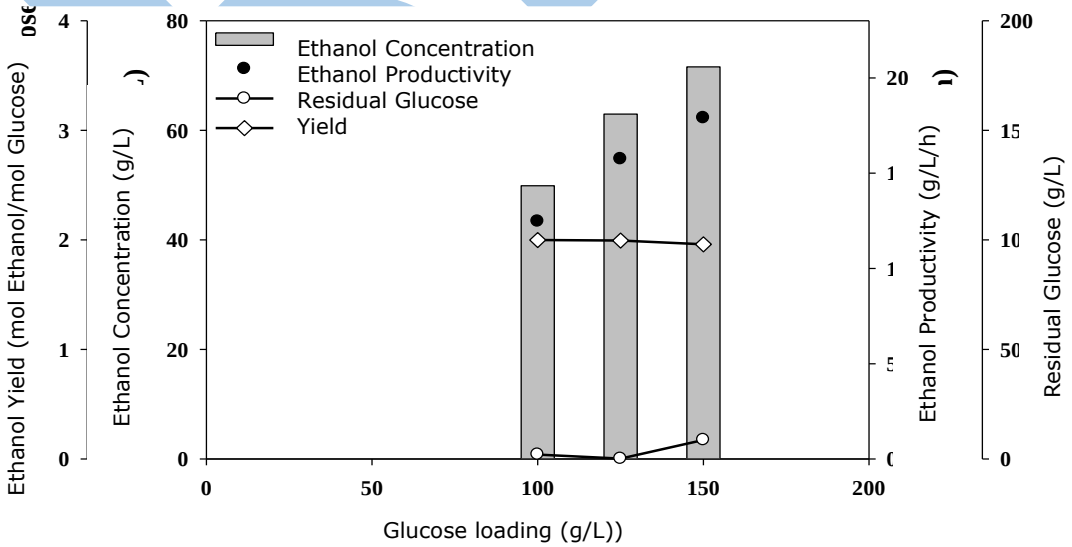

Fig. 4 Ethanol fermentation performance of PVA-immobilized *Zymomonas mobilis* ATCC 29191 cells based on the effect of glucose initial loading on ethanol production

Table 1 Comparison of the performance of bioethanol production on this study with related studies using immobilized-cell systems

Glucose Loading (g/L)	Microorganisms	Operating Mode	Ethanol Productivity (g/L/h)	Ethanol Production (g/L)	Glucose Conversion (%)	References
20	ST1 *Yeast*	Batch	1.37	8.2	82	(Zain *et al*., 2011)
50	*Z. mobilis*	CSTR	3.2	16	65	(Wirawan, 2012).
78.6	*S. cerevisiae*	Batch	1.61	38.6	98	Vucurovic *et al*., (2009)
80	*S. cerevisiae*	CSTR	8.7	34.38	86	Janiszyn *et al*., (2007)
86.7	*S. cerevisiae*	Batch	1.65	39.6	91	Vucurovic *et al*., (2009)
113	*S. cerevisiae*	Batch	3.1	48	85	Vucurovic *et al*., (2009)
141	*S. cerevisiae*	Fed-batch	1.46	40.7	58	Janiszyn *et al*., (2007)
150	*S. cerevisiae*	CSTR	6.71	47	63	Najafpour *et al*., 2004).
100	*Z. mobilis*	CSTR	12.47	49.89	99.8	This study
125	*Z. mobilis*	CSTR	15.74	62.97	100	This study
150	*Z. mobilis*	CSTR	17.89	71.59	95.5	This study

Table 1 compares the performances achieved in this study to related works on bioethanol production from sugars. It exhibits that the results obtained from the current study are more acceptable compared to the previous ones. In brief, the PVA-immobilized *Z. mobilis* cells are preferable cell immobilization system for ethanol production.

4. Conclusion

Continuous ethanol production in an immobilized-cells fermentor was successfully carried out with high sugar concentration. The advantage of immobilized fermentor was that the inhibition of substrate and product are not apparent even with 150 g/L of glucose solution in the fresh feed. Regarding to fermentor stability in producing ethanol, an initial glucose concentration of 100 g/L with cells loading of 20% (w/v) was considered as the optimum conditions to maintain the stability of ethanol production and further can be applicable on industrial scale. Likewise to cells loading effect, the cells loading of 40% (w/v) was selected as the optimum condition because of its ability to increase the fermentation rate in order to obtain high ethanol concentration and productivity. According to study result of effect of dilution rate on ethanol production, the 4 hour of HRT (0.25 h^{-1} of dilution rate) was selected as the optimum condition with ethanol production, ethanol productivity and residual glucose obtained were 49.89 g/L, 12.47 g/L/h, and 2.04 g/L, respectively. Meanwhile, the other test was investigated to determine ethanol inhibition limitation by employing glucose initial loading of 150 g/L, the result displayed that the remained glucose was at high concentration (8.68 g/L) because of stuck or sluggish fermentation which significantly compromised ethanol yield. This phenomenon may be overcome by integrated separation system (i.e. *in-situ* membrane separation) for further study.

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