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Short Communication

Effects of initial pH value of the medium on the alcoholic fermentation performance of *Saccharomyces cerevisiae* cells immobilized on nipa leaf sheath pieces

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Abstract

Immobilized yeast on nipa leaf sheath pieces was applied to ethanol fermentation using the medium with different initial pH values (5.1, 4.5, 4.0, and 3.5). Control samples with the free yeast were also carried out under the same conditions. Low pH value of 4.0 or 3.5 significantly reduced yeast growth and increased the residual sugar level in the fermentation broths for both the immobilized and free cells. In all cases, the ethanol content produced and ethanol formation rate of the immobilized yeast were 13-33% and 35-69%, respectively, higher than those of the free yeast. In addition, the residual sugar content in the immobilized yeast cultures was 2.1-20.5 times lower than that in the free yeast cultures. The yeast immobilized on nipa leaf stem pieces exhibited higher alcoholic fermentation performance than the free yeast in medium with low pH value. This support was potential for further research for application in ethanol industry.

Keywords: ethanol, fermentation, nipa leaf sheath, pH, yeast

1. Introduction

Ethanol has re-emerged as an alternative to petroleum-based liquid fuels. Many studies have been performed for improvement in ethanol yield and productivity (Bai *et al.*, 2008). According to Roehr (1996), the initial pH value of the medium is usually adjusted to 5.2 as *Saccharomyces cerevisiae* cells are used in ethanol industry. In practice, producers could lower the initial pH value of the medium for prevention of contamination. However, reduction in pH value inhibited yeast growth and prolonged the fermentation time (Buzás *et al.*, 1989).

Ethanol production with immobilized cell systems has attracted great attention due to high productivity and protection of yeast cells from inhibitions (Phisalaphong *et al.*, 2007). From the last decade, different cellulosic supports have been used in yeast immobilization for ethanol production because of low cost, environmental friendliness, worldwide availability, and relatively high resistance in alcoholic fermentation conditions (Querol and Fleet, 2006).

Nipa (*Nypa fruticans*) is one of the most useful palms in the restricted mangrove forests of Southeast Asia and Oceania. Its leaf sheath, fruit, juice, or sap from its inflorescence stalk (peduncle) are a source of products for indigenous people living near these regions since historical times (Hamilton and Murphy, 1988). Recently, nipa leaf sheath was used as heavy metal adsorbent due to its highly porous structure (Wankasi *et al.*, 2005) and pulping raw material (Jahan *et al.*, 2006). Chemical composition analysis showed that nipa leaf sheath pieces contained high level of cellulose, hemicelluloses, and lignin (Tamunaidu, 2011). According to Yu *et al.* (2010), cellulosic material with high porous structure

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could be used as support for yeast immobilization. In this study, nipa leaf sheath pieces were applied as support for yeast immobilization for ethanol fermentation. The objective of this study was to evaluate the effects of initial pH value of the medium on the growth and ethanol formation of the immobilized yeast on nipa leaf sheath pieces. The free yeast was also used as the control in order to clarify the extent of improvement in fermentation performance of the immobilized yeast.

2. Materials and Methods

2.1 Materials

2.1.1 Yeast

Saccharomyces cerevisiae TG1 from the culture collection of the Department of Food Technology, Ho Chi Minh City University of Technology, S.R. of Vietnam, was used in this research.

2.1.2 Support

The leaf sheaths of nipa (*Nypa fruticans*) were collected from a local farm in Ho Chi Minh City. Nipa leaf sheaths were washed with potable water to remove adhering dirt and cut into cubic shape with 0.5 cm in height, 2 cm in wide and 3 cm in long axis. The nipa leaf sheath pieces were sterilized at 121°C for 20 min before used for cell immobilization.

2.1.3 Media

Chemical composition of the medium for culture preparation was as follows: 30 g/L glucose, 5 g/L yeast extract, 1 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4.7H_2O$. The medium for yeast immobilization and ethanol fermentation was similar to that for preculture preparation except that the glucose concentration was adjusted to 120 g/L and 200 g/L, respectively. All media were sterilized at 121°C for 20 min before use. Chemicals used in this study were provided by Merck Corp. (U.S.A).

2.2 Experimental methods

2.2.1 Preculture preparation

Yeast cell propagation was carried out by two successive inoculations: 1) in a 50 mL Erlenmeyer flask containing 10 mL of growth medium and 2) in a 250 mL Erlenmeyer flask containing 90 mL of growth medium. For both periods, the preculture was grown at 30°C and 150 rpm for 24 hrs. The cell suspension was then centrifuged at 5°C and 5,000 rpm for 20 min. The biomass obtained was used for cell immobilization.

2.2.2 Yeast immobilization

The yeast biomass was re-suspended in the medium for yeast immobilization to achieve the cell concentration of 3.0×10^7cfu/mL; 20 g support was then added into 250 mL shake flask containing 100 mL yeast suspension and the mixture was incubated in a thermostate-shaker at 30°C for 20 hrs. Finally, the nipa leaf sheath pieces with the immobilized cells were removed and washed with sterile water three times. The cell density was 7.5×10^9cfu/g wet support. The immobilized yeast obtained was ready for ethanol fermentation.

2.2.3 Fermentation

Static fermentation was conducted in 1 L Erlenmeyer flasks containing 500 mL of medium. The initial pH value of the fermentation medium was varied: 5.1 (non-adjusted pH value), 4.5, 4.0 and 3.5. The inoculum size was fixed at 1.0×10^7 cfu/mL. Control samples with the free yeast were simultaneously performed under the same conditions. The fermentation was considered completed when the residual sugar level in the culture remained constant during 12 consecutive hours.

2.3 Analytical methods

Viable cell numbers in the free yeast culture were quantified by plate count agar with the incubation at 30°C for 48 hrs, whereas the viable cell numbers in the immobilized yeast culture were quantified by a method proposed by Mallouchos *et al.* (2002) with slight modifications: 1.0 g of the support with the immobilized cells and 9.0 mL of distilled water were ground in a grinder at 3,500 rpm for 5 min; the cell number in the obtained suspension was determined by plate count agar method with the incubation at 30°C for 48 hrs.

Glucose concentration was determined by spectrophotometric method with 3,5-dinitrosalicylic acid reagent (Miller, 1959). Ethanol concentration was evaluated by enzymatic method using ethanol kit with a reflectometer model 116970 (Merck KG, Germany). Under the catalytic effect of alcohol dehydrogenase, ethanol is oxidized by NAD to acetaldehyde. In the presence of an electron transmitter, the NADH formed in the process reduces a tetrazolium salt to a blue formazan that is determined reflectometrically.

Specific growth rate (h^{-1}) of the immobilized and free yeast was calculated by the formula described elsewhere (Slininger *et al.*, 1982). Average sugar uptake rate R_s (g/L.h) and ethanol formation rate R_p (g/L.h) of the immobilized and free yeast were calculated using the following formulas:

$$R_s = (S_1-S_2)/t \quad (1)$$

$$R_p = (P_2-P_1)/t \quad (2)$$

where S_1 and S_2 were the initial and residual sugar concentration in the culture, respectively (g/L), P_1 and P_2 were the initial and final ethanol concentration in the culture, respectively (g/L), and t was the fermentation time (h). Ethanol yield (g/g) was calculated as the ratio of the weight of ethanol produced and the weight of glucose assimilated by the yeast in the fermentation.

2.4 Statistical analysis

All experiments were performed in triplicate. Mean values were considered significantly different when $P<0.05$. Multi-way analyses of variance were performed using the software Statgraphics Centurion XV.

3. Results and Discussion

3.1 Effects of initial pH value of the medium on yeast growth

Table 1 presents specific growth rate and maximum cell density in the cultures with the immobilized and free cells.

The yeast growth was not changed as the initial pH value of the medium reduced from 5.1 to 4.5. However, the specific growth rate and maximum cell density were significantly reduced for both the immobilized and free yeast when the pH value decreased from 4.5 to 3.5. Roehr (1996) stated that low pH value in the medium reduced the pH value in the cellular cytoplasm and lowered catalytic activity of the intracellular enzymes. This phenomenon inhibited yeast growth. When the initial pH value of the medium was 5.1, 4.5, 4.0, and 3.5, the final pH value was 4.2, 3.8, 3.4 and 3.0, respectively for the immobilized yeast and 4.4, 3.9, 3.5 and 3.1, respectively for the free yeast. As a result, the lower the initial pH value of the medium was, the lower the final pH value of the culture was. That would increase the inhibition effect for the yeast growth.

In all cases, the immobilized yeast on nipa leaf sheath pieces always grew faster and higher than the free yeast. Formerly, Phisalaphong *et al.* (2007) observed a higher cell growth for the immobilized yeast in alginate gel in comparison with the free yeast when the initial pH value of the medium was 5.0. Immobilization would prevent the inhibition effects from the cultures and that resulted in a better growth of the immobilized cells (Buzás *et al.*, 1989).

3.2 Effects of initial pH value of the medium on glucose assimilation

For the immobilized yeast, low residual glucose level was observed when the initial pH value of the medium was 5.1, 4.5, and 4.0 (Table 2). At the pH value of 3.5, the residual sugar concentration in the culture sharply augmented. It can be concluded that the pH value of 3.5 significantly inhibited the sugar uptake of the immobilized yeast on nipa leaf sheath

Table 1. Effects of initial pH value of the medium on the growth of the free and immobilized yeast on nipa leaf sheath pieces.

pH	Specific growth rate (h^{-1})		Maximum cell density (10^7cfu/mL)	
	Immobilized cells	Free cells	Immobilized cells	Free cells
5.1	0.62 ± 0.006^{e}	0.46 ± 0.029^{c}	13.8 ± 0.3^{m}	6.6 ± 0.2^{k}
4.5	0.61 ± 0.014^{e}	0.44 ± 0.013^{c}	14.4 ± 0.4^{m}	6.6 ± 0.3^{k}
4.0	0.53 ± 0.008^{d}	0.36 ± 0.013^{b}	10.2 ± 0.2^{l}	5.0 ± 0.2^{j}
3.5	0.37 ± 0.023^{b}	0.15 ± 0.009^{a}	6.5 ± 0.2^{k}	4.0 ± 0.3^{f}

Various superscripts in table indicate significant differences ($p<0.05$).

Table 2. Effects of initial pH value in the medium on residual glucose concentration and glucose uptake rate of the free and immobilized yeast on nipa leaf sheath pieces.

pH	Residual glucose content (g/L)		Glucose uptake rate (g/L.h)	
	Immobilized cells	Free cells	Immobilized cells	Free cells
5.1	3.40 ± 0.39^{b}	13.53 ± 0.74^{d}	3.93 ± 0.07^{l}	2.97 ± 0.03^{k}
4.5	0.97 ± 0.11^{a}	10.18 ± 1.12^{c}	5.00 ± 0.08^{m}	2.97 ± 0.05^{k}
4.0	1.02 ± 0.13^{a}	20.95 ± 2.15^{e}	4.00 ± 0.05^{l}	2.68 ± 0.05^{i}
3.5	36.77 ± 2.49^{f}	77.56 ± 0.62^{g}	2.63 ± 0.08^{j}	1.72 ± 0.01^{h}

Various superscripts in table indicate significant differences ($p<0.05$).

pieces. Similar inhibition was observed for *Saccharomyces cerevisiae* cells immobilized on bacterial cellulose support as the medium with pH value of 3.5 was used in wine fermentation (Ton *et al.*, 2010).

In all cases, the residual glucose level for the immobilized yeast was 2.1-20.5 times lower than that for the free yeast. High residual sugar content in the fermentation broth decreased the ethanol concentration in the culture and that led to a reduced economic efficiency of ethanol production. Analysis of variance showed that reduction in pH value from 4.5 to 4.0 did not change the level of assimilated sugar for the immobilized cells but nearly doubled the level of residual sugar for the free cells. Consequently, the immobilized yeast on nipa leaf sheath pieces was more resistant to low pH value than the free yeast.

The lowest sugar uptake rate for both the immobilized and free cells was also observed at the pH value of 3.5. Formerly, Bajpai and Margaritis, (1987) used immobilized cells *Kluyveromyces marxianus* in alginate gel for ethanol fermentation and demonstrated low sugar assimilation rate in the pH range from 3.0 to 3.5. Nevertheless, the sugar uptake rate of the immobilized yeast on nipa leaf sheath pieces was 1.32-1.68 times higher than that of the free yeast when the initial pH of the medium varied from 3.5 to 5.1. Our results proved that the immobilized yeast on nipa leaf sheath pieces fermented sugar faster than the free yeast.

3.3 Effects of initial pH value of the medium on ethanol formation

Table 3 shows that the final ethanol concentration in the immobilized yeast culture was 13-33% higher than that in the free yeast culture. This observation was completely appropriate to the residual glucose level in the cultures with the immobilized and free cells (Table 1). In addition, the immobilized yeast utilized more sugar and produced more ethanol than the free yeast. The ethanol formation rate of the immobilized yeast was 35-69% higher than that of the free yeast. Recently, Chandel *et al.* (2009) demonstrated that the cells *Saccharomyces cerevisiae* immobilized on wide sugarcane pieces produced ethanol faster than the free cells. According to Phisalaphong *et al.* (2007), immobilization protected microbial cells against the possible toxic effects in the fermentation. As a result, the fermentation performance of the immobilized yeast was improved in comparison with that of the free yeast.

In our study, the ethanol yield of the immobilized and free cells was nearly similar (Table 3). Similar observation was also reported by Chandel *et al.* (2007) who compared ethanol yield of the free and immobilized yeast on wide sugar pieces.

4. Conclusion

When the initial pH value decreased from 5.1 to 4.5, the growth of both the free and immobilized yeast on nipa leaf sheath pieces did not change. However, further reduction in pH value from 4.5 to 3.5 lowered the yeast growth. In all cases, the immobilized yeast always grew better, converted glucose to ethanol faster and produced more ethanol than the free yeast. The immobilized yeast on nipa leaf sheath pieces exhibited higher fermentation performance than the free yeast under pH stress.

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Table 3. Effects of initial pH value of the medium on the final ethanol concentration, ethanol formation rate and ethanol yield of the free and immobilized yeast on nipa leaf sheath pieces.

pH	Final ethanol content (% v/v)		Ethanol formation rate(g/L.h)		Ethanol yield (g/ g)	
	Immobilized cells	Free cells	Immobilized cells	Free cells	Immobilized cells	Free cells
5.1	11.40 ± 0.06^{f}	10.08 ± 0.77^{d}	1.67 ± 0.04^{l}	1.24 ± 0.01^{j}	0.423 ± 0.001^{m}	0.416 ± 0.004^{n}
4.5	11.82 ± 0.02^{f}	10.46 ± 0.13^{e}	2.09 ± 0.04^{k}	1.24 ± 0.02^{j}	0.416 ± 0.001^{n}	0.416 ± 0.002^{n}
4.0	11.81 ± 0.02^{f}	9.44 ± 0.16^{c}	1.66 ± 0.02^{i}	1.11 ± 0.02^{h}	0.413 ± 0.001^{n}	0.411 ± 0.001^{n}
3.5	8.51 ± 0.12^{b}	6.42 ± 0.04^{a}	1.08 ± 0.03^{h}	0.71 ± 0.01^{g}	0.411 ± 0.002^{n}	0.411 ± 0.001^{n}

Various superscripts in table indicate significant differences ($p<0.05$).

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