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Fermentation performance of free and immobilized yeast on cork (*Sonneratia caseolaris*) root – application of immobilized yeast to repeated batch ethanol fermentation

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Abstract

The immobilized yeast on cork (*Sonneratia caseolaris*) root grew better and exhibited higher fermentation rate than the free yeast. In the repeated batch fermentation, the immobilized cell system could be reused at least for ten cycles. The sugar uptake rate of the immobilized yeast gradually increased from 2.35 g/L.h (cycle 1) to 3.31 g/L.h (cycle 10). Similarly, the ethanol production rate of the fixed yeast augmented during the ten cycles and achieved maximum level of 1.38 g/L.h at cycle 10.

Introduction

During the last decade, cell immobilization techniques have become increasingly important and are being successfully applied to production of ethanol, beer, wine, cider, vinegar, and dairy products. Cell immobilization offers different advantages including prolonging activity and stability of the biocatalyst, reducing fermentation time, feasibility of continuous processing, reducing cost for equipment and energy demands (Strehaiano *et al*., 2006).

Ethanol has many uses in industry as well as in daily life. It is a raw material in liquor production, a popular solvent in many industries and an environmentally friendly fuel. The development of the ethanol industry leads to the demand for effective and economical fermentation technique to improve ethanol yield and devalue prices (Kosaric, 1996). Application of immobilized yeast to ethanol fermentation has attracted considerable interest (Strehaiano *et al*., 2006).

Yeast cell immobilization by adsorption seems to be more reasonable than cell entrapment within a porous matrix and cell aggregation without external support because of the facts that the yeast cell growth is not significantly affected, and some yeast cells can be washed out of the fermentation system and be continuously renewed (Verbelen *et al*., 2006; Bai *et al*., 2008). Cells have been immobilized by the adsorption on a variety of natural and synthetic supports (Yu *et al.*, 2007). Many reports have proposed various immobilization supports for ethanol fermentation such as sorghum bagasse (Yu *et al*., 2007), wild sugarcane (Chandel *et al*., 2009), orange-peel (Plessas *et al.*, 2007). The immobilized yeast could be reused for some cycles of the fermentation and this is one of the important advantages of microbial immobilization (Strehaiano *et al.*, 2006).

In this study, cork (*Sonneratia caseolaris*) root was selected for yeast immobilization because of abundant carrier in tropical countries and simple immobilization procedure. The objectives of this research, hence, were i) to compare fermentation performance of the free and immobilized yeast on cork root in batch fermentation, 2i) to investigate the substrate assimilation rate and ethanol production rate of the fixed yeast during the repeated batch fermentation for ethanol production.

Materials and Methods

Microorganism

In this study, *Saccharomyces cerevisiae* BT1 from the collection of Microbiological Laboratory, Department of Food Technology, Ho Chi Minh City University of Technology was used.

Procedure of yeast immobilization

Saccharomyces cerevisiae was grown on medium proposed by Kourkoutas *et al.* (2002). The medium

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consisted of 4% glucose, 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 and 0.5% $MgSO_4.7H_2O$. Yeast was incubated in the erlenmeyers in thermostate-shaker at 30°C for 24 hours. Yeast cells were then collected by centrifugation at 4°C, 5000 rpm for 20 min. For yeast immobilization, medium included 12% glucose, 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 and 0.5% $MgSO_4.7H_2O$ was used (Kourkoutas *et al*., 2002). The cell concentration in the yeast suspension was 50×10^6 cells/mL. This yeast suspension was mixed with the support cylinders with 2 cm diameter and 1.8 cm height in 500 mL erlenmeyers. The ratio of support to yeast suspension was 25 g support/100 mL yeast suspension. After the incubation period of 12 hours in a thermostate-shaker, the culture was decanted and the carriers were washed twice with sterile water. The number of viable yeast cells on cork root support was about 3.9×10^9 cells/gram of the dried support. The biocatalyst obtained was immediately used for ethanol fermentation.

Alcoholic fermentation

Chemical composition of the medium for ethanol fermentation was as follows: glucose - 200 g/L, yeast extract - 4 g/L, $(NH_4)_2SO_4$ - 1 g/L, KH_2PO_4 - 1g/L, MgSO4.7H2O - 5g/L. Batch fermentation by immobilized and free yeast was carried out at 30°C. The inoculum size was 10×10^6 viable cells/mL. During the fermentation, the samples were taken for analysis of yeast growth, reducing sugar assimilation and ethanol formation. In the repeated batch fermentation, the immobilized yeast cells were used for ten consecutive cycles. At the end of each cycle, the immobilized biocatalyst was separated from the culture and washed with sterile water. Then the immobilized yeast was ready to reuse for the next cycle. Fermentation was considered as completed when the fermentation degree achieved 98%. For each fermentation cycle, the content of reducing sugars assimilated by the fixed yeast and the content of ethanol produced were evaluated.

Analytical methods

For counting yeast cells immobilized on cork root, the support was blended with sterile water in a blender machine (Ton *et al.*, 2010). Yeast concentration in the sample obtained was then determined by haemocytometry using Thoma counter at 40× magnification with an Olympus Eclipse light microscope (model CX21FS1). Yeast viability was determined by methylene blue test (Patkova *et al.*, 2000). For counting the free yeast cells, haemocytometry with Thoma counter was also used. Reducing sugar was quantified by spectrophotometric method, using 3,5 dinitrosalicylic acid (Miller, 1959). Ethanol was evaluated by gas chromatography (Varian 430-GC) using a flame ionization detector and a HP-FFAP column (19091F-413) with 30 m length, 0.25 μm film thickness and 0.32 mm internal diameter. The working conditions were as follows: injection temperature was 250°C, oven temperature was maintained at 40°C for 5 min, then increased to 70°C with the rate of 5°C/min, augmented to 160°C with the rate of 10°C/min, continued to be increased to 230°C with the rate of 30°C/min and hold for 2 min, detector temperature was 250°C. The carrier gas was nitrogen with the inlet pressure of 12 psi (Horwitz and Latimer, 2010). The carrier was examined using scanning electron microscope (SEM) technique (Speers *et al*., 1993).

Calculation formulae

Sugar assimilation rate (g/L.h): $(S_1-S_2)/t$; where S_1 and S_2 was the sugar concentration (g/L) in the culture at the beginning and at the end of the fermentation, respectively; t was the fermentation time (h). Ethanol formation rate (g/L.h): $(P_2-P_1)/t$; where P_1 and P_2 was the ethanol concentration (g/L) in the culture at the beginning and at the end of the fermentation, respectively; t was the fermentation time (h). Average growth rate of yeast (cells/mL.h): $(X_2-X_1)/t$; where X_1 and X_2 was the yeast cell number (cells/mL) in the culture at the beginning and at the end of the fermentation, respectively; t was the fermentation time (h).

Statistical treatment

Each presented result was the average of three independent experiments. The data were analyzed for statistical significance by analysis of variance. Multiple Range Test with the Least Significant Difference (LSD 0.05) was applied in order to determine which means are significantly different from which others by using Statgraphics plus software, version 3.0.

Result and Discussion

Comparison of fermentation performance of the free and immobilized yeast on cork root in batch fermentation

Figure 1 presents the growth curves of the immobilized and free yeast during the fermentation. With the same inoculum size of 10×10^6 cells/mL, the immobilized yeast grew significantly faster than the free yeast. Maximum number of yeast cells in the fixed yeast culture was approximately 80% higher than that in the free yeast culture. At the end of the fermentation, the viable cells in the

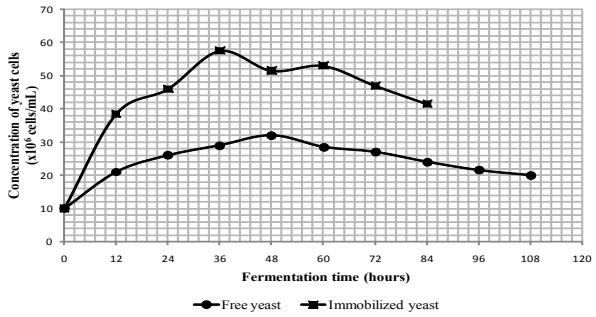

Figure 1. Growth curves of the free and immobilized yeast on cork root during batch fermentation

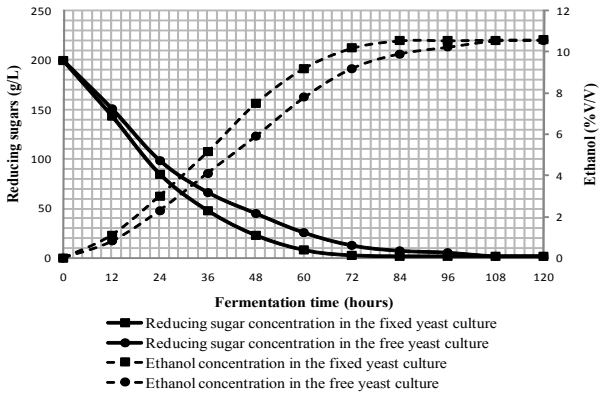

Figure 2. Reducing sugar assimilation and ethanol formation by the free and immobilized yeast on cork root during batch fermentation

fermentation broth with the fixed and free yeast were 98% and 91%, respectively. It was probably due to protection role of the carrier against osmotic and ethanol stress in the culture. Similar result was also reported by Ton and Le (2011) who used immobilized yeast on bacterial cellulose for ethanol fermentation in winemaking.

Figure 2 shows the evolution of reducing sugar and ethanol levels in the immobilized and free yeast cultures during the fermentation. The fixed yeast fermented sugar faster than the free yeast. The immobilized cells completely assimilated sugars during the first 84 h while reducing sugars in the free yeast culture were exhausted just after 104 h. At the end of the fermentation, the ethanol concentration in the two cultures was nearly similar. It can be noted that the sugar uptake rate and ethanol formation rate of the immobilized cells was 28.9% and 28.5%, respectively higher than those of the free cells. Similar phenomenon was also observed by Yu *et al.*, (2007) who compared the ethanol productivity of the free and immobilized yeast on sorghum bagasse in ethanol fermentation. In addition, the analysis of variance showed that the ethanol yield of the fixed yeast on cork root and the free yeast was similar (42.0 ± 0.2%).

It can be concluded that cell immobilization on cork root improved yeast growth and that resulted in higher fermentative activities of the biocatalyst

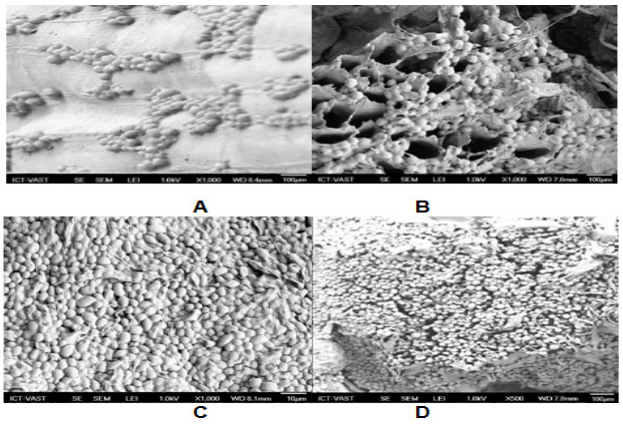

Figure 3. SEM pictures of the immobilized yeast on cork root support (A): cells inside the support at the start of cycle 1; (B): cells on the surface of the support at the start of cycle 1; (C): cells inside the support at the start of cycle 10; (D): cells on the surface of the support at the start of cycle 10.

and shorter fermentation time. The fermentation performance of the fixed yeast was therefore investigated during the repeated batch fermentation in the next experiment.

Application of the immobilized yeast on cork root to the repeated batch ethanol fermentation

Our experimental results showed that the average growth rate of the immobilized yeast in cycle 1 was the lowest (1.3 $\times 10^6$ cells/mL.h). During the repeated batch fermentation, the average growth rate of the fixed cells gradually increased and reached maximum (3.3×10^6 cells/mL.h) in cycle 10. It was due to the adaptation of the immobilized yeast to the fermentation conditions (Ton and Le, 2011). As a consequence, lag-phase could be shortened.

It should be noted that the inoculum size of the immobilized yeast was slightly increased from the previous cycle to the next cycle during the repeated batch fermentation because of the yeast growth on the carrier. High inoculum size led to higher maximum cell density in the immobilized yeast culture. Maximum yeast cell number increased from 9.5×10^7 cells/mL in cycle 1 to 15×10^7 cells/mL in cycle 10. Figure 3 shows the yeast cells on the support surface and in the porous structure of the support. It is clearly observed that the yeast cell number on the cork root before cycle 10 was significantly higher than that before cycle 1.

Table 1 indicates that during the repeated batch fermentation, the fermentation performance of the immobilized yeast on cork root was gradually improved. The sugar uptake rate and ethanol formation rate of the immobilized yeast in cycle 10 were 40.9% and 39.4%, respectively higher than those in cycle 1. Consequently the fermentation time

Table 1. Fermentation characteristics of the repeated batch fermentation with the immobilized yeast on cork root

Cycle	Sugar uptake rate	Ethanol concentration	Ethanol production rate	Fermentation time
	(g/L.h)	(%v/v)	(g/L.h)	(h)
1	$2.35^{i} \pm 0.04$	$10.55^{e} \pm 0.015$	$0.99^{i} \pm 0.016$	$84.33^{b} \pm 1.52$
2	$2.42^{h} \pm 0.01$	$10.59^{d} \pm 0.010$	$1.01^{h} \pm 0.007$	$82.33^{c} \pm 0.57$
3	$2.54^{g} \pm 0.03$	$10.64^{b} \pm 0.021$	$1.07^{g} \pm 0.013$	$79.00^{d} \pm 1.00$
4	$2.58^{g} \pm 0.03$	$10.68^{a} \pm 0.014$	$1.09^{g} \pm 0.014$	$77.00^{d} \pm 1.00$
5	$2.64^{f} \pm 0.02$	$10.59^{d} \pm 0.019$	$1.11^{f} \pm 0.009$	$74.67^{e} \pm 0.57$
6	$2.72^{e} \pm 0.03$	$10.60^{c} \pm 0.022$	$1.14^{e} \pm 0.015$	$73.00^{f} \pm 1.00$
7	$2.83^{d} \pm 0.02$	$10.60^{c} \pm 0.015$	$1.18^{d} \pm 0.009$	$70.33^{g} \pm 0.57$
8	$3.01^{c} \pm 0.04$	$10.56^{e} \pm 0.013$	$1.26^{c} \pm 0.019$	$66.00^{h} \pm 1.00$
9	$3.10^{b} \pm 0.02$	$10.52^{f} \pm 0.015$	$1.30^{b} \pm 0.011$	$63.67^{i} \pm 0.57$
10	$3.31^{a} \pm 0.03$	$10.51^{g} \pm 0.021$	$1.38^{a} \pm 0.013$	$59.67^{j} \pm 0.57$

Various superscripts in each column indicate significant difference ($p < 0.05$)

Table 2. Some volatile compounds in the culture in the repeated batch ethanol fermentation (cycle 1,3,5,7 and 10) with the immobilized yeast on cork root

Sample	Acetaldehyde (ppm)	Ethylacetate (ppm)	1-Propanol (ppm)	Isobutyl alcohol (ppm)	Amyl alcohol (ppm)	Methanol (ppm)
1	102.8^{c}	56.3^{b}	26.4^{b}	11.6^{c}	79.4^{ab}	103.3^{d}
3	101.9^{c}	58.9^{b}	23.5^{c}	15.2^{b}	88.3^{a}	100.5^{e}
5	105.1^{b}	66.3^{a}	24.6^{b}	16.5^{a}	77.9^{b}	99.9^{e}
7	99.6^{c}	54.5^{b}	22.1^{c}	16.1^{a}	86.1^{a}	105.8^{c}
10	109.7^{b}	53.8^{b}	21.1^{c}	15.8^{b}	76.3^{b}	109.8^{a}

Various superscripts in each column indicate significant difference ($p < 0.05$)

gradually reduced from cycle 1 to cycle 10. It was due to a reduction in lag-phase time and a gradual increase in the cell number on the support during the reuse of the biocatalyst. This result is in accordance with the findings of Ton *et al*., (2010) who reported that the sugar uptake rate and ethanol formation rate of the immobilized yeast on bacterial cellulose were gradually enhanced during the repeated batch fermentation in winemaking. In addition, Hilge-Rotmann and Rehm (1991) showed that the ratio of saturated fatty acids in cellular membrane of the fixed yeast was higher than that of the free yeast. Consequently, the improvement in metabolic activities can be explained by some changes in chemical composition of the immobilized yeast cells.

There was a very slight difference in ethanol concentration in the fermentation broth in ten cycles (Table 1). This difference did not affect notably the ethanol productivity in industrial scale. The same observation was also mentioned in the study of Chandel *et al.* (2009) who applied the immobilized yeast on *Saccharum spontaneum* stalks to ethanol fermentation and reused the biocatalyst for 8 cycles. Table 2 shows that the level of each volatile compound in the fixed yeast culture varied in a narrow range during the repeated batch fermentation. This observation confirmed the stability of the immobilized yeast during the reuse process.

Conclusion

Immobilization of yeast cells on cork root enhanced the yeast growth and ethanol production rate. During ten cycles of the repeated batch fermentation, the fermentation performance of the immobilized yeast on cork roots was gradually improved. Cork root was therefore a potential support for yeast in repeated batch ethanol fermentation. Application of immobilized yeast to repeated batch fermentation for ethanol production would enhance the economic effectiveness of the production-line because of cost reduction in inoculum preparation and increase in fermentation performance of the immobilized yeast.

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