Application of immobilized yeast in bacterial cellulose to the repeated batch fermentation in wine-making

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Abstract: This study focused on wine fermentation by immobilized yeast in bacterial cellulose. During 10 consecutive cycles of the repeated batch fermentation, the sugar uptake rate of the immobilized yeast increased from 1.71 g/L.h (cycle 1) to 3.28 g/L.h (cycle 7) and then reduced to 2.75 g/L.h (cycle 10). Similarly, the ethanol production rate of the fixed yeast augmented during the first 7 cycles, achieved maximum level of 1.21 g/L.h and subsequently decreased during the last 3 cycles. However, the sugar uptake rate and ethanol production rate of the immobilized yeast during the repeated batch fermentation were 16.4 - 91.8% and 19.6 - 116.1%, respectively higher than those of the free yeast. In addition, the fixed yeast in bacterial cellulose did not affect negatively the sensory quality of the final product.

Keywords: Bacterial cellulose, immobilization, repeated batch fermentation, yeast, wine

Introduction

Cellulose is the most abundant earth biopolymer, recognized as the major component of plant biomass. In the field of cell immobilization, various supports with high cellulosic content such as cashew apple baggasse (Pacheco *et al*., 2010), sorghum baggasse (Yu *et al*., 2007), wild sugarcane (Chandel *et al*., 2009), corn stems (Vucurovic *et al*., 2008) were used. These supports exhibit many advantages: low cost, high reusability, freedom from toxicity problems, high mechanical strength in industrial fermentation conditions (Chandel *et al*., 2009). However, these natural organic supports are not stable in chemical composition and have non-uniform structure (Yu *et al*., 2007). As a result, each cellulosic support requires a special treatment procedure before use.

During the last decade, the application of bacterial cellulose to industrial processes has widely attracted attention (Rezaee *et al*., 2008). Bacterial cellulose is mostly synthesized by *Acetobacter* strains. It is free of lignin, pectin, hemicelluloses, as well as biogenic products, which are associated with plant cellulose (Hong *et al*., 2001). After the fermentation, the treatment procedure for bacterial cellulose is very simple and inexpensive. Bacterial cellulose displays unique physical, chemical and mechanical properties including high crystallinity, high water holding capacity, large surface area, elasticity, mechanical strength and biocompatibility (Astley *et al*., 2001). These features proved that bacterial cellulose could be used as a novel cellulosic support for cell immobilization (Rezaee *et al*., 2008).

Recently, immobilization of wine yeast in bacterial cellulose was reported (Nguyen *et al*., 2008). The immobilized yeast in bacterial cellulose exhibited much higher metabolic activity and higher resistance to unfavourable conditions during the wine fermentation process in comparison with the free yeast (Ton *et al*., 2010).

Reuse of the fixed cells for some cycles of production is one of the important advantages of microbial immobilization (Strehaiano *et al*., 2006). There have been no studies on the reuse of immobilized yeast in bacterial cellulose in wine fermentation. The objective of this research was to determine the metabolic activities of the immobilized yeast in bacterial cellulose during repeated batch fermentation for wine-making. In addition, the fermentation performance of the fixed yeast during the reuse process was also compared to that of the free yeast in a batch fermentation.

Materials and Methods

Materials

Yeast

A strain of *Saccharomyces cerevisiae* from the microbial collection of Food Technology Department (Ho Chi Minh city University of Technology) was used in this study. Grape juice was used for yeast multiplication. Preculture was prepared by two succesive inoculations: 1) in 250 mL erlenmeyer shake flask containing 100 mL of grape juice, and 2) in a 2 L erlenmeyer shake flask containg 0.5 L of grape juice. For both periods, the inoculum was grown at 28°C, 250 rpm and for 24 h. The biomass was then separated by centrifugation at 4°C, 3000 rpm for 15 min and used for immobilization and fermentation.

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Bacterial cellulose

Bacterial celulose (support for yeast immobilization) was produced by the procedure previously described elsewhere (Nguyen, 2006). A strain of *Acetobacter xylinum* from the microbial collection of Biotechnology Department (Ho Chi Minh city University of Technology) was used for bacterial cellulose synthesis.

Medium

The grape juice was prepared from *Vitis vinefera* (Red Cardinal variety) originated from Ninh Thuan, Vietnam. The pH value of must was adjusted to 4.0 by adding tartaric acid or sodium bicarbonate. Glucose and ammonium phosphate were supplemented to must for increasing the hexose and assimilable nitrogen levels to 240 g/L and 195 ppm, respectively. The medium was pasteurized by sulfitation; the sulfure dioxide content in must before fermentation was 112 ppm.

Experimental methods

Yeast immobilization

The biomass of wine yeast obtained from the preculture was subsequently immobilized in bacterial cellulose by adsorption-incubation method. The immobilization procedure was previously described elswhere (Nguyen *et al*., 2009).

Fermentation

The fermentation with immobilized yeast was carried out in erlenmeyer flask containing 500 mL of must at 25±2^{o}C. The inoculating rate for the first cycle of the repeated batch fermentation was 5.0x10^{6} cells/mL. The fermentation was considered as completed when the attenuation reached 97.5% (The attenuation was the ratio between the content of reducing sugars assimilated by yeast during the fermentation and the initial content of reducing sugars in the medium). Ten cycles of the repeated batch fermentation were realized. After each cycle, the immobilized yeast was removed and subsequently washed twice by sterile water at 4^{o}C during 10 min. The immobilized yeast was then reused for the next cycle of the repeated batch fermentation. During each cycle, samples were taken for examining the fermentation kinetics and young wine quality. A batch fermentation with the free yeast was also conducted as the control.

Analytical methods

Yeast cell number in the culture was quantified by haemocytometry, using Thoma counting chamber. For counting yeast cells immobilized in the bacterial cellulose pieces, the support was blended with sterile water in a blender machine (Ton *et al*., 2010). Reducing sugar was determined by spectrophotometric method, using 3,5-dinitrosalicylic reagent (Miller, 1959). Volatile compounds such as ethanol, 1-propanol, isoamyl alcohol, ethyl acetate and acetaldehyde were determined by gas chromatography (Agilent technologies 6890N) using a flame ionization detector (FID) 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 200^{0}C, oven temperature was maintained at 45^{0}C for 2 min, then increased to 150^{0}C with the rate of 7^{0}C/min, hold for 2 min, detector temperature was 200^{0}C. The carrier gas was hydrogen (Gil *et al*., 2006). The immobilized yeast cells in bacterial cellulose were examined under the scanning electron microscope (FESEM, 7410F, Jeol, Japan). The samples were washed with sterile water, dried overnight at 30^{0}C and then sputtered with gold and photographed (Kopsahelis *et al*., 2007).

Statistical treatment

Each presented result was the average of three independent experiments. The data was analyzed for statistical significance by Analysis of Variance (ANOVA). 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.

Results and Discussion

Yeast growth

Figure 1 presents the kinetics of yeast growth during the fermentation. With the same inoculum size of 5.0x10^{6} cells/mL, the immobilized yeast during cycle 1 grew higher and faster than the free yeast. The cell density in the immobilized yeast culture (cycle 1) achieved maximum of 9.5x10^{7} cells/mL after 60 h while the maximum cell density in the control only reached 5.5x10^{7} cells/mL after 72 h. It can be explained that bacterial cellulose protected the wine yeast under unfavourable conditions such as high osmotic pressure, low pH value (Ton *et al*., 2010). As a consequence, the growth of the immobilized yeast was better than that of the free yeast. This phenomenon was previously reported in ethanol fermentation with the immobilized yeast in calcium alginate gel (Bui and Le, 2008).

It should be noted that the cell density in the

bacterial cellulose pieces gradually increased due to yeast growth. Figure 2 demonstrates the yeast cells inside the bacterial cellulose pieces before cycle 1 and 10. In this study, the grape juice volume used in 10 cycles of the repeated batch fermentation was similar. As a result, the initial cell concentration in the medium augmented during the reuse of the fixed yeast. Figure 1 shows that the cell concentration in the medium at the start of the fermentation for cycle 3, 5 and 7 was $2.0x10^7$, $5.0x10^7$ and $7.0x10^7$ cells/mL, respectively. Increase in initial cell concentration in must enhanced the maximum cell density in the immobilized yeast culture. Nevertheless, the initial and maximal cell densities in the immobilized yeast culture of cycle 7 and 10 were nearly similar.

Figure 3 demonstrates that the average growth rate of the immobilized yeast in bacterial cellulose was always higher than that of the free yeast. This observation was also in agreement with the findings of Bui and Le (2008) who applied immobilized yeast in calcium alginate gel to ethanol fermentation. In the repeated batch fermentation, the average growth rate of the immobilized yeast in cycle 1 was the lowest. The average growth rate increased from cycle 1 to cycle 5 and remained nearly constant from cycle 5 to cycle 7. After 7 cycles, the average growth rate of the fixed yeast was gradually reduced. However, the average growth rate in the last cycle (cycle 10) was still higher than that in cycle 1 and 2.

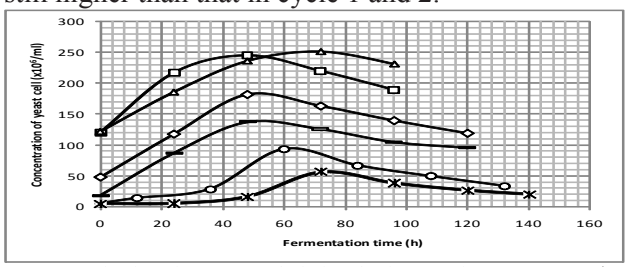

Figure 1. Kinetics of yeast growth during the fermentation Free yeast: (✳); Immobilized yeast with cycle 1: (O), cycle 3: (⊏), cycle 5: (◊), cycle 7: (□), cycle 10: (Δ)

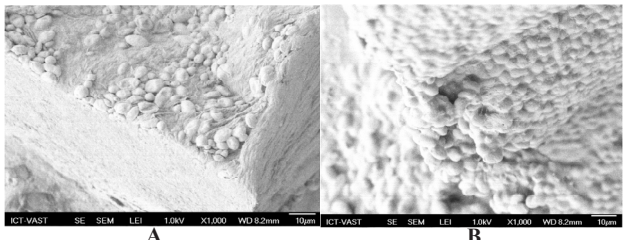

Figure 2. Electron micrographs of the immobilized yeast in bacterial cellulose support (A: before cycle 1, B: before cycle 10)

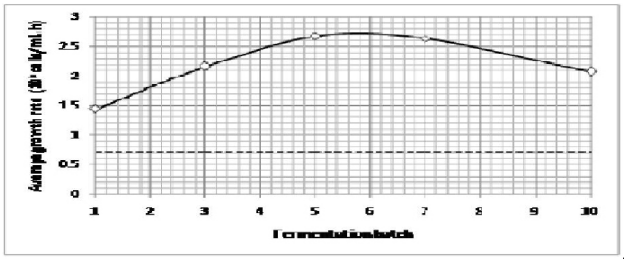

Figure 3. Average growth rate of the immobilized yeast in 10 cycles of the repeated batch fermentation (Immobilized yeast: bolt line, Free yeast (control): dotted line)

Substrate assimilation

The content of sugars assimilated by yeast during the fermentation is visualized in Figure 4. The immobilized yeast in bacterial cellulose utilized sugars significantly faster than the free yeast. Similar results were also demonstrated when the immobilized yeast on delignified cellulosic material was used in wine fermentation (Bardi and Koutinas, 1994). Table 1 indicates that during the repeated batch fermentation, the sugar uptake rate of the immobilized yeast increased from 1.71 g/L.h (cycle 1) to maximum level of 3.28 g/L.h (cycle 7). It was due to a gradual adaptation of the fixed cells to the fermentation conditions. From cycle 8 to cycle 10, the sugar assimilation rate was reduced from 3.18 g/L.h to 2.75 g/L.h. However, the sugar uptake rate of the fixed yeast during the reuse process was 16.4 - 91.8% higher in comparison with that of the free yeast. Gradual increase in substrate consumption rate of the immobilized yeast during the first cycles of the repeated batch fermentation was also mentioned in the study of Diep and Le (2009) who applied the immobilized yeast in pineapple pieces to pineapple wine fermentation.

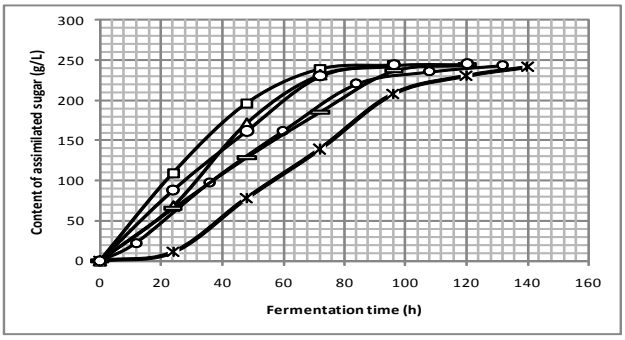

Figure 4. Kinetics of sugars assimilated by yeast during the fermentation Free yeast: (✳); Immobilized yeast with cycle 1: (O), cycle 3: (⊏),cycle 5: (◊),cycle 7: (□), cycle 10: (Δ)

Ethanol formation

The evolution of ethanol concentration in the cultures is given in Figure 5. Immobilization of wine yeast in bacterial cellulose enhanced ethanol formation. Table 1 shows that the ethanol concentration in the immobilized yeast cultures increased from 0 to 21.0% in comparison with that in the free yeast culture. During the repeated batch fermentation, the ethanol production rate of the fixed yeast increased from 0.67 g/L.h (cycle 1) to 1.21 g/L.h (cycle 7) and then reduced to 1.04 g/L.h (cycle 10). Nevertheless, the ethanol production rate of the fixed yeast during the reuse process was 19.6 - 116.1% higher than that of the free yeast. Our results are in accordance with Bakoyianis *et al*. (1997) who reported that the immobilized yeast on inorganic supports such as kissiris and γ-alumina exhibited greater ethanol production rate than the free yeast in wine-making.

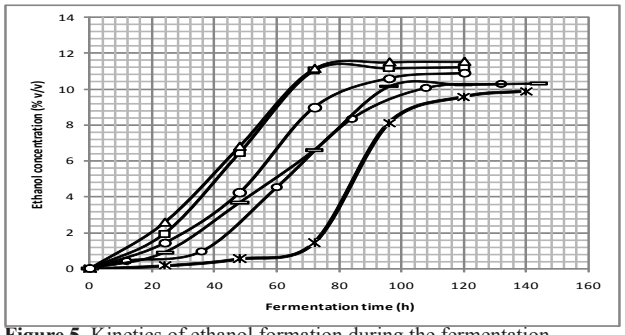

Figure 5. Kinetics of ethanol formation during the fermentation
Free yeast: (✳); Immobilized yeast with cycle 1: (O), cycle 3: (▭),cycle 5: (◊), cycle 7: (□), cycle 10: (Δ)

Increase in sugar uptake rate and ethanol production rate proved that the metabolic activities of the immobilized yeast in wine fermentation were significantly enhanced in comparison with those of the free yeast. The improvement in metabolic activities can be explained by many changes in the morphology of yeast cells inside the supports (Martynenko and Gracheva, 2003). Moreover, Sarishvili and Kardash (1980) reported that the immobilized yeast exhibited a greater activity of some endocellular enzymes and that led to an increase in metabolic reaction rate.

Table 1 illustrates that the fermentation time in the immobilized yeast cultures was notably shortened as compared with that in the free yeast culture. During the repeated batch fermentation, the fermentation time of the fixed cells decreased 39.2% from cycle 1 to cycle 7. However, from cycle 8 to cycle 10, the fermentation time gradually augmented because of the reduction in sugar uptake rate and ethanol production rate of the immobilized yeast.

Table 1. Fermentation characteristics of the repeated batch fermentation

Cycle	Fermentation time (h)	Sugar uptake rate (g/L.h)	Ethanol production rate (g/L.h)	Ethanol concentration in the young wine (% v/v)
FY	140.0^{j}	1.71^{a}	0.56^{a}	9.56^{a}
IY-1	120.6^{i}	1.99^{b}	0.67^{b}	10.28^{c}
IY-2	111.6^{h}	2.15^{c}	0.71^{c}	10.01^{ab}
IY-3	103.1^{g}	2.33^{d}	0.79^{d}	10.31^{c}
IY-4	95.0^{f}	2.53^{e}	0.85^{e}	10.19^{bc}
IY-5	87.0^{e}	2.76^{f}	0.99^{g}	10.87^{d}
IY-6	77.8^{c}	3.09^{h}	1.11^{i}	10.95^{de}
IY-7	73.3^{a}	3.28^{j}	1.21^{k}	11.18^{f}
IY-8	75.6^{b}	3.18^{i}	1.18^{j}	11.17^{f}
IY-9	82.8^{d}	2.90^{g}	1.11^{i}	11.57^{g}
IY-10	87.2^{e}	2.75^{f}	1.04^{h}	11.51^{g}

FY: Free yeast; IY-1,2,3,4,5,6,7,8,9,10: Immobilized yeast with cycle 1,2,3,4,5,6,7,8,9,10
Various superscripts in each column indicate significant difference ($p< 0.05$)

By-product formation

The levels of certain volatile compounds in some young wine samples obtained in the experimentation are given in Table 2. According to Clarke and Bakker (2004), ethyl acetate is an important flavour component of wine. This ester contributes a fruity aroma to wine (Clarke and Bakker, 2004). The immobilized yeast in bacterial cellulose during the repeated batch fermentation produced much higher ethyl acetate level than the free yeast and improved the organoleptic property of the final product. This result is in agreement with previous study focusing on repeated batch fermentation with immobilized yeast in pineapple pieces (Diep and Le, 2009).

Propanol and isoamyl alcohol are main components in fusel alcohol group in wine. At a high concentration (>300 mg/L), fusel alcohols are negative quality factors; but at lower levels, they add to the desirable aspects of wine flavour (Clarke and Bakker, 2004). Table 3 shows that the concentration of propanol and isomyl alcohol in the immobilized yeast cultures was similar or lower than that in the free yeast culture. Similar result was reported by Mallios *et al*. (2004) when using immobilized yeast on pear pieces for wine fermentation. Acetaldehyde is usually regarded as an off-odour. Its threshold level is 120 ppm (Clarke and Bakker, 2004). The immobilized yeast in bacterial cellulose produced higher level of acetaldehyde than the free yeast during the repeated batch fermentation. However, the acetaldehyde content in all young wine samples obtained was much lower than its threshold. In summary, the immobilized yeast in bacterial cellulose did not affect negatively the sensory quality of the final product during the repeated batch fermentation.

Table 2. Some volatile compounds (mg/L) in young wine in the repeated batch fermentation

Cycle	Ethyl acetate	Propanol	Isoamyl alcohol	Acetaldehyde
FY	10.6^{a}	43.2^{a}	73.4^{a}	13.9^{a}
IY-1	39.8^{b}	26.0^{b}	10.7^{b}	52.9^{b}
IY-3	70.4^{c}	33.2^{c}	75.1^{a}	61.2^{c}
IY-7	57.5^{d}	33.3^{c}	42.5^{c}	75.1^{d}

FY: free yeast; IY-1,3,7: immobilized yeast with cycle 1,3,7
Various superscripts in each column indicate significant difference ($p< 0.05$)

Conclusion

Application of immobilized yeast to repeated batch fermentation in wine-making enhances the economic effectiveness of the production-line because of cost reduction in inoculum preparation and simple separation of yeast at the end of the fermentation. In 10 cycles of the repeated batch fermentation, the fixed yeast in bacterial cellulose always exhibited higher metabolic activities than the free yeast. Bacterial cellulose was therefore a potential support for wine yeast in wine fermentation.

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