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Optimization of *Saccharomyces cerevisiae* immobilization in bacterial cellulose by 'adsorption- incubation' method

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Abstract: Bacterial Cellulose (BC) was used as a carrier for *Saccharomyces cerevisiae* immobilization. The immobilization method included two steps: adsorption and incubation. In the adsorption step, initial cell concentration in the yeast suspension and immobilization time were optimized by Face Centered Central Composite Design (FCCCD) for maximizing cell immobilization yield. In the incubation step, incubation time was examined for increasing yeast cell number in BC. The yeast cell number could reach $1.4x10^9$ cells/g BC. In wine fermentation, the metabolic activities of the immobilized yeast in BC were much higher than those of the free yeast.

Key words: Adsorption, bacterial cellulose, immobilization, incubation, *Saccharomyces cerevisiae*

Introduction

Application of immobilized yeast in wine fermentation has been an attractive and rapidly expanding research area because of its technical and economical advantages compare to free cell system (increasing the substrate uptake rate, reducing the fermentation time, and resulting in expected concentration of volatile constituents for product) (Kourkoutas *et al.,* 2002). Researches have proposed many supports for yeast immobilization in wine production such as porous glass, fruit pieces (apple, quince), and alginates. However, inorganic supports are considered as inconvenient for food production (Kourkoutas *et al.,* 2004); immobilization by adsorption on surface of the supports such as porous glass, fruit pieces leads to low immobilization yield (Bardi, 1994); while alginate gel, a popular support in wine fermentation, is gradually broken during the reutilization of the biocatalyst due to the release of carbon dioxide by yeast (Nguyen, 2006).

BC is mostly synthesized by *Acetobacter xylinum*, does not require the treatment to remove unwanted polymers and contaminants (lignin, hemicellulose). Therefore BC retains a great degree of polymerisation. In native state, BC also has great hydration, holding over a hundred times its own weight in water. So BC can be considered as a good support for cell immobilization (Serafica, 1997).

'Adsorption- incubation' has been a potential method for cell immobilization in BC. Nguyen (2006) immobilized lactic acid bacteria in BC pieces by this method. In lactic acid fermentation, this biocatalyst could be reused for many batches without significant change in metabolic activity. This method includes 2 steps: adsorption and incubation. Firstly, microbial biomass and BC pieces are suspended in a medium for cell adsorption onto the support. The substrates diffused partially into the internal structure of BC pieces. Then the BC pieces are isolated and incubated in a sterile empty vessel at a suitable temperature. During the second step, microbial growth occurs inside the structure of BC and the cell number per 1 g of BC increases.

This study focused on optimization of wine yeast immobilization in BC by 'adsorption- incubation' method for maximizing the cell number in BC.

Materials and methods

Materials

A strain of *S. cerevisiae* used in this study was supplied by Food Technology Department, Ho Chi Minh City University of Technology, Vietnam. Yeast was propagated at 30°C for 2 days, and then the biomass was separated by centrifugation at 4°C, 3000 rpm for 15 minutes and used for immobilization.

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Table 1. Experimental ranges and levels of the independent variables for response surface of the immobilization yield

Independent variables	Symbol	Range and levels		
		-1	0	1
Cell concentration in yeast suspension (x10^8 cells/ml)	X_1	1	2	3
Immobilization time (h)	X_2	4	5	6

Table 2. Face Centered Central Composite Design and response of the dependent variable (Y) for cell immobilization yield to independent variables (X1: initial cell concentration in yeast suspension, X2: immobilization time)

Exp.No.	X_1	X_2	Response, Y
1	+1	+1	16.67
2	-1	+1	23.44
3	+1	-1	22.92
4	-1	-1	35.94
5	+1	0	31.77
6	-1	0	48.44
7	0	+1	39.84
8	0	-1	56.25
9	0	0	60.16
10	0	0	61.72
11	0	0	59.38
12	0	0	60.16

With experiment number 1 to 4: factorial runs; 5 to 8: axial points with α =1; 9 to 12: center points in cube

A variety of *Vitis vinifera* was used. The initial sugar concentration in must was adjusted by adding D-glucose up to 240 g/l. This medium was sterilized at 121°C for 20 minutes and used for yeast propagation and immobilization. BC from *A. xylinum* was produced by the procedure previously described elsewhere (Krystynowics *et al.,* 2002; Nguyen, 2006).

Yeast immobilization in BC by 'adsorption- incubation'

In the adsorption step, yeast biomass was suspended in grape must to reach a proper cell concentration. Then a determined weight of BC pieces which had an appropriate size was added to the cell suspension. Agitation was continuously realized during the adsorption step. Finally, the liquid was decanted and the immobilized biocatalyst was washed with sterile water. In the incubation step, BC pieces containing yeast cells were incubated in a sterile empty erlenmeyer at 30°C for a proper time.

Optimization of technological parameters in the yeast adsorption step

The objective of this experiment was determining optimal parameters in the yeast adsorption step for maximizing the cell immobilization yield on BC pieces. Firstly, six variables as factors affecting cell immobilization yield were alternatively examined: initial cell concentration in the yeast suspension, size of BC pieces, amount of BC in 1 l yeast suspension, pH of yeast suspension, agitation rate and immobilization time (this part does not include in this paper). Secondly, two independent variables including initial cell concentration in the yeast suspension (X_1) and immobilization time (X_2) were chosen and optimized by FCCCD for maximizing the cell immobilization yield (Y). The experimental design was shown in Table 1. With two variables coded to three levels of -1, 0, +1, a set of 12 runs was designed with four factorial runs, four axial points and four center point in cube. In addition, alpha and center point in axial

were 1 and 0, respectively. The optimum conditions of cell immobilization were obtained by using FCCCD of the coded variables, using the design presented in Table 2.

Influence of incubation time on the yeast cell number in BC

After adsorption step, the obtained biocatalysts were alternatively incubated for 0, 1, 2, 3 and 4 days. Then cell concentration in BC was quantified.

Wine fermentation by immobilized yeast in BC

Grape must was used as a medium. $Na_2S_2O_5$ was added to grape must (110 mg/l) in order to inhibit its microbial flora. The pitching rate was 5 x 10^6 cells per 1 ml grape must. Batch fermentation was carried out without agitation at 20°C, using 4 samples of the immobilized yeast in BC. The incubation time of the 4 biocatalysts in the incubation step was 0, 1, 2 and 3 days. In the control sample, free yeast cells were used. At the end of the fermentation, the sugar uptake rate, ethanol uptake rate and some physico-chemical characteristics of young wine (content of ethanol, residual sugar, total and volatile acidity) were determined.

Analytical methods

Cell immobilization yield of the adsorption step: Y (%) = N_1/N_2; where: N_1: cell number in the support at the end of the adsorption step, N_2: cell number in the yeast suspension at the beginning of the adsorption step. For determining exactly the cell immobilization yield and preventing yeast growth during the adsorption step, an acetate buffer (pH 4) was replaced for grape must in the immobilization procedure. Yeast cell number in BC pieces was quantified by a method described elsewhere (Leboffe and Pierce, 2006).

Reducing sugar was measured by spectrophotometric method using 3,5 dinitrosalycylic acid reagent. Alcohol was distilled and measured by using a Gay-Lussac alcoholmeter. Volatile acidity was estimated by titration of distillate that was obtained by steam distillation of young wine sample, using 0.1 M NaOH solution.

Statistical treatment

Each presented result was the average of three independent experiments. The obtained results were subjected to analysis of variance (ANOVA) with p value <0.05, using Statgraphics plus software, version 3.2.

Results and discussion

Optimization of technological factors in the yeast adsorption step

The suitable technological factors for yeast adsorption on BC pieces were as follows: initial cell concentration in the yeast suspension: 2 x 10^8 cells/ml; size of BC pieces: 1 x 1 cm x cm; amount of BC in 1 l of yeast suspension: 200 g; pH of yeast suspension: 4; agitation rate: 200 rpm, and immobilization time: 5 h.

After fitting the experimental data (Table 3), the results showed that linear coefficients (X_1, X_2) and pure quadratic coefficients (X_1^2, X_2^2) were significant, but the interaction coefficient was not (p=0.276). The statistical significance of the quadratic model equation was evaluated by the analysis of variance (ANOVA) in Table 4. The influence of cell concentration in the yeast suspension and immobilization time on the immobilization yield were calculated and expressed in quadratic model by Eq. 1. Considering the model equation, both factors affected the immobilization

Table 3. Estimated values, standard errors, T and P-value of coefficients for cell immobilization yield

Variables	Estimated value of coefficient	Standard error	T	P
Intercept	60.86	1.19	51.07	0.000
X_1	-6.08	1.07	-5.70	0.001
X_2	-5.86	1.07	-5.50	0.002
$X_1.X_2$	-1.56	1.31	1.20	0.276
X_1^2	-21.78	1.60	-13.62	0.000
X_2^2	-13.84	1.60	-8.65	0.000

S=2.611; -R^2= 98.7%

Table 4. Analysis of variance for the model representing the immobilization yield (Y)

Source	DF	SS	MS	F-value	P
Regression	5	3036.90	607.38	89.10	0.000
Linear	2	427.59	213.80	31.36	0.001
Square	2	2599.54	1299.77	190.68	0.000
Interaction	1	9.77	9.77	1.43	0.276
Residual error	6	40.90	6.82		
Lack of fit	3	38.01	12.67	13.15	0.031
Pure error	3	2.89	0.96		
Total	11				

DF: degrees of freedom; SS: sum of square; MS: mean square

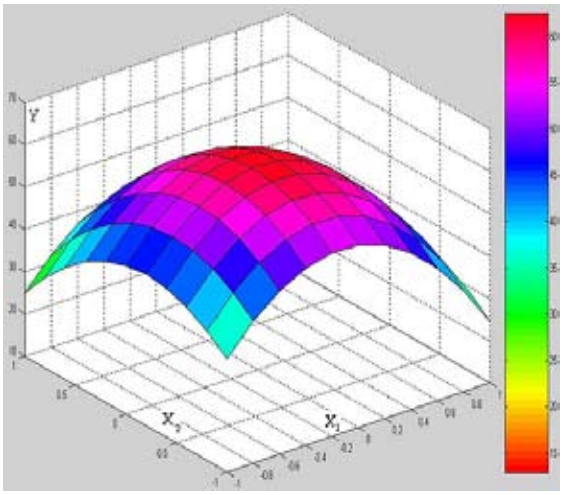

Figure 1. Response surface plot described by Eq. 1 for maximizing cell immobilization yield (Y), X_1: initial cell concentration in yeast suspension, X_2: immobilization time

yield but the effect of cell concentration in the yeast suspension was higher.

$$Y = 60.86 - 6.08X_1 - 5.86X_2 - 21.78X_1^2 - 13.84X_2^2 \quad (1)$$

with $-1 \le X_1, X_2 \le 1$, Y: immobilization yield, X_1: cell concentration in the yeast suspension, X_2: immobilization time.

The response surface plot for the effect of initial cell concentration in the yeast suspension and immobilization time was presented in Figure 1. The paraboloid plot showed that too low or too high values of each variable also leaded to low immobilization yield. It was explained that the proper mass of BC only adsorbed the proper number of cells during the appropriate time.

Optimal parameters were determined by setting the partial derivatives of the model equation to zero. The stationary point is a maximum. Critical values of the two factors were X_1= -0.139, X_2= -0.212, respectively (X_1: initial cell concentration in the yeast suspension, X_2: immobilization time, with $-1 \le X_1, X_2 \le 1$). Actual values of the two factors against critical values were 1.9 x 10^8 cells/ml of initial cell concentration in the yeast suspension and 4 hours 45 minutes of immobilization time. In these conditions, the immobilization yield in buffer solution reached maximum value, 62.61%. This value was equivalent to 5.8 x 10^8 cells immobilized in 1 g BC. If grape must was used for replacing buffer solution in the immobilization procedure, 1 g BC contained approximately 6 x 10^8 cells. It was due to yeast growth during the adsorption step.

Table 5. Technological characteristics of wine fermentation by the immobilized yeast in BC and free yeast. The initial sugar concentration in grape must was 240 g/l

	Immobilized cells in BC Incubation time (days) in the immobilization method 'adsorption- incubation'				Free cells (Control sample)
	0	**1**	**2**	**3**	
Fermentation time (h)	$121.7^{a} \pm 2.3$	$102.4^{b} \pm 1.8$	$94.5^{c} \pm 1.6$	$99.5^{b} \pm 2.1$	$132.1^{d} \pm 2.5$
Sugar uptake rate (g/lh)	$1.92^{a} \pm 0.08$	$2.29^{b} \pm 0.07$	$2.48^{c} \pm 0.06$	$2.35^{bc} \pm 0.07$	$1.77^{d} \pm 0.08$
Residual sugar in young wine (g/l)	$6.20^{a} \pm 0.21$	$3.52^{b} \pm 0.27$	$3.27^{b} \pm 0.18$	$3.59^{b} \pm 0.19$	$7.81^{c} \pm 0.31$
Ethanol in young wine (% v/v)	$11.97^{a} \pm 0.05$	$12.02^{a} \pm 0.17$	$12.05^{a} \pm 0.30$	$11.97^{a} \pm 0.13$	$11.94^{a} \pm 0.19$
Ethanol production rate (g/lh)	$0.76^{a} \pm 0.05$	$0.91^{b} \pm 0.04$	$0.99^{b} \pm 0.05$	$0.93^{b} \pm 0.02$	$0.75^{a} \pm 0.02$
Total acidity (g tartaric acid / l young wine)	$6.89^{a} \pm 0.07$	$6.05^{b} \pm 0.12$	$5.79^{c} \pm 0.06$	$6.13^{b} \pm 0.04$	$6.98^{a} \pm 0.05$
Volatile acidity (g acetic acid / l young wine)	$0.24^{a} \pm 0.01$	$0.23^{b} \pm 0.01$	$0.20^{c} \pm 0.01$	$0.23^{b} \pm 0.01$	$0.25^{a} \pm 0.01$

Various small letters in each row represent statistically significant difference at the level of p=0.05

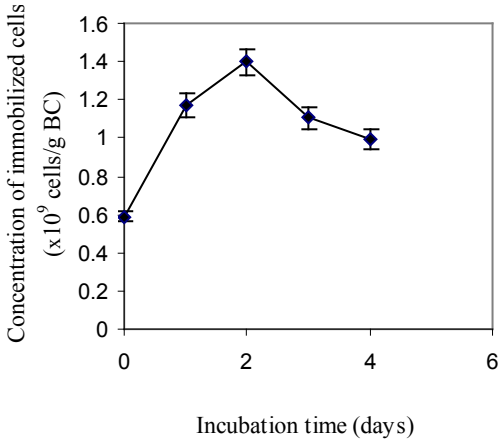

Figure 2. Effect of incubation time on yeast cell number in BC

Influence of incubation time on the yeast cell number in BC

The effect of incubation time on yeast cell number in BC is showed in Figure 2. Increase in incubation time from 0 to 2 days augmented the yeast cell number in the BC pieces 2.33 times. During the adsorption step of the immobilization procedure, yeast cells adsorbed mostly on the surface of BC pieces, while the substrates from the grape must could diffuse partially to the inside structure of the carrier. Then during the incubation step of the immobilization procedure, some yeast cells diffused from the surface to the inside structure of BC pieces for substrate uptake and yeast growth occurred inside the BC. After 2 day incubation, the yeast cell number in the biocatalyst reached maximum. The image from scanning electron microscope (SEM) in Figure 3 proved an important increase in cell number in the inside structure of BC pieces after 2 day incubation. However, if the incubation time was longer than 2 days, yeast cell number in the biocatalyst decreased due to the lack of substrates for yeast metabolism.

Wine fermentation using immobilized yeast in BC

The results are presented in Table 5. Application of immobilized yeast in BC decreased the fermentation time from 7.8 to 28.4% in comparison with the control sample. The sugar uptake rate of the immobilized yeast was always higher than that of the free yeast. The same phenomenon was also observed by different researchers when using immobilized yeast in alginate gel (D'Amore, 1989; Galazzo and Bailey, 1990), on pear pieces (Mallios, 2004). In addition, the biosynthesis of volatile acids of the immobilized yeast was lower than that of the free yeast. Low volatile acidity in young wine improved the flavour of the final product. According to Bardi *et al.* (1994) and Mallouchos *et al.* (2003), wine fermented by yeast immobilized on delignified cellulose or grape skin had a better flavour in comparison with the control sample using the free yeast.

When using the immobilization method 'adsorption- incubation', the incubation of the BC pieces containing yeast cells in an empty erlenmeyer improved significantly the fermentation performance of the biocatalyst in comparison with the sample

without incubation. It should be affirmed that 2 day incubation of the biocatalyst resulted in the shortest fermentation time, the highest glucose uptake rate and ethanol production rate, the lowest volatile acidity in young wine. Therefore, two day incubation was regarded as the most appropriate time in the immobilization method 'adsorption- incubation'.

Conclusion

Immobilization of wine yeast in BC by 'adsorption- incubation' method resulted in high cell number in the biocatalyst. The immobilization procedure was very simple, easy realizing and inexpensive. The application of immobilized yeast in BC in wine fermentation was therefore very hopeful. In addition, 'adsorption- incubation' method can be used for immobilization of different microbial species in BC.

References

Bardi, E. P. and Koutinas, A. A. 1994. Immobilization of yeast on delignified cellulosic material for room temperature and low-temperature wine making. Journal of Agriculture and Food Chemistry 42: 221-226.

D'Amore, T., Russell, I. and Stewart, G. G. 1989. Sugar utilization by yeast during fermentation. Journal of IndustriaI Microbiology 4: 315-324.

Galazzo, J. L. and Bailey, J. E. 1990. Growing *Saccharomyces cerevisiae* in calcium-alginate beads induces cell alterations which accelerate glucose conversion to ethanol. Biotechnology and Bioengineering 36: 417-426.

Kourkoutas, Y., Douma, M., Koutinas, A. A., Kanellaki, M., Banat, I. M. and Marchant, R. 2002. Continuous winemaking fermentation using quince-immobilized yeast at room and low temperatures. Process Biochemistry 39: 143-148.

Kourkoutas, Y., Bekatorou, A., Banatb, I. M., Marchant, R. and Koutinas, A. A. 2004. Immobilization technologies and support materials suitable inalcohol beverages production: a review. Food Microbiology 2: 377-397.

Krystynowics, A. and Czaja, W. 2002 Factors affecting the yield and properties of bacterial cellulose. Industrial Microbiology and Biotechnology 29: 189-195.

Leboffe, M. J. and Pierce, B. E. 2006. Microbiology: Laboratory theory and application. 2th edn. Colorado: Morton.

Mallios, P., Kourkoutas, Y., Iconomopoulou, M., Koutinas, A. A., Psarianos, C., Marchant, R. and Banat, I. M. 2004. Low-temperature wine-making using yeast immobilized on pear pieces. Journal Agriculture and Food Chemistry 84: 1615–1623.

Mallouchos, A., Skandamis, P., Loukatos, P., Komaitis, M., Koutinas, A. A. and Kanellaki, M. 2003. Volatile compounds of wines produced by cells immobilized on grape skins. Journal Agriculture and Food Chemistry 51: 3060-3066.

Nguyen, T. H. 2006. Selection and improvement of strains of *Acetobacter xylinum* to synthesize bacterial cellulose in production and application at pilot scale. Ho Chi Minh City, Vietnam: The University of Natural Sciences- Ho Chi Minh City National University, PhD Thesis.

Serafica, G. C. 1997. Production of bacterial cellulose using a rotating disk film bioreactor by *Acetobacter xylinum*. New York, America: Rensselaer Polytechnic Institute, PhD Thesis.