International Food Research Journal 17: 743-749 (2010)

Influence of initial pH and sulfur dioxide content in must on wine fermentation by immobilized yeast in bacterial cellulose

Ton, N.M.N., Nguyen, M.D., Pham, T.T.H. and *Le, V.V.M.

Department of Food Technology, Ho Chi Minh City University of Technology, Vietnam

Abstract: In this study, wine fermentation was carried out by yeast immobilized in bacterial cellulose (BC). The initial pH and sulfur dioxide content in must were adjusted from 3.5 to 4.5, and from 12 ppm to 312 ppm, respectively. The results indicated that the maximum specific growth rate of the immobilized yeast was 1.17 – 1.39 times more than that of the free yeast. Besides, the sugar uptake rate and ethanol production rate of the immobilized yeast were 1.04 - 1.41 times and 1.05 – 1.35 times, respectively, more than those of the free yeast. However, the ethanol production yield of the immobilized yeast was similar or slightly lower than that of the free yeast in must with low initial pH or high sulfur dioxide content. Application of immobilized yeast to must fermentation produced wine with low pH and volatile acid content and this led to an improvement in physico-chemical, microbiological and sensory quality of the final product.

Keywords: Bacterial cellulose, immobilization, fermentation, pH, sulfur dioxide, yeast

Introduction

Wine production is a very old finding and wine is defined as an alcoholic beverage obtained from grape juice using yeast as a fermenting organism. Cell immobilization in alcoholic fermentation is a rapidly expanding research area because of its technical and economic advantages compared to the conventional free cell system including: prolonging activity and stability of the biocatalyst, reducing fermentation time, feasibility of continuous processing, reducing cost for equipment and energy demands (Kourkoutas *et al*., 2004; Reddy *et al*., 2008).

Various supports have been proposed for potential use in fermentation of wine, including inorganic supports such as mineral kissiris and γ-alumina (Bakoyianis *et al*., 1997), but none of them fulfilled the food-grade properties. Food-grade natural supports such as delignified cellulose materials, gluten pellets (Loukatos et al., 2003), calcium alginate (Bakoyianis *et al*., 1997) and also fruit pieces like pear and apple (Kourkoutas *et al*., 2002; Mallios *et al*., 2004) were successfully used as immobilization supports for wine production. However, for winemaking industry, it is important to identify a suitable immobilization support which is food-grade, readily available and cost-effective (Reddy *et al*., 2008).

Combined treatment of must by pH adjustment and sulfur dioxide addition has been considered as an appropriate technique to prevent microbial spoilage in wine fermentation because low pH can improve the pasteurization effect of sulfur dioxide and gives the winemaker important information about how much sulfur dioxide is needed to control microbes effectively (Vine *et al*., 1997). However, low pH and high sulfur dioxide content in must can inhibit the growth and metabolic activities of wine yeast. Moreover, high sulfur dioxide content in must negatively affects human health.

There have been many researches on the effects of pH and sulfur dioxide on the fermentation characteristics of yeast immobilized on different supports (Bui *et al*., 2005; Ton *et al*., 2008a and b). However, there have been no studies on the influence of these technological factors on the growth and metabolic activities of yeast immobilized in BC. BC produced by acetic acid bacteria is far superior to its counterpart from plants, because of its exceptional purity, ultra fine network structure, high biodegradability and unique mechanical strength (Takayasu *et al*., 1997). The aim of this research was to investigate the influence of initial pH and sulfur dioxide in must on wine fermentation by yeast immobilized in BC.

Corresponding author.
Email: *lvvman@hcmut.edu.vn*
Tel: (00) (84) (8) 38 64 62 51 **;** Fax: (00) (84) (8) 38 63 75 04

Materials and Methods

Yeast

A strain of *Saccharomyces cerevisiae* used in this study was supplied by Food Technology Department, Ho Chi Minh City University of Technology. Yeast was prepared by two successive inoculations: 1) in 250 mL Erlenmeyer shake flasks containing 100 mL of grape juice for 24 hours, and 2) in a 2 L Erlenmeyer shake flask containing 500 mL of grape juice. For both periods, the inoculum was grown at 30°C and 200 rpm. The biomass was then separated by centrifugation at 4°C, 3000 rpm for 15 minutes and used for immobilization and fermentation.

Bacterial cellulose (BC)

BC from *A. xylinum* was produced by the procedure previously described elsewhere (Nguyen, 2006).

Must

A variety of *Vitis vinifera* was used. Must was prepared for inoculation and fermentation. For inoculation, must was adjusted to 195ppm of ammonium nitrogen and sterilized at 121°C for 20 minutes. For fermentation, must was adjusted to 240 g/L of glucose and 195ppm of ammonium nitrogen; initial pH value was regulated by adding tartaric acid or sodium bicarbonate; initial sulfur dioxide content was adjusted by adding $Na_2S_2O_5$.

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 the size of 1x1 cm^2 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 48 hours. (Nguyen *et al*., 2009).

Fermentation

Fermentation was conducted at 22-25°C in Erlenmeyers containing 500mL of grape must. The inoculating rate was $5x10^6$ cells/mL. Free yeast cells were used in the control sample. When the pH value of must was varied from 3.5 to 4.5, the initial sulfur dioxide content in must was fixed at 112ppm. And when the initial sulfur dioxide content in must varied from 12 ppm to 312 ppm, the pH value of must was adjusted to 4.0.

Analytical methods

Yeast cell number was quantified by haemocytometry, using Thoma counting chamber. For counting yeast cells immobilized in the BC pieces, the support was blended with sterile water in a blender machine (Leboffe *et al*., 2006).

Reducing sugar and ammonium were measured by spectrophotometric method using 3,5 dinitrosalycylic acid and phenol reagents, respectively (Helrich, 1992). Alcohol was distilled and measured by using a Gay-Lussac alcoholmeter (Helrich, 1992).

Volatile acidity was estimated by titration of distillate that was obtained by steam distillation of young wine sample, using 0.1 M NaOH solution (Vine *et al*., 1997).

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 3.0.

Results and Dicussion

Effect of pH and sulfur dioxide on yeast growth

Figure 1 indicates that when the initial pH value of must reduced from 4.5 to 3.5, the maximum specific growth rate and maximum cell density of the immobilized yeast culture decreased 35.4 % and 8.5 %, respectively, while those of the free yeast culture decreased 53.0 % and 15.5%. According to Vine *et al*. (1997), decrease in initial pH value of must inhibited metabolic activities of contaminated bacteria during wine fermentation. The growth of wine yeast was therefore facilitated. However, too low pH value could inhibit wine yeast. It can be affirmed that the growth of the immobilized yeast in BC was always better than that of the free yeast in all cases. The maximum specific growth rate and maximum cell density of the immobilized yeast culture were 29.0% – 77.0 % and 3.7% - 12.3 %, respectively, higher than those of the free yeast culture.

From Figure 2, it can be noted that when the initial SO_2 content in must augmented from 12 ppm to 112 ppm, the maximum specific growth rate and maximum cell density of the immobilized and free yeast cultures remained unchanged or increased slightly. On the contrary, when the initial SO_2 content in must increased from 112 ppm to 312 ppm, both maximum specific growth rate and maximum cell density of the immobilized and free yeast cultures

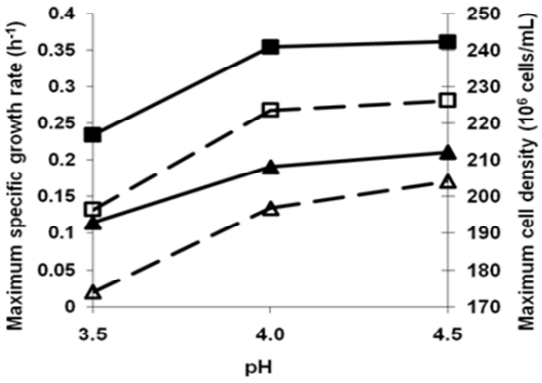

Figure 1. Effect of pH on maximum specific growth rate and maximum cell density. Symbol: (□■), maximum specific growth rate, (△▲), maximum cell density. Opened symbol: free yeast, filled symbol: immobilized yeast

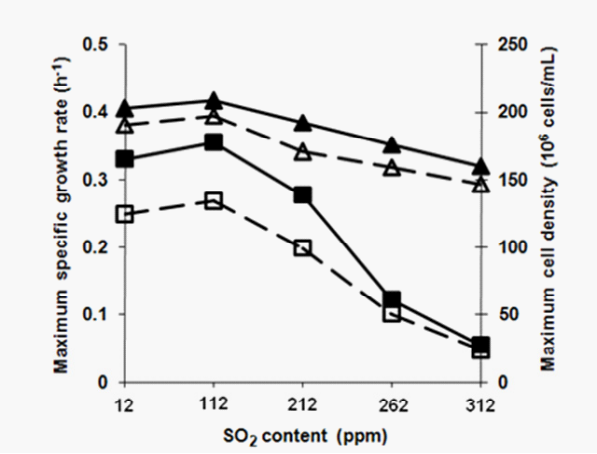

Figure 2. Effect of SO_2 on maximum specific growth rate and maximum cell density. Symbol: (□■), maximum specific growth rate, (△▲), maximum cell density. Opened symbol: free yeast, filled symbol: immobilized yeast

decreased sharply. Nevertheless, the maximum specific growth rate and maximum cell density of the immobilized yeast culture was always higher than those of the free yeast culture except that in medium with 312ppm sulfur dioxide, these values were insignificantly different. Sulfur dioxide was used for controlling growth of the contaminated bacteria and wild yeast (Vine *et al*., 1997; Reddy *et al*., 2008). However, high content of sulfur dioxide in must could inhibit growth of the inoculum.

In summary, low initial pH value and high initial SO_2 content in must decreased the maximum specific growth rate and maximum cell density of the immobilized and free yeast cultures. However, the immobilized yeast in BC grew similar or better than the free yeast. This result was not in agreement with the studies of some researchers such as Banyopadhyay *et al*. (1982) with yeast adsorbed on pore–glass and Doran *et al*. (1986) with yeast covalently linked with gelatin. According to these authors, the growth of the immobilized yeast was lower in comparison with that of the free yeast because the formation of the daughter cells by the immobilized yeast could not proceed as readily as with the freely suspended cells. In our study, it can be explained that yeast immobilized in BC by "adsorption-incubation" method could grow not only on the surface of the support but also in the support structure (Nguyen, 2006; Nguyen *et al*., 2009). Moreover, the immobilized cells in BC had a longer adaptation period to the substrates in must during the incubation step (Nguyen *et al*., 2009). These phenomena improved the growth of the immobilized cells in BC in comparison with that in other supports.

Effects of pH and sulfur dioxide on fermentation rate

Table 1 shows that when the initial pH value of must decreased from 4.5 to 3.5, the fermentation time of the free yeast was 3.5h - 37.8h longer than that of the immobilized yeast in BC. In this study, the fermentation time was recorded when the fermentation degree that was the ratio between the utilized sugar content during the fermentation and the initial sugar content in the medium reached 90%. Similarly, when the initial sulfur dioxide content in must augmented from 12 ppm to 312 ppm, the fermentation time of the free yeast was 8.9h - 29.7h higher than that of the immobilized yeast (Table 2). It can be concluded that the immobilized yeast in BC fermented more quickly than the free yeast.

In winemaking, evaluation of fermentation rate of inoculum is based on sugar and ammonium uptake rates and ethanol production rate. From Table 1, it can be seen that reduction in initial pH value from 4.5 to 3.5 decreased both sugar and ammonium uptake rate. However, the sugar and ammonium uptake rates of the immobilized yeast were 4.4% – 27.3% and 4.1% – 29.3 %, respectively higher than those of the free yeast. Similar phenomenon was also observed by Doran *et al*. (1986) and Galazzo *et al*. (1988). In their study, the immobilized cells in gelatin and alginate gel consumed glucose twice as fast as the free cells.

Concerning with effect of sulfur dioxide, when its content in must increased from 12 ppm to 112 ppm, the sugar and ammonium uptake rates of the immobilized yeast changed insignificantly and those of the free yeast increased slightly (Table 2). Frivik *et al.* (2003) also recognized that there was not a significant effect on the progress of the fermentation at the low levels of the sulfur dioxide additions from

Table 1. Effect of initial pH on the fermentation ability of immobilized and free yeast

Initial pH value	State of yeast	Fermentation time (h)	Average sugar uptake rate (g/L.h)	Average ammonium uptake rate (mg/L.h)	Average ethanol production rate (g/L.h)	Ethanol production yield (mole of ethanol/ mole of glucose)
3.5	Immobilized	$103.2^{c} \pm 1.7$	$2.09^{b} \pm 0.03$	$1.88^{b} \pm 0.04$	$0.822^{b} \pm 0.011$	$1.536^{a} \pm 0.018$
	Free	$141.0^{d} \pm 2.0$	$1.52^{a} \pm 0.04$	$1.33^{a} \pm 0.03$	$0.611^{a} \pm 0.015$	$1.565^{b} \pm 0.015$
4.0	Immobilized	$79.6^{a} \pm 1.2$	$2.71^{d} \pm 0.06$	$2.44^{e} \pm 0.05$	$1.116^{e} \pm 0.022$	$1.609^{c} \pm 0.017$
	Free	$88.5^{b} \pm 1.5$	$2.30^{b} \pm 0.10$	$2.07^{c} \pm 0.05$	$1.005^{c} \pm 0.018$	$1.611^{c} \pm 0.015$
4.5	Immobilized	$78.5^{a} \pm 1.3$	$2.75^{d} \pm 0.05$	$2.46^{e} \pm 0.04$	$1.141^{f} \pm 0.013$	$1.623^{d} \pm 0.020$
	Free	$82.0^{a} \pm 1.3$	$2.63^{d} \pm 0.08$	$2.36^{d} \pm 0.03$	$1.091^{d} \pm 0.017$	$1.620^{d} \pm 0.015$

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

Table 2. Effect of initial sulfur dioxide content on the fermentation ability of immobilized and free yeast

Initial SO_2 content (ppm)	State of yeast	Fermentation time (h)	Average sugar uptake rate (g/L.h)	Average ammonium uptake rate (mg/L.h)	Average ethanol production rate (g/L.h)	Ethanol production yield (mole of ethanol/ mole of glucose)
12	Immobilized	$80.6^{a} \pm 1.1$	$2.68^{f} \pm 0.07$	$2.41^{i} \pm 0.05$	$1.092^{i} \pm 0.015$	$1.594^{bc} \pm 0.015$
	Free	$99.3^{c} \pm 0.9$	$2.17^{d} \pm 0.06$	$1.94^{f} \pm 0.03$	$0.902^{f} \pm 0.022$	$1.603^{cd} \pm 0.019$
112	Immobilized	$79.6^{a} \pm 0.4$	$2.71^{f} \pm 0.05$	$2.44^{i} \pm 0.03$	$1.116^{j} \pm 0.022$	$1.609^{cd} \pm 0.018$
	Free	$88.5^{b} \pm 0.6$	$2.44^{e} \pm 0.11$	$2.19^{h} \pm 0.05$	$1.005^{h} \pm 0.018$	$1.612^{cd} \pm 0.015$
212	Immobilized	$94.9^{c} \pm 0.9$	$2.27^{d} \pm 0.05$	$2.03^{g} \pm 0.03$	$0.936^{g} \pm 0.015$	$1.608^{cd} \pm 0.011$
	Free	$115.4^{e} \pm 1.4$	$1.87^{c} \pm 0.08$	$1.63^{d} \pm 0.04$	$0.771^{e} \pm 0.011$	$1.613^{d} \pm 0.019$
262	Immobilized	$109.2^{d} \pm 0.7$	$1.97^{c} \pm 0.09$	$1.73^{e} \pm 0.02$	$0.640^{d} \pm 0.014$	$1.594^{bcd} \pm 0.015$
	Free	$138.7^{f} \pm 1.0$	$1.56^{b} \pm 0.07$	$1.4^{c} \pm 0.05$	$0.566^{c} \pm 0.012$	$1.609^{cd} \pm 0.023$
312	Immobilized	$150.5^{g} \pm 3.0$	$1.43^{b} \pm 0.06$	$1.18^{b} \pm 0.04$	$0.484^{b} \pm 0.011$	$1.543^{a} \pm 0.011$
	Free	$180.2^{h} \pm 2.3$	$1.20^{a} \pm 0.09$	$0.97^{a} \pm 0.03$	$0.432^{a} \pm 0.009$	$1.580^{b} \pm 0.012$

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

0 to 150 mg/L. However, when the initial sulfur dioxide content in must increased from 112 ppm to 312 ppm, both sugar and ammonium uptake rates of the immobilized and free yeasts clearly decreased; and the immobilized cells in BC consumed substrates more rapidly than the free cells. The results obtained in this experiment justified why the immobilized yeast in BC grew better than the free yeast.

Decrease in initial pH value from 4.5 to 3.5 or increase in initial sulfur dioxide content in must from 112 ppm to 312 ppm reduced ethanol production rate for both immobilized yeast in BC and free yeast (Table 1 and 2). The same phenomenon was also observed in some previous researches. Ton *et al*. (2008) affirmed that low pH value decreased ethanol biosynthesis in yeast cells immobilized calcium alginate gel. According to Frivik *et al*. (2003), sulfur dioxide inhibited aldehyde dehydrogenase and decreased ethanol concentration in the culture. Nevertheless, in medium with initial pH value from 3.5 to 4.5, the ethanol production rate of the immobilized yeast was 11% – 35 % higher than that of the free yeast. And when the initial sulfur dioxide content in must varied from 12 ppm to 312 ppm, the immobilized yeast in BC produced ethanol as 11% – 21% faster than the free yeast. Holcberg (1981) also recognized that the rate of ethanol production by the immobilized cells in agar, carrageenan and alginate gels was 20-25% higher

than that of the free cells. According to Kourkoutas *et al*. (2004), the immobilization support may act as a protective agent against physico-chemical effects in the culture, and that was the reason why ethanol biosynthesis in immobilized yeast was accelerated in medium with high acidity and sulfur dioxide content.

Ethanol production yield is one of the important technological characteristics of yeast in ethanol fermentation. It is determined as ratio of the ethanol content in wine to the sugar content utilized by yeast during the fermentation. Table 1 and 2 show that the ethanol production yield of the free and immobilized yeasts was similar except in must with initial pH value of 3.5 or initial sulfur dioxide content of 312 ppm. For these cultures, the analysis of variance shows that the ethanol production yield of the free yeast was higher than that of the immobilized yeast in BC; however, the difference in ethanol production yield between the immobilized and free yeast cultures was merely varied from 1.9% to 2.3%.

Effects of pH and sulfur dioxide on volatile acid content in the product

When the initial pH value in must was varied from 4.5 to 3.5, the evolution of pH value during the fermentation is given in Figure 3. pH value in the immobilized yeast culture decreased faster than that in the free yeast culture. At the end of the fermentation, the pH value of wine fermented by the immobilized yeast was always lower than that of wine fermented by the free yeast. The same phenomenon was also observed when the initial sulfur dioxide content in must varied from 12 ppm to 312 ppm (Figure 4). Vine *et al*. (1997) affirmed that pH strongly affects several important wine properties; wine with low pH value has high color intensity, colloidal and microbiological stability. Application of immobilized yeast in BC to wine fermentation could therefore improve sensory, physico-chemical and microbiological quality of the final product.

Among organic acids produced by wine yeast during the fermentation, volatile acids such as acetic acid influenced negatively sensory characteristics of wine. Besides, volatile acid content in wine increased sharply when acetic bacteria and wild yeasts were contaminated in the culture (Fleet, 1993). Figure 5 indicates that the free yeast produced wine with higher volatile acid content than the immobilized yeast in BC. This phenomenon agreed with the research of Mallouchos *et al*. (2003) who compared the content of volatile acids in wines produced by immobilized yeast on grape skins and free yeast. Low volatile acid content in wine ameliorated the product flavor.

Conclusion

It can be confirmed that bacterial cellulose was a suitable support for wine yeast immobilization. The immobilized yeast in BC was more resistant to low pH value and high sulfur dioxide content in must than the free yeast. Moreover, pH value and volatile acid content in wine fermented by the immobilized yeast were lower, and this phenomenon improved the sensory properties, colloidal and biological stability of the final product.

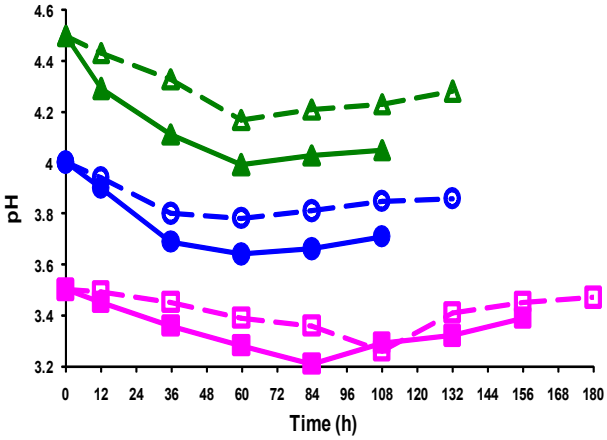

Figure 3. Change in pH value during must fermentation. The initial pH value of must was varied: 3.5 (□■), 4.0 (○●), 4.5 (△▲). Opened symbol: free yeast, filled symbol: immobilized yeast

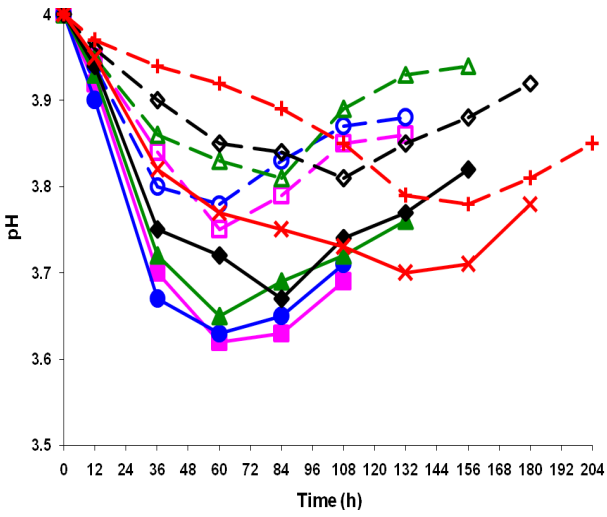

Figure 4. Effect of initial SO_2 content in must on pH value during the fermentation: 12ppm (□■), 112ppm (○●), 212ppm (△▲), 262ppm (◊♦), 312ppm (＋×). Opened symbol and (＋): free yeast, filled symbol and (×): immobilized yeast

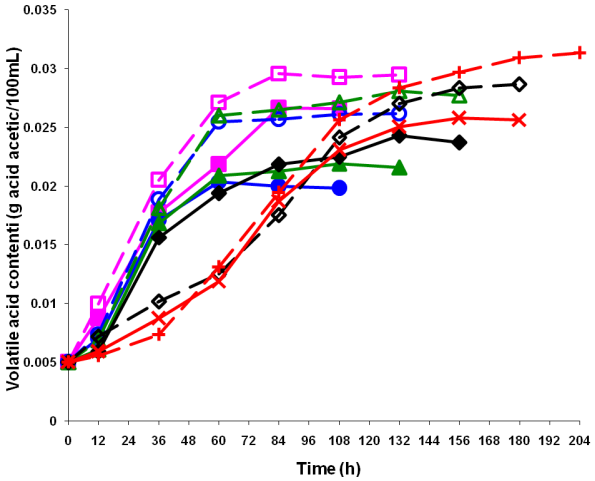

Figure 6. Effect of initial SO_2 content in must on volatile acid content during the fermentation: 12ppm (□■), 112ppm (○●), 212ppm (△▲), 262ppm (◇◆), 312ppm (+×). Opened symbol and (+): free yeast, filled symbol and (×): immobilized yeast

References

Bandyopadhyay, K.K. and Ghose, T.K. 1982. Studies on immobilized *Saccharomyces cerevisiae* III. Physiology of growth and metabolism on various supports. Biotechnology and Bioengineering 24(6): 805-815.

Bakoyianis, V., Koutinas, A. A., Agelopoulos, K. and Kanellaki, M. 1997. Comparative study of kissiris, γ-alumina, and calcium alginate as supports of cells for batch and continuous wine-making at low temperatures. Journal of Agriculture and Food Chemistry 45: 4884-4888.

Bui, T.H. and Le, V.V.M. 2005. Growth and metabolic activities of immobilized yeast cells in calcium alginate gel during alcoholic fermentation. In Ha, D.T. (Editor). Proceedings of Regional Symposium on chemical Engineering, pp. 153-157. Hanoi, Vietnam

Doran, P. M. and Bailey, J.E. 1986. Effects of immobilization on growth, fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin. Biotechnology and Bioengineering 28: 73-87.

Fleet, G.H. 1993. Wine microbiology and biotechnology. Camberwell: Harwood Academic Publishers.

Frivik, S.K. and Ebeler, S.E. 2003. Influence of sulfur dioxide on the formation of aldehydes in white wine. Journal of American Society of Enology and Viticulture 54: 32-38.

Galazzo, J. L. and Bailey, J. E. 1989. In vivo nuclear magnetic resonance analysis of immobilization effects on glucose metabolism of yeast *Saccharomyces cerevisiae*. Biotechnology and Bioengineering 33: 1283-1289.

Helrich, K. 1992. Official methods of analysis of the association of official analytical chemists, Virginia: AOAC Inc.

Holcberg, I.B. and Margalith, P. 1981. Alcoholic fermentation by immobilized yeast at high sugar concentrations. European Journal of Applied Microbiology and Biotechnology 13: 133-140.

Kourkoutas, Y., Bekatoroua, A., Banatb, I.M., Marchantb, R. and Koutinasa, A.A. 2004. Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiology 2: 377-397.

Kourkoutas, Y., Koutinas, A.A., Kanellaki, M. and Banat, I.M. 2002. Continuous wine fermentation using a psychrophilic yeast immobilized on apple cuts at different temperatures. Food Microbiology 19: 127-134.

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

Loukatos, P., Kanellaki, M., Komaitis, M., Athanasiadis, I. and Koutinas, A. A. 2003. A new technological approach proposed for distillate production using immobilized cells. Journal of Bioscience and Bioengieering 95: 35-39.

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 of the Science of Food and Agriculture 84: 1615–1623.

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

Nguyen, D. N., Ton, N. M. N. and Le, V. V. M. 2009. Optimization of *Saccharomyces cerevisiae* immobilization in bacterial cellulose by "adsorption-incubation" method. International Food Research Journal 16: 59-64

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: University of Natural Sciences, PhD Thesis.

Reddy, L. V., Reddy, Y. H. K., Reddy, L. P. A. and Reddy, O. V. S. 2008. Wine production by novel yeast biocatalyst

prepared by immobilization on watermelon *(Citrullus vulgaris)* rind pieces and characterization of volatile compounds. Process Biochemistry 43: 748–752.

Takayasu, T. and Fumihiro, Y. 1997. Production of bacterial cellulose by agitation culture systems. Pure and Applied Chemistry 69: 2453-2458.

Ton, N.M.N., Le, N.L., Nguyen, T.H.L. and Le, V.V.M. 2008a. Effect of initial pH value of must on kinetics of wine fermentation, using yeast immobilized in calcium alginate gel. In Le, D.D. (Editor). Proceedings of the 4th National Scientific Conference on Biochemistry and Molecular Biology for Agriculture, Biology, Medicine and Food industry, pp. 383-386. Hanoi, Vietnam

Ton, N.M.N., Le, N.L., Nguyen, T.H.L. and Le, V.V.M. 2008b. Effect of initial sulfur dioxide in must on the kinetics of wine primary fermentation, using yeast immobilized in calcium alginate gel. Journal of Science and Technology Development 11: 83-89.

Vine, R.P., Harkness, E.M., Browning, T. and Wagner, C. 1997. Winemaking: from grape growing to marketplace, New York: Chapman and Hall, 439p.