Immobilization of Yeast on Delignified Cellulosic Material for Room Temperature and Low-Temperature Wine Making

E. P. Bardi and A. A. Koutinas*

Department of Chemistry, University of Patras, Patras, Greece

The immobilization of *Saccharomyces cerevisiae* strain AXAZ-1 on delignified cellulosic material was examined by electron microscope. Using the biocatalyst prepared by immobilization, repeated batch fermentations (about 55) were done for glucose and separately for wine making employing must, without any loss of the activity. This biocatalyst caused about a 3-fold increase of the fermentation rate as compared with free cells. Furthermore, it was found that the delignified cellulosic material supported biocatalyst reduces the activation energy, E_a. Repeated batch fermentations performed at low temperatures gave average wine and alcohol productivities at 16 °C about equal to those at 30 °C; productivities at 10 °C were 35% lower than at 30 °C and at 0 °C were at the level of a fermentor of 1000 L producing 1000 L of dry wine in 1 month.

INTRODUCTION

Considerable attention was given in the past decade to the cell immobilization of microorganisms convenient for alcoholic fermentation. The interest was mainly focused on the use of immobilized cells for potable and fuel grade alcohol production (Kennedy *et al.*, 1976; Siton and Gaddy, 1980; Margaritis *et al.*, 1981; Rouxhet *et al.*, 1981; Tyagi and Ghose, 1982; Margaritis and Bajpai, 1982; Arcuri, 1982; Bland *et al.*, 1982; Margaritis and Rowe, 1983; van Haecht *et al.*, 1984, 1985; Mozes and Rouxhet, 1985; Bajpai and Margaritis, 1986; Koutinas *et al.*, 1988).

Wine making in contrast to potable alcohol production has additional prerequisites: final alcohol content of at least 11.5% v/v and a support of food grade purity. Therefore, the biocatalyst prepared for wine making by immobilization of yeast cells on solid supports has to be alcohol resistant. White wine with 11% v/v alcohol was obtained by yeast cells fixed in sodium alginate gel, through which must was passed for alcoholic fermentation (Shimobayashi and Tominaga, 1986). Likewise, white wine was produced in a batch fermentation by immobilized *Saccharomyces cerevisiae* OC-2 on calcium alginate gel, κ-carrageenan, agar, and pectic acid (Nakanishi and Yokotsuka, 1987). Furthermore, immobilization of yeast strains on alginates was used to produce fruit (Mori, 1987) and sparkling wine (Fumi *et al.*, 1987). *S. cerevisiae* immobilized on glass beads (Hamdy, 1990) and minerals such as polygorskite, montmorilonite, and hydromica (Ageeva *et al.*, 1985) produced wine by a rapid fermentation. Mineral kissiris was used for yeast immobilization to obtain continuous low-temperature wine making (Bakoyianis *et al.*, 1992). To immobilize yeast cells on cellulose for continuous wine making, researchers covered it with sodium alginate gel (Otsuka, 1980) and the product DEAE-cellulose by anion-exchange resin (Lommi and Advenainen, 1990). After the presentation of the supports used for wine making is concluded, further research is needed to obtain cell immobilization on a support that is more hygienic for food production, cheap, and abundant in nature and which gives an alcohol-resistant biocatalyst by immobilization suitable for low-temperature wine making.

The aim of the present study was to investigate the immobilization of cells on delignified cellulosic material to obtain a biocatalyst suitable for room temperature and low-temperature wine making.

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

AXAZ-1, an alcohol-resistant and psychrophile *S. cerevisiae* strain isolated (Argiriou *et al.*, 1992) from the Greek agricultural area, was used in the present study. It was grown on complete medium. Pressed wet weight cells (15–20 g) were prepared as in the aforementioned reference and employed directly in the fermentations. The batch culture media in the case of glucose solutions had an initial °Be density in the range 7.1–11.4 and contained 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.5% $MgSO_4 \cdot 7H_2O$ in distilled water. These complete media were sterilized at 130 °C for 15 min.

All musts were prepared by crushing an amount grape (cultivar Sideritis); the initial °Be density was adjusted to 11.5, 12.1, 12.3, 15.6, or 17.1 by the addition of a 1:1 mixture of glucose and fructose. The pH of must was 3.6 and total acidity 4.2 g of tartaric acid/L.

Alcoholic degrees were obtained after distillation of samples using a Gay–Lussac alcohol meter. The determination of ethanol enabled us to calculate the ethanol productivity, defined as the grams of ethanol per liter liquid volume produced per day. Wine productivity was calculated as grams of wine per liter total volume produced per day.

Total acidity was estimated by titration of samples by 0.1 N NaOH solution and volatile acidity by titration with 0.1 N NaOH of distillates obtained by steam distillation of wine samples (Zoeklein *et al.*, 1990).

Residual sugar was determined in all samples by the Lane–Eynon method (Egan *et al.*, 1981).

Wet free cell concentrations were determined using the absorbance experimental prodedure (Klein and Kressdorf, 1983; Bajpai and Margaritis, 1986) and are given in grams wet weight per liter being determined using standard curves.

Preparation of Delignified Cellulosic Material and Immobilization. In a beaker of 5 L were placed 300 g of wood sawdust and 3 L of a 1% sodium hydroxide solution. The slurry was heated for 3 h at the boiling point, and the volume of water during the heating was held constant by the addition of water. It was then filtered in a Büchner funnel, and the delignified cellulosic material obtained was washed several times with hot water (80 °C). The product was pressed on the funnel by a beaker to remove the water. This preparation gave a wet delignified cellulosic material used as follows for cell immobilization.

In a culture medium of 800 mL containing 12% glucose with a pH of 4.8 adjusted by the addition of sulfuric acid was spread 16 g wet weight cells of the *S. cerevisiae* strain AXAZ-1. This was mixed with 170 g of wet delignified cellulosic material and allowed to ferment for 6 h. After that time the liquid was decanted and the solid was washed two times, each time with 400 mL of culture medium containing glucose. After filtration and pressing on the Büchner funnel, the delignified cellulosic material supported biocatalyst was obtained, which was used directly for wine making by repeated batch fermentations.

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Repeated Batch Fermentations for Wine Making at Room and Low Temperatures. The amount of delignified cellulosic material supported biocatalyst, prepared as described above by the immobilization of cells, was introduced in 400 mL of liquid medium containing glucose (pH adjusted to 5.6) or must, in a 1-L glass cylinder. The glass cylinder for each fermentation batch was incubated for glucose and must at 30, 16, 10, and 0 °C as indicated in Tables 1 and 2, and the fermentations were carried out without agitation. Before the fermentation was completed, the liquid was filtered by a Büchner funnel and the support was washed three times, each time with 400 mL of liquid medium containing glucose or must in the case of must treatment. The biocatalyst was pressed on the funnel so as to remove the liquid. After that, the biocatalyst was used for the next fermentation batch. At the end of every batch sample were collected and analyzed for ethanol, residual sugar, free cells, and total and volatile acidity.

When the weight of the support was reduced by more than 10%, the apropriate amount was added.

All values were the mean of three repetitions. The standard deviation for ethanol concentration was $<\pm 0.2$, for ethanol productivity $<\pm 10$, for residual sugar $<\pm 2$, for cell concentration $<\pm 0.5$, and for volatile acidity $<\pm 0.1$.

Estimation of Immobilized Cells. To compare the ethanol production rate of free cells with that of immobilized cells, the estimation of immobilized cells was necessary. In a glass cylinder of 500 mL the immobilization of cells was performed according to the method aforementioned. Simultaneously, a fermentation by the same strain and cell concentration at similar liquid media and conditions, in the absence of delignified cellulosic material, was also performed. After 6 h of fermentation time, the liquids of both cylinders were agitated and liquid samples of them collected. Then, the number of cells to both samples was determined by a microscope using a blood cytometer; there were found to be 13×10^4 immobilized cells/mL. The estimation of immobilized cells was obtained by the difference in the number of cells between the liquid media fermented in the presence of delignified cellulosic material and those in the absence of it. The calculation of immobilized cells as gram wet weight cells per gram of delignified cellulosic material was made by taking into account liquid volume, the weight of delignified cellulosic material, and the number of cells per milliliter of a liquid culture which the cell concentration as wet weight grams per liter is known. Therfore, the immobilized biomas was found to be 4.9 g of wet weight cells/100 g of this support material.

Effect of Temperature on Alcoholic Fermentation by Delignified Cellulosic Material Supported Biocatalyst. Must obtained from Sideritis grapes was fermented simultaneously in batch systems by free cells and separately with immobilized cells at temperatures of 15 and 30 °C. Fermentations using free cells and cells immobilized on delignified cellulosic material were performed in two glass cylinders of 500 mL. For those of free cells the liquid contained 20 g/L of wet weight cells and had an initial °Be density of 12.3. For the fermentation employing immobilized cells on delignified cellulosic material, the immobilization was made as aforementioned. The prepared biocatalyst was filtered, washed with liquid media containing glucose, and transferred to the glass cylinder, and after the addition of grape must, the fermentation took place in a parallel way with those of free cells. The concentration of free cells on a wet weight basis was equal to the concentration of the immobilized ones. In every pair of samples (immobilized and free) three replicate fermentations were carried out at every temperature studied.

Fermentations by Free Cells in the Presence of Powdered Cellulose and Delignified Cellulosic Material and Without Them. Must obtained from grapes and having initial °Be density of 12 was fermented simultanuously in three batch systems by free cells: (i) in the presence of powdered cellulose; (ii) with delignified cellulosic material; (iii) without either of them. Into each must was spread 10 g/L (wet weight) yeast cells in a pressed form. Into one of them was added 88 g/L powdered cellulose and in another delignified cellulosic material in wet form containing 88 g/L dry mass. All cylinders were incubated at 20 °C. Kinetics of fermentations were performed by measuring °Be density at various time intervals; for every sample three replicate fermentations were performed.

Electron Microscope Study. For the electron microscope studies, the delignified cellulosic material supported biocatalyst was washed with must and the delignified cellulosic material without immobilized cells dried overnight at room temperature. The above materials and an amount of maternal wood sawdust separately were placed on an aluminum plate. They were coated with gold in a Balzers SCD 004 sputter coater for 2 min, so as to obtain an increase in the electron conductivity. The prepared samples were studied in a Cambridge Stereoscan 120 scanning electron microscope.

RESULTS AND DISCUSSION

To increase the surface as well as to obtain gelatinous material and hence to accommodate immobilization of cells by going through the pores, sawdust was treated with hot sodium hydroxide solution for the removal of lignin (Koutinas *et al.*, 1981). The delignified cellulosic material obtained was mixed with a liquid culture of an alcohol-resistant and psychrophile yeast strain for its immobilization and using the biocatalyst prepared for fermentation at room and low temperatures. Then the biocatalyst obtained was used for repeated batch fermentations of liquid culture media containing glucose. The latter was made for 13 batches to see if the activity of the biocatalyst was reduced or not. After the fermentation of glucose, the biocatalyst was used for repeated batch fermentation of grape must for wine making at room and low temperatures. The results are summarized in Tables 1 and 2.

Specifically, Table 1 shows repeated batch fermentations of glucose and grape must at 30 °C. The first 13 repeated batch fermentations using glucose were carried out without any significant increase in the fermentation time at similar initial °Be densities. Furthermore, initial °Be densities of grape must were in the range 11.5–12.3 up to the 34th batch, and wines produced contained alcohol at concentrations about similar with those of dry table wines. After that, six repeated batch fermentations were performed employing higher initial °Be density and gave relatively high alcohol concentrations in the range 14.2–15.3% v/v. Residual sugar concentrations obtained show that this parameter can be obtained at the content needed for dry wines. Likewise, wine and ethanol productivities are at least 10-fold higher than those of natural fermentation. Free cell concentrations at temperatures of 16 and 30 °C were in the range 1.5–5.5 g/L; total and volatile acidities are similar to those found in dry wines. Table 2 also shows repeated batch fermentations of grape must at low temperatures. At all temperatures studied the ethanol concentrations were also at the level of dry wines. Wine and ethanol productivities obtained at 16 °C were not significantly reduced compared with those obtained at 30 °C. At 10 °C wine average productivity was only 35% lower than that at 30 °C, and according to results obtained at 0 °C it is concluded that a fermentor of 1000-L capacity produces an equal number of liters of dry wine at about 1 month. At low temperatures free cell concentration was very low and total acidity was in the range of those found in wines. Volatile acidity at 16 °C was lower than 0.50 g of CH_3COOH/L, and at the lower temperatures studied it increased greatly.

Furthermore, Figure 1 shows the system of fermentation performed for the present study. In the figure are observed the bubbles of the fermentation, and the clarity of the solution confirms the very low free cell concentations found mainly at low temperatures. To verify immobilization of cells on delignified cellulosic material, a biocatalyst pre-

Table 1. Fermentation Parameters Obtained at 30 °C in Repeated Batch Fermentation with Immobilized Cells of the Strain AXAZ-1 on Delignified Cellulosic Material

raw material	repeated fermentation batches	initial °Be density	fermentation time (h)	daily wine productivity (g/L)	ethanol concn (% v/v)	daily ethanol productivity (g/L)	residual sugar (g/L)	final concn of free cells (g/L)	total acidity (g of tartaric acid/L)	volatile acidity (g of CH_3COOH/L)
glucose	1	7.7	12.0		6.5		32.0			
glucose	5	7.1	12.5		7.5		4.1			
glucose	6	7.1	12.5		7.3		4.8			
glucose	7	11.4	16.5		8.3		66.0			
glucose	9	11.0	22.0		9.8		33.0			
glucose	11	10.7	20.0		9.5		48.0			
glucose	13	10.7	23.0		9.8		33.0			
grape must	14	11.5	23.5	910	11.2	82	21.2		8.3	0.72
grape must	15	11.5	23.0	900	11.1	80	22.4		8.7	0.75
grape must	17	11.5	24.0	880	11.3	80	15.7		8.6	0.80
grape must	19	11.5	25.0	860	11.6	80	20.0		9.4	0.60
grape must	23	12.1	22.0	990	11.5	91	37.0		6.4	0.52
grape must	25	12.1	23.0	940	11.3	85	10.2		6.2	0.40
grape must	26	12.1	23.5	910	11.6	84	4.1		7.1	0.60
grape must	27	12.1	23.0	900	11.8	85	3.4	5.5	6.9	0.55
grape must	28	12.1	23.0	900	12.1	87	2.5	5.1	6.5	0.53
grape must	31	12.1	22.0	990	12.4	98	2.8	5.3	6.8	0.58
grape must	32	12.3	21.0	1040	12.5	104	3.0	4.8	7.0	0.50
grape must	33	12.3	19.0	1150	12.2	112	5.3	3.6	7.6	0.34
grape must	34	12.3	24.0	880	12.5	88	2.2	2.1	8.2	0.16
grape must	35	15.6	22.5	960	14.2	109	13.2	4.3	6.5	0.50
grape must	36	15.6	24.0	880	14.4	101	18.9	3.0	5.3	0.52
grape must	37	15.6	26.0	830	14.2	94	26.8	2.1	5.2	0.47
grape must	38	15.6	27.0	800	14.9	95	26.0	1.7	8.3	0.56
grape must	39	15.6	40.0	520	15.0	39	12.2	2.8	4.8	
grape must	40	17.1	96.0	190	15.3	23	41.9	4.1		0.50
grape must	41	12.1	30.0	710	11.5	65	15.2	3.5	5.3	0.60
grape must	42	12.1	30.0	710	11.6	66	13.7	3.0	5.5	0.55

Table 2. Fermentation Parameters Obtained at Low-Temperature Wine Making by Repeated Batch Fermentation with Immobilized Cells of the Strain AXAZ-1 on Delignified Cellulosic Material

raw material	temp (°C)	repeated fermentation batches	initial °Be density	fermentation time (h)	daily wine productivity (g/L)	ethanol concn (% v/v)	daily ethanol productivity (g/L)	residual sugar (g/L)	final concn of free cells (g/L)	total acidity (g of tartaric acid/L)	volatile acidity (g of CH_3COOH/L)
grape must	16	42	12.1	44	470	12.0	45	2.1	2.7	8.5	0.39
grape must	16	43	12.1	31	690	11.9	66		2.5	8.8	0.37
grape must	16	44	12.1	24	880	11.2	79	12.7	2.0	7.5	0.42
grape must	16	45	12.2	24	880	12.2	86	5.6	2.1	6.5	0.40
grape must	16	46	12.1	23	900	13.0	94	5.5	1.7	7.2	0.50
grape must	16	47	12.1	20	1090	12.4	108	6.9		8.4	0.38
grape must	16	48	12.1	20	1090	12.4	108	6.5	1.5	8.3	0.38
grape must	10	49	12.1	32	660	12.2	64	13.8	1.6	7.9	0.58
grape must	10	50	12.1	40	520	12.6	52	9.5	1.8	8.1	0.60
grape must	10	51	12.1	33	640	11.8	60	28.3	0.4	6.9	0.90
grape must	10	52	12.1	33	640	12.0	61	20.5	0.4	7.0	0.85
grape must	0	53	12.1	672	30	12.5	3	8.7	0.3	6.3	0.80
grape must	0	54	12.1	665	29	12.4	3	7.6	0.3	6.5	0.77
grape must	0	55	15.6	2640	9	15.3	1	10.2	0	6.0	0.65

pared under Materials and Methods was washed three times with must and used for the electron microscope study. Figure 2C clearly shows cells immobilized on the surface of this support. To show that the removal of lignin creates holes or pores on sawdust and therefore accommodates the entrapment of cells as well as their going through the pores, samples of sawdust (Figure 2A) and its delignified product (Figure 2B) were studied by the electron microscope. Thus, Figure 2A,B proves this possibility.

It was observed that batch fermentations of must at various temperatures performed by delignified cellulosic material supported biocatalyst were faster than those of free cells with equal cell concentrations. These equal concentrations were obtained after calculation of immobilized cells according to the procedure of the estimation of immobilized cells given under Materials and Methods. This calculation of the actual value of immobilized cells on delignified cellulosic material was performed as follows: initial cell concentration, 25×10^4 cells/mL. Cells contained in 800 mL weighed 16 g on a wet weight basis. Likewise, it was found that after immobilization on delignified cellulosic material, the free cell concentration of the liquid was 18×10^4 cells/mL , and in the parallel fermentation without delignified cel lulosic material, 31×10^4 cells/mL. Therefore, immobilized will be 13×10^4 cells/mL and weigh $16 \times 13 \times 10^4 \times 800/25 \times 10^4 \times 800 = 8.32$ g. These grams of cells were immobilized on 170 g of delignified cellulosic material and on 100 g of the support $8.32 \times 100/170 = 4.89$ g of cells (wet weight).

A fermentation kinetic at 0 °C is shown in Figure 3. This figure indicates that free cells of the strain ferment at 0 °C, a fact that can support that the strain be psychrophilic. Likewise, the figure shows a drastic increase of the fermentation rate in the case of delignified cellulosic material supported biocatalyst, as compared to that of free cells. These results are additionally confirmed by the fermentation kinetics presented in Figure 4. Likewise, this promotion is in agreement with the increase of ethanol production observed in the alcoholic fermentation of glucose and molasses carried out with free cells

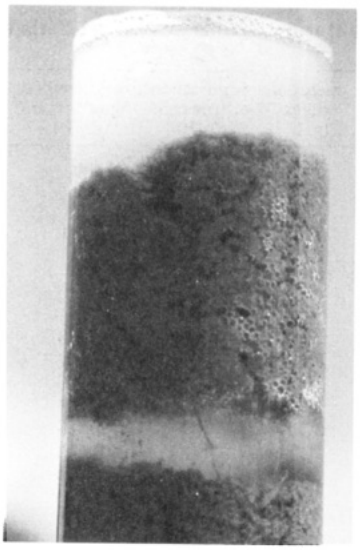

Figure 1. Delignified cellulosic material supported biocatalyst in the fermentation of grape must.

and in the presence of delignified cellulosic material (Iconomou and Koutinas, 1993).

To examine the catalytic effect of the cellulose on alcoholic fermentation, experiments by free cells in the presence of powdered cellulose and delignified cellulosic material and in their absence were organized and are presented in Figure 5. This figure shows that powdered cellulose increases the ethanol production rate and delignified cellulosic material increases even more the rate of the fermentations as compared with the fermentation without them. These results contribute to the idea that cellulose promotes action in the alcoholic fermentation.

The promotion effect observed in the fermentation of must by delignified cellulosic material supported biocatalyst was the reason to undertake experiments to investigate its effect on activation energy (E_a) in the case of alcoholic fermentation. Thus, fermentations of must at 15 and 30 °C were performed in the presence of delignified cellulosic material supported biocatalyst and separately in the presence of free cells in an equal concentration with those of immobilized. The results are presented in Figure 4. This figure illustrates that the delignified cellulosic material supported biocatalyst causes an increase in the fermentation time of 158% and free cells of 331% as the temperature of the fermentation drops from 30 to 15 °C. This means that the proportion of reaction speed constants at 30 and 15 °C K_{30}/K_{15} becomes lower in the case of the presence of delignified cellulosic material supported biocatalyst as compared with those of free cells. Taking into account the Arrhenius equation

$$\log \frac{K_2}{K_1} = \frac{E_a}{2.303R} \frac{T_2 - T_1}{T_2 - T_1}$$

the activation energy E_a is reduced in the case of delignified cellulosic material supported biocatalyst. The latter shows that this biocatalyst behaves as a catalyst or as a promoter of the catalytic activity of the enzymes involved in the process (Giannacoudakis, 1964).

The removal of the phenolic insoluble compound lignin from cellulose converts sawdust to a gelatinous material with more holes and pores. This accommodates cells going through and its immobilization by entrapment or by van der Waals forces or upon chemical bonding (Kennedy *et al.*, 1976) and hydrogen bonding between hydroxyl groups of cellulose and carboxyl, hydroxyl, and amino groups of the cell wall. The immobilization of yeast cells for the

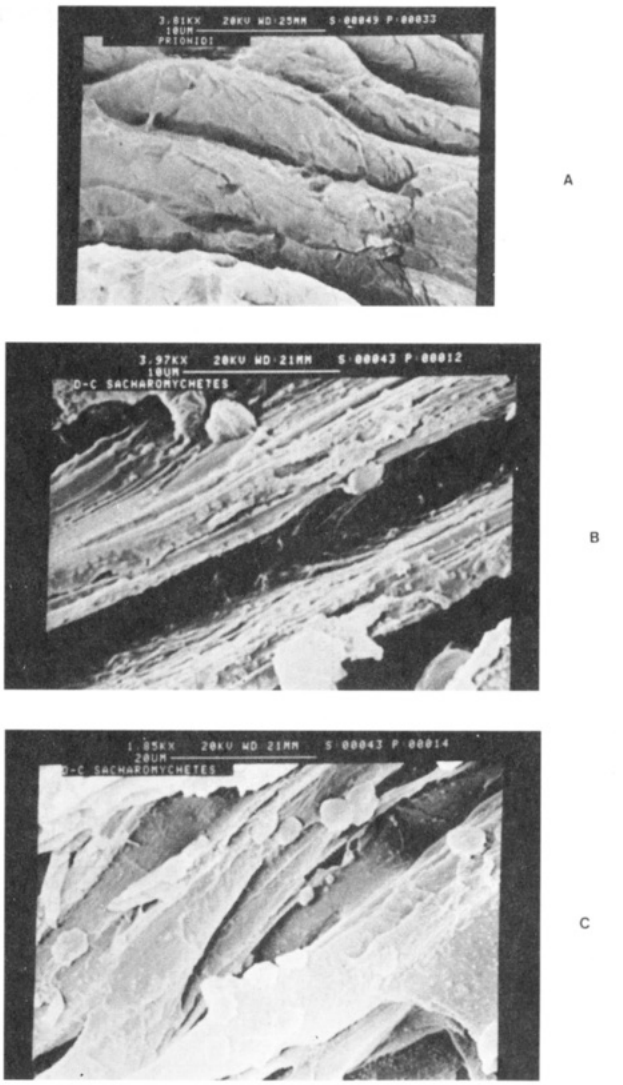

Figure 2. Electron micrographs: (A) at ×3.81 KX showing maternal nondelignified cellulosic material (sawdust), (B) its delignified product at ×3.97 KX; and (C) immobilized cells on delignified product at ×1.85 KX. (Figure is reproduced here at 50% of its original size.)

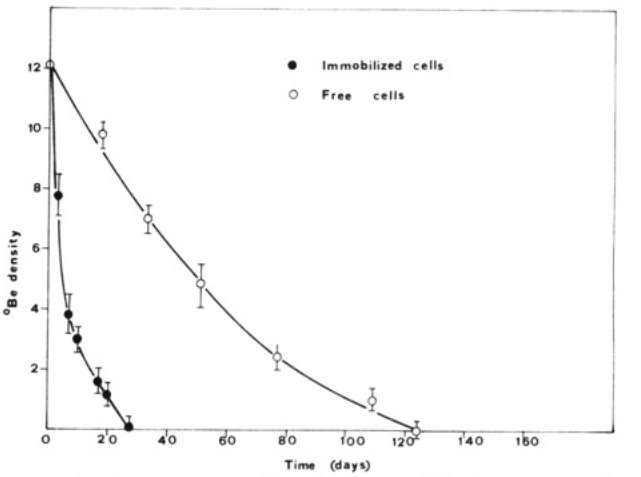

Figure 3. Fermentations kinetics observed in the fermentation of must at 0 °C using immobilized yeast cells on delignified cellulosic material as compared with free ones.

preparation of delignified cellulosic material supported biocatalyst is proved by the repeated batch fermentation obtained, the appearance of immobilized cells in the electron micrographs, and the observation of fermentation

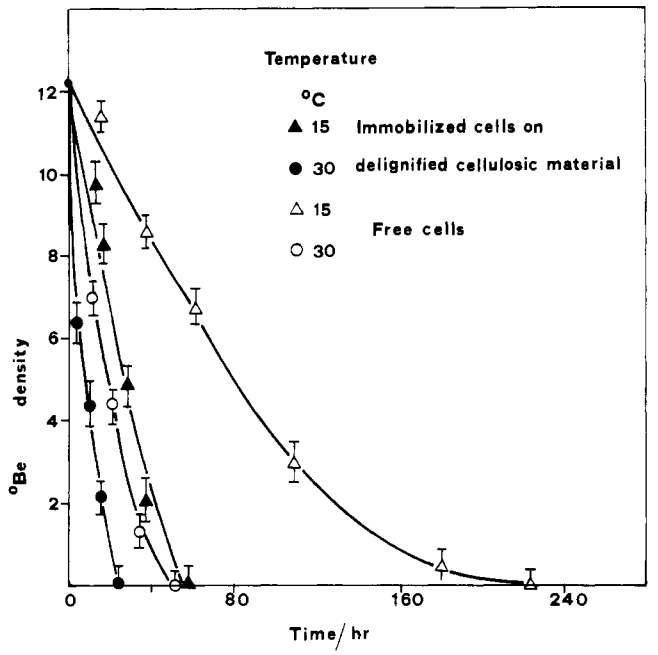

Figure 4. Fermentation kinetics of must observed by delignified material supported biocatalyst and free cells at temperatures of 15 and 30 °C.

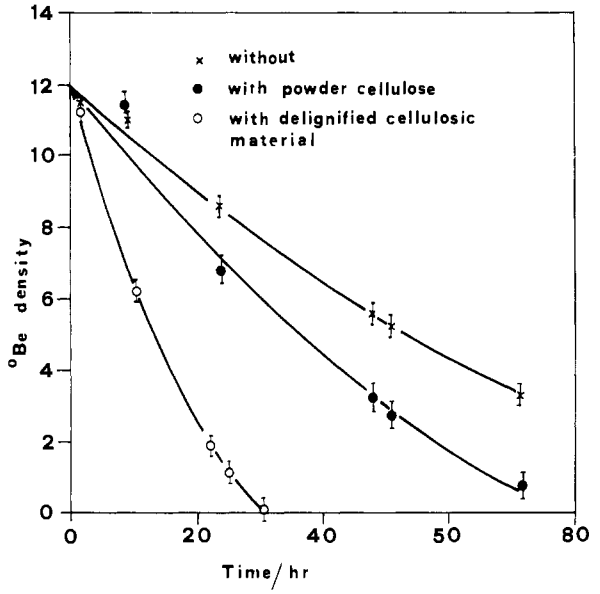

Figure 5. Fermentation kinetics of must observed for free cells in the presence of powdered cellulose and delignified cellulosic material as compared with the absence of them.

done in clear fermentation broth. The relatively high productivity obtained shows that the fermentation was carried out mainly by immobilized cells, since the low free cell contents found in all batches cannot give the reason for the vigorous fermentation that was observed. The large number of fermentation batches obtained and the production in each batch of wines having relatively high alcohol content (about 12%) without any loss of the activity prove that the delignified cellulosic material supported biocatalyst is alcohol tolerant. The increase of the fermentation rate as compared with those obtained by free cells reduces the wine-making cost. The high increase of the fermentation rate at low-temperature wine making reduces the cost and makes safe the fermentation for wine production even at the very low temperature of 0 °C. The increased activity found at low temperatures in the case of delignified cellulosic material supported biocatalyst can be explained by the reduction of the activation energy E_a caused by it. Likewise, the higher increase of the ethanol production rate observed in the fermentations with free cells by the presence of delignified cellulosic material as compared with those of powdered cellulose can be attributed to pores of delignified cellulosic material and therefore an increase of the active surface.

Wines produced by this biocatalyst had the alcohol content of dry table wines, and their total and volatile acidities were at a normal level. Wines prepared at 0 °C were very clear. The latter contributes the idea that wines produced at very low temperatures may not need any treatment to obtain their clarity.

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