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Effect of Freeze-Dried Immobilized Cells on Delignified Cellulosic Material in Low-Temperature and Ambient-Temperature Wine Making

M. Ikonomopoulou, M. Kanellaki, M. Soupioni, and A. A. Koutinas*

Food Biotechnology Group, Department of Chemistry, University of Patras, GR-26500 Patras, Greece, E-mail: A.A.Koutinas@upatras.gr

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Abstract

In this article, we report on wine making by freeze-dried immobilized cells on delignified cellulosic material for ambient and low temperatures. Biocatalyst supported by freeze-dried delignified cellulosic (FDC) material recovered after the first repeated-batch fermentations the fermentation efficiency and startup, which become about equal to those of biocatalyst supported by wet delignified cellulosic material. The FDC biocatalyst was suitable for wine making at low temperatures (5–15°C), and produced wine of 12% alcoholic degree, with the main volatiles contained in the wine and reduced by a decrease in temperature. The fermentation efficiency was not affected by total acidity of must, while an increase in initial °Be density improved percentages of higher alcohols and ethyl acetate. The quality of the wine was validated by a preliminary taste test to be in the range of acceptable to excellent.

Index Entries: Freeze drying; immobilization; wine making; delignified cellulosics; low-temperature fermentation.

Introduction

For the last 20 yr many investigations have been conducted and articles published on the cell immobilization of yeasts concerning wine making and the influence on wine quality. However, the technical and industrial experience in relation to industrialization of immobilized cells creates

*Author to whom all correspondence and reprint requests should be addressed.

additional prerequisites for a cost-effective application of immobilized cells on an industrial scale. These prerequisites include the need to train personnel mainly in small enterprises about the technical problems of this technology, the need for a new bioreactor system for easy filling and emptying by the support, and the need for high operational stability of the bioreactor.

In particular, cryotolerant and ethanol-resistant yeast cells immobilized on food-grade purity supports, such as gluten pellets and delignified cellulosic (DC) material, have been used to produce wine by low-temperature fermentations. The products had an improved taste and aroma and in batch fermentation resulted in higher productivity *(1–5)*. Continuous wine making by biocatalyst supported by DC material was also studied *(2)*. When, e.g., an immobilized yeast bioreactor or different immobilization techniques have been used for successful industrial applications *(6)*, the freeze-dried immobilized cells seem to be quite convenient for the substitution of free freeze-dried wine yeasts and traditional natural fermentation. In addition, successful freeze-drying experiments with cells immobilized on DC material already have been performed in alcoholic fermentation of synthetic media containing glucose *(7)*.

To overcome the prerequisite of adapting the new technology of cell immobilization in wine making, the use of the freeze-dried immobilized cells are proposed. This biocatalyst could be manufactured by new enterprises, and, thus, the product could be used as a substitute for the freeze-dried wine yeasts. However, the new biocatalyst needs to obtain acceptable fermentation rates and quality of the wine.

Low-temperature fermentations lead to improvement of quality of the wine, by improving organoleptic character and reducing toxicity of some compounds *(4,5)*. Therefore, the aim of the present work was to study the suitability of FDC material biocatalyst in low-temperature wine making. In addition, because DC material is of food-grade purity, abundant, and inexpensive, we evaluated biocatalyst supported by FDC material for the possibility of industrial application in wine making.

Materials and Methods

Yeast Strain and Support

AXAZ I, an alcohol-resistant and cryotolerant *Saccharomyces cerevisiae* strain that was isolated locally *(8)*, was used in all experiments. It was grown in a complete medium. Pressed wet wt cells as described in the aforementioned reference were employed. DC was used as support for cell immobilization. It was prepared as described in a previous study *(1)*.

Fermentations were performed by using red grape must. It was sterilized in an autoclave at 130°C for 15 min. The pH was adjusted to 3.6 using tartaric acid, and the initial total acidity was 4 g of tartaric acid/L.

Preparation of Biocatalyst Supported by FDC Material

Biocatalyst supported by DC material was prepared by immobilizing AXAZ I cells on gluten pellets as described previously *(1)*.

To prepare freeze-dried immobilized cells for wine making, the biocatalyst supported by DC material without protective media was cooled at a cooling rate of 3°C/min. It was frozen to –40°C and finally freeze-dried overnight at $15–5 \times 10^{-3}$ bar and at –50°C in a Freeze Dry System, Freezone 4.5 (Labconco, Kansas City, MO). Likewise, free freeze-dried cells (FFDCs) were also prepared as just described using wet biomass produced after cell growth in a complete medium.

Fermentations

The effects of initial °Be density, temperature, and total acidity were examined. To study the effect of initial °Be density, for each initial °Be density, three 500-mL Erlenmeyer flasks were used with each containing 300 mL of grape must. The experiments were performed using initial °Be densities of 8, 10, 12, 14, and 16. In each experiment, 100 g wet DC material–supported biocatalyst holding 4.9 g wet wt cells were freeze dried and subsequently pretreated with must of the same density, and the must was fermented. This was added into the first Erlenmeyer flask. In the same way, 4.9 g of wet wt free cells were freeze dried and then placed into the second flask. Into the third flask was placed 100 g of biocatalyst supported by wet DC material having also 4.9 g of wet wt immobilized cells. The flasks were incubated at 30°C, and the fermentations were carried out without agitation.

To study the effect of temperature the preceding process was repeated with must of 12 °Be density. Batch fermentations were performed at temperatures of 5, 10, 15, 20, 25, and 30°C. All fermentations were carried out by using initial wet wt cell concentration of 16.3 g/L. Likewise, a second repeated batch was fermented by using the same biocatalyst supported by DC material. Before the completion of fermentation, the liquid was filtered using a Buchner funnel, and the biocatalyst supported by DC material was washed once with 200 mL of grape must.

To study the effect of total acidity, musts of 12 initial °Be density were employed and batch fermentations were carried out at 30°C for three different total acidities of 4, 6, and 8 g of tartaric acid/L. The fermentations were carried out by using an initial wet wt cell concentration of 11.7 g/L, which is the same and in the cases of F_1DC and FFDC.

Repeated-batch fermentations of grape must were also carried out by freeze-dried immobilized cells on DC material. Fermentation kinetics were performed by measuring the °Be density at various time intervals. Samples were collected and analyzed for ethanol concentration, residual sugar, and volatile byproducts. All values were the mean of two repeats.

Analysis of Alcohol and Residual Sugar

Ethanol and residual sugar concentrations were obtained by high-performance liquid chromatography analysis. A Shimadzu liquid chromatograph with a high-pressure pump model LC-9A, a constant temperature oven C-R 6A, and a refractive index detector RID-6A connected with an

integrator C-R 6A was used. A column SCR-101N packed with a cationic resin was used. The temperature was set at 60°C, and an aqueous mobile phase with a flow rate of 0.8 mL/min was used. Ethanol productivity was expressed as grams of ethanol/(liter of substrate·d).

Analysis of Volatiles

Ethanol, ethyl acetate, propanol-1, isobutyl alcohol, and amyl alcohols were determined using a stainless steel column packed with Escatro 5905 (consisting of 5% squalene, 90% Carbowax 300, and 5% [v/v] bis[2-ethylhexyl]sebacate) with N_2 as the carrier gas (20 mL/min). The injection port and detector temperatures were 210°C, and the column temperature was 60°C. The internal standard was pentanol-1 at a concentration of 0.5% (v/v). Two-microliter samples of wine were injected directly into the column.

Sensory Evaluation

Wines produced by using freeze-dried immobilized cells on DC material (30 and 5°C) and FFDCs (30°C) were evaluated by seven taste and aroma panelists. The evaluation was performed on a scale of 0 to 10. Tables 1 and 2 present the product characterization and results.

Results

This study was undertaken to contribute to the production of a new form of commercial biocatalyst in wine making. The biocatalyst was prepared after immobilization of cells on porous DC material and then freeze drying of wet immobilized cells. To examine the efficiency of the new biocatalyst in grape must fermentation, the effects of initial °Be density, temperature, and total acidity were studied. Most of the experiments were performed simultaneously with FFDCs and WDC. The results are presented in Tables 3–5 and Fig. 1.

Table 3 shows the effect of initial °Be density on fermentation kinetic parameters for FDC, FFDCs, and WDC. Freeze-dried immobilized cells at 30°C resulted in similar fermentation time and ethanol and wine productivities compared with FFDCs. At the high 16 °Be density, FDC clearly improved fermentation time and productivities. However, WDC resulted in better startup, fermentation time, and productivities as compared with freeze-dried cells (FDC and FFDCs). FDC resulted in lower startup as compared with FFDCs. Conversion was high and about the same in all cases studied.

Table 4 illustrates FDC, FFDC, and WDC fermentations in the temperature range of 5–30°C. The efficiency of fermentations performed by FDC and FFDCs was about the same for all kinetic parameters except startup, where FDC was more effective. WDC led to an improvement in all kinetic parameters in comparison with freeze-dried immobilized cells. However, Fig. 1 indicates faster fermentation rate of FDC as compared with

Table 1
Preliminary Taste Test Results of Wines Produced by Using FDC at 30 and 5°C

Sample	Scale (0–10)[a]	Tester 1	2	3	4	5	6	7	8
FDC (30°C)									
Taste	10								
	9						X		
	8								X
	7	X							
	6			X		X			
	5		X		X				
	4								
	3								
	2							X	
	1								
	0								
Flavor	10								
	9								
	8						X		
	7				X				X
	6					X			
	5		X	X				X	
	4								
	3	X							
	2								
	1								
	0								
FDC (5°C)									
Taste	10								
	9					X			
	8		X				X		
	7								X
	6			X					
	5				X			X	
	4	X							
	3								
	2								
	1								
	0								
Flavor	10								
	9								
	8		X			X			
	7	X		X			X	X	
	6				X				X
	5								
	4								
	3								
	2								
	1								
	0								

[a]0, unacceptable; 1, very bad; 2, bad; 3, might be bad; 4, acceptable; 5, medium; 6, might be good; 7, good; 8, very good; 9, excellent; 10, wonderful.

Table 2
Preliminary Taste Test Results of Wines Produced by Using FFDCs at 30°C

		Tester							
Sample	Scale (0–10)[a]	1	2	3	4	5	6	7	8
FFDC (30°C)									
Taste	10								
	9								X
	8				X	X			
	7		X						
	6								
	5							X	
	4			X			X		
	3	X							
	2								
	1								
	0								
Flavor	10								
	9								
	8				X				
	7					X		X	X
	6			X			X		
	5	X	X						
	4								
	3								
	2								
	1								
	0								

[a]0, unacceptable; 1, very bad; 2, bad; 3, might be bad; 4, acceptable; 5, medium; 6, might be good; 7, good; 8, very good; 9, excellent; 10, wonderful.

FFDCs and a sharp increase in the fermentation rate of FDC in the second repeated-batch fermentation. In this case, the fermentation time decreased from 120 to 45 h, close to the fermentation time obtained by WDC (Table 3).

Table 5 shows the effect of total acidity on fermentations performed by FDC and FFDCs. The results clearly show that the startup, fermentation time, ethanol concentration, and residual sugar were not affected by the total acidity in the range of 6–10 g of tartaric acid/L.

To evaluate the produced wine by freeze-dried immobilized cells on DC material, the volatiles with the higher concentration in wines were analyzed. Since the quality of the wine is positively affected by the low temperatures, wines were produced in the broad temperature range of 5–30°C. Repeated-batch fermentations were performed using a new freeze-dried immobilized biocatalyst at each temperature. The results are presented in Table 6. It is clearly indicated that propanol-1 and isobutyl alcohol were reduced as the temperature was decreased. Amyl alcohols were also diminished up to 15°C. This reduction in the higher alcohols by the drop in

Table 3
Effect of Initial °Be Density on Fermentation Kinetic Parameters Observed at 30°C in Batch Wine Making by Biocatalyst Supported by FDC Material (F_1DC), FFDCs, and Biocatalyst Supported by WDC

Biocatalyst	Initial °Be density	Startup (h)	Fermentation time (h)	Ethanol concentration (% v/v)	Ethanol productivity (g/[L·d])	Wine productivity (g/[L·d])	Residual sugar (g/L)	Conversion (%)
F_1DC	8	18	62	7.9	24.0	258	4.3	96.8
	10	12	90	9.8	19.2	178	8.4	95.0
	12	16	116	12.0	19.2	138	2.0	99.0
	14	20	145	13.6	16.8	110	18.2	92.3
	16	20	168	13.2	14.4	95	40.6	85.0
FFDCs	8	24	84	8.1	18.0	190	3.9	97.1
	10	26	95	10.9	21.4	168	5.6	96.7
	12	48	110	12.0	20.4	145	7.6	96.2
	14	31	140	12.9	17.0	114	22.1	90.7
	16	48	240	13.1	10.0	67	65.0	76.1
WDC	8	0.2	14	8.0	105.6	1142	0.8	99.4
	10	0.2	18	9.9	100.8	889	2.1	98.7
	12	0.2	23	12.0	96.0	696	2.9	98.5
	14	0.2	34	13.8	74.4	471	3.6	98.4
	16	0.4	48	14.9	57.6	333	18.6	93.1
	18	0.4	106	15.3	26.4	151	47.9	84.3

Table 4
Effect of Temperature on Fermentation Kinetic Parameters Observed at 12 °Be Grape Must Fermentation by Biocatalyst Supported by FDC Material (F_1DC), FFDCs, and Biocatalyst Supported by WDC

Biocatalyst	Temperature	Startup (d)	Fermentation time (d)	Ethanol concentration (% v/v)	Ethanol productivity (g/[L·d])	Wine productivity (g/[L·d])	Residual sugar (g/L)	Conversion (%)
F_1DC	5	4	65	11.3	1.2	10.2	9.7	95.2
	10	7	34	11.4	2.4	19.6	10.1	95.0
	15	2	17	11.6	5.3	39.2	6.2	96.9
	20	1	8	11.6	11.3	83.3	3.4	98.3
	25	0.9	6	11.7	15.1	111.1	4.4	97.8
	30	0.6	5	12.0	19.4	139.0	2.0	99.0
FFDCs	5	8	66	11.4	1.2	10.1	7.6	96.2
	10	8	36	11.4	2.4	18.5	6.7	96.7
	15	3	15	11.7	6.0	44.4	5.5	97.3
	20	3	8	11.5	11.0	83.3	5.6	97.2
	25	2	8	11.9	11.7	83.3	—	—
	30	2	5	12.0	20.4	148.0	7.6	96.2
WDC	5	0.5	12	11.7	7.4	55.5	4.2	97.9
	10	0.4	5	11.8	18.2	133.3	3.8	98.1
	15	0.4	3	11.8	30.5	222.2	3.0	98.5
	20	0.3	2	11.9	42.0	303.0	2.1	98.9
	25	0.2	2	11.8	45.6	333.3	3.0	98.5
	30	0.2	1	12.0	96.0	666.6	2.9	98.5

Table 5
Effect of Total Acidity on Kinetic Parameters in Repeated-Batch Wine Making by Biocatalyst Supported by FDC Material and FFDCs at 30°C

	6 g/L				8 g/L				10 g/L			
	Startup (h)	Fermentation time (h)	Ethanol concentration (% v/v)	Residual sugar (g/L)	Startup (h)	Fermentation time (h)	Ethanol concentration (% v/v)	Residual sugar (g/L)	Startup (h)	Fermentation time (h)	Ethanol concentration (% v/v)	Residual sugar (g/L)
FDC	26	116	11.8	5.1	26	114	11.9	3.4	26	114	11.8	4.1
FFDCs	24	102	11.8	2.8	24	99	11.9	3.1	24	102	11.9	2.8

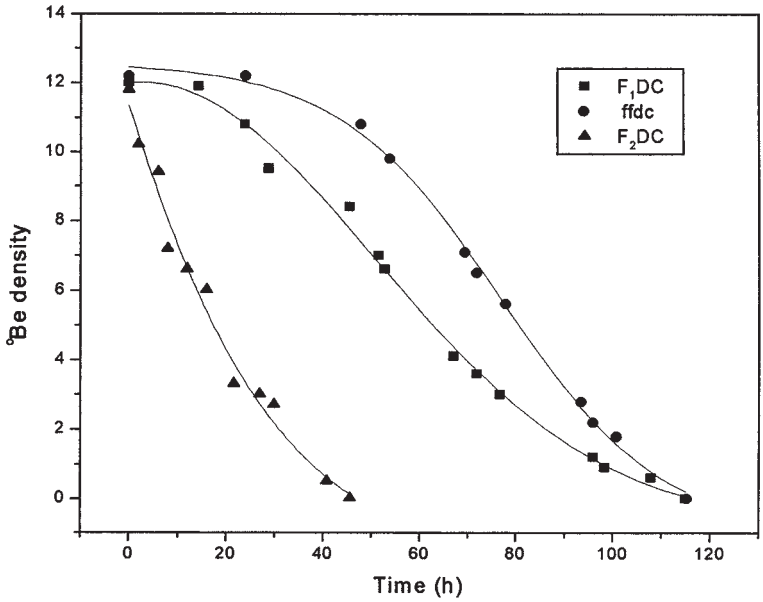

Fig. 1. Kinetics at 30°C in wine making by biocatalyst supported by FDC material ($F_{1,2}$DC) and FFDC (in which subscripts 1 and 2 refer to the first and second repeated batches, respectively).

temperature agrees with previous results of biocatalyst supported by WDC materials *(4)*. In addition, ethyl acetate was decreased as the temperature dropped.

Discussion

The results showed that FDC and FFDCs in the first batch had about the same fermentation time and productivities. However, this study proved that in repeated-batch fermentations the fermentation time was gradually decreased from batch to batch whereas productivities were increased (Table 7). The reduced fermentation time and increased productivities as well as startup were close to the important values of wet immobilized cells (Tables 3 and 4). This recovery of productivity by the freeze-dried biocatalyst is of commercial importance, because the industrialization of immobilized cells requires high productivity and operational stability in repeated-batch fermentations to reduce production costs. Likewise, the results in Table 7 are evidence for an important operational stability of freeze-dried immobilized biocatalyst. The similarity of the results of FDC and WDC in repeated-batch fermentations is also important because biocatalyst supported by WDC material dropped sharply the fermentation time as compared with free cells *(1)*.

The reduction in the concentration of higher alcohols as the temperature was decreased improved organoleptic quality because these compounds cause poor taste quality. Low-temperature fermentation led to a

Table 6
Effect of Temperature on Formation of Volatiles in Repeated-Batch Wine Making by Biocatalyst Supported by FDC Material Using 12 °Be Initial °Be Density

Temperature (°C)	Repeated-batch wine making	Fermentation time (h)	Ethanol concentration (% v/v)	Ethanol (mg/L)	Ethyl acetate (mg/L)	Propanol-1 (mg/L)	Isobutyl alcohol (mg/L)	Amyl alcohols (mg/L)	Total volatiles determined (mg/L)
5	1-2	839	11.3	62	76	25	18	158	339
10	1-3	338	11.4	48	82	28	21	—	—
15	1-3	176	11.6	29	108	34	23	127	321
20	1-4	91	11.6	26	116	42	32	151	367
30	1-6	54	12.0	28	132	41	37	152	390

Table 7
Operational Stability of Grape Must Fermentation by Biocatalyst Supported by FDC Material at 30°C and 12 °Be Initial Density

Biocatalyst	Batch	Startup (h)	Fermentation time (h)	Ethanol concentration (% v/v)	Ethanol productivity (g/[L·d])	Wine productivity (g/[L·d])	Residual sugar (g/L)	Conversion (%)
F_1DC	1	15	116	12.0	19.4	139	2.0	99.0
F_1DC	2	0.2	72	11.7	30.4	222	3.9	98.0
F_1DC	3	0.2	53	11.6	40.9	302	4.3	97.8
F_1DC	4	0.2	46	12.0	48.8	348	—	—
F_1DC	5	0.2	40	11.4	53.3	400	3.1	98.4
F_1DC	6	0.2	36	11.8	61.0	444	—	—
F_1DC	7	0.2	34	11.9	65.5	470	1.5	99.2
F_1DC	8	0.2	30	11.8	73.6	533	2.8	98.6

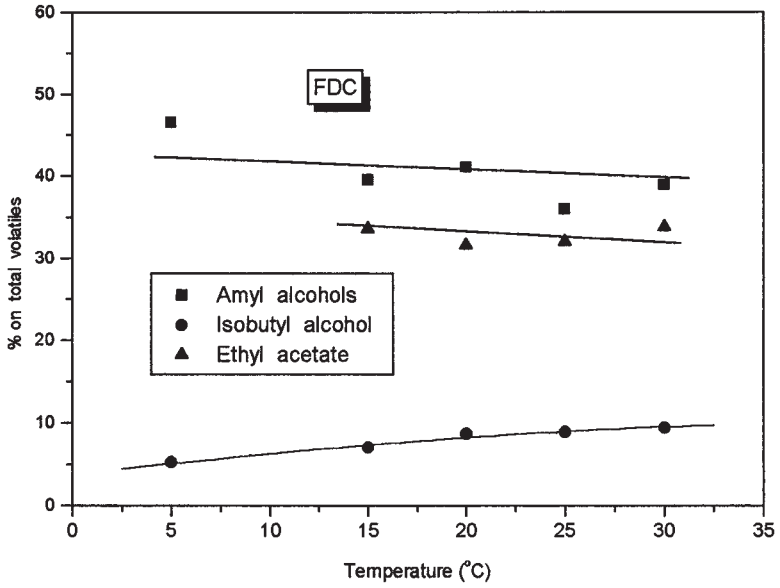

Fig. 2. Effect of temperature on percentage of ethyl acetate, isobutyl alcohol, and amyl alcohols on total volatiles in wine making by biocatalyst supported by FDC material.

decrease in the percentage of isobutyl alcohol on total volatiles and constant percentages of amyl alcohols (Fig. 2), whereas the percentage of ethyl acetate in total volatiles determined was increased. This contributes to the conclusion that the aroma of the wine produced by freeze-dried immobilized cells was improved as the temperature was decreased; a fine aroma was observed in the wines produced. The increase in initial °Be density resulted also in a decrease in the percentage of amyl alcohols on total volatiles determined and an increase in percentages of ethyl acetate, which also results in an improvement in the organoleptic quality of the wine (Fig. 3). The decrease in higher alcohols reduced the toxicity of the wine.

Table 1 shows that the FDC produced wines with a taste characterized by most of the panelists in the range of acceptable to excellent. The same taste range was also given for the wine produced by FFDCs (Table 2). However, flavor was improved at 5°C, which complies with the results obtained in Fig. 2.

Biocatalyst supported by FDC material resulted in a fermentation efficiency of grape must about similar to that of the biocatalyst supported by WDC material. It produced wine with the necessary alcohol concentration as well as characteristic byproducts. It was a suitable biocatalyst for low-temperature wine making in the range of 5–15°C. Taking into account the abundance in nature of the support, its food-grade purity, and the acceptable quality of the wine produced, which was validated by taste tests, the scale-up of the process for this freeze-dried immobilized biocata-

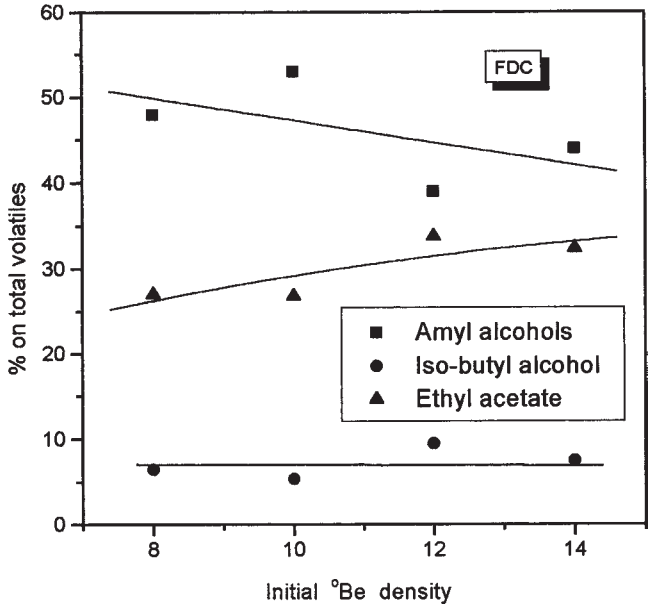

Fig. 3. Effect of initial °Be density on percentage of ethyl acetate, isobutyl alcohol, and amyl alcohols on total volatiles in wine making by biocatalyst supported by FDC material.

lyst could be designed. The drop in temperature improves concentrations of byproducts responsible for organoleptic quality of the wine.

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