Delignified Cellulosic Material Supported Biocatalyst as Freeze-Dried Product in Alcoholic Fermentation

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Freeze-dried delignified cellulosic (DC) material supported biocatalyst is proposed as a suitable form of biocatalyst to be preserved. The alcoholic fermentation of glucose using freeze-dried immobilized cells is reported. Freeze-dried immobilized baker's yeast cells on DC material do not need any protective medium during freeze-drying. The effect of initial glucose concentration and temperature on the alcoholic fermentation kinetic parameters is reported in the present study. It was found that the freeze-dried immobilized cells ferment more quickly than free freeze-dried cells and have a lower fermentation rate as compared with wet immobilized cells. However, repeated batch fermentations showed freeze-dried immobilized cells to ferment at about the same fermentation rate as wet immobilized cells. The results indicate that the freeze-dried immobilized cells must be further studied to establish a process for the preservation of immobilized cells.

Keywords: *Delignified cellulosics; freeze-dried; alcoholic fermentation; immobilization*

INTRODUCTION

Cell immobilization has been extensively studied during the past two decades. However, its application in industry has not been possible. This is because the industrialization of immobilized cells has prerequisites that must be satisfied to produce marketable products and cost-effective processes. For beverage production these prerequisites are as follows: (i) The support should be of food grade purity, low cost, and abundant in nature. (ii) The support should not be a destructible material, such as some gels. (iii) A process should be available for the preservation of immobilized cells. (iv) A biocatalyst is needed to improve the quality of products and productivity [In the framework of some of the aforementioned prerequisites, cryotolerant and ethanol-resistant yeast cells immobilized on delignified cellulosic (DC) material have been used to produce wine and beer of improved taste and aroma. The DC supported biocatalyst improved productivity in batch fermentation (Bardi and Koutinas, 1994; Iconomou et al., 1995; Bardi et al., 1996a−c).] Continuous wine-making by DC material supported biocatalyst was also studied (Iconomou et al., 1996). Maturation of beer was achieved by an immobilized yeast bioreactor (Linko et al., 1998; Yamauchi et al., 1995). The immobilization technique was employed to protect starter culture during freeze-drying of fermented meat (McLoughlin and Champagne, 1994). When such a technique starts to be applied, the problem of supplying wineries and/or breweries with preserved and marketable ready-to-use immobilized cells may have been solved. Freeze-drying is a process for food preservation and microorganism preservation as well. It is considered to be a valuable process for products of high added value. Loureiro (1990) has performed freeze-drying experiments of cells immobilized by gel occlusion. The improvement of structural and mechanical properties of alginate beads by freeze-drying was studied (Tal et al., 1997). Stabilization of invertase enzyme (Schebor et al., 1996) and loss of cell viability (Lange and Weber, 1995) after freeze-drying were studied.

It is obvious from the above literature review that the freeze-drying of immobilized cells needs much further research to produce a marketable biocatalyst on the basis of immobilized cells for industrial use. The aim of this investigation is to study the suitability of freeze-dried (DC) material supported biocatalyst in alcoholic fermentation and to evaluate its possible industrial application.

MATERIALS AND METHODS

The preparation of DC material supported biocatalyst was made by the immobilization of baker's yeast on DC material as described previously (Bardi and Koutinas, 1994). Briefly, DC material was prepared after lignin removal from sawdust with sodium hydroxide solution. The fermentations were performed in synthetic media containing glucose, 1 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 5 g/L $MgSO_4$, and 4 g/L yeast extract.

The ethanol and residual sugar concentrations were obtained by HPLC 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. An SCR-101N column 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 and sugar utilization were expressed as grams of ethanol per liter of substrate per hour and grams of sugar utilized per 100 g of initial sugar, respectively.

Freeze-Drying and Protective Media. Two protecting media were used in the freeze-drying of immobilized cells to compare with fermentations performed by the use of freeze-dried immobilized cells without protective media. The composition used in the first medium was 20% skim milk, 5% honey, and 5% glutamate, and the second medium was a mixture of 10% skim milk and 5% glutamate. Samples of DC material supported biocatalyst without protective media and with each of the two aforementioned protective media were cooled at a cooling rate of 3 °C/min and frozen to −40 °C. The samples

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Table 1. Start-up of the Alcoholic Fermentation at 30 °C Using Freeze-Dried Immobilized Cells on DC Material As Related with Protective Agent

protective agent	fermentation medium	start-up, h	fermentation time, h
without	glucose (12%)	5	110
without	grape must (12 °Be)	5	130
20% skim milk 5% honey 5% glutamate	grape must (12 °Be)	6	100
10% skim milk 5% glutamate	grape must (12 °Be)	5	98

Table 2. Start-up and Fermentation Times of the Fermentation of 23% Glucose after an Adaptation at Low Temperatures of Free Freeze-Dried Cells, Freeze-Dried Immobilized Cells, and Wet Immobilized Cells

	free freeze-dried cells		freeze-dried immobilized cells		wet immobilized cells	
temp, °C	start-up, h	fermentation time, h	start-up, h	fermentation time, h	start-up, h	fermentation time, h
15	10	100	10	95	0.1	48
10	18	600	6	672	1	156
15	12	105	8	98	0.1	48
10	15	670	6	696	1.5	144
5	22	2050	12	1724	5	670

were freeze-dried overnight at $(15-5) \times 10^{-3}$ mbar and at −40 °C in a freeze-dry system, Freezone 4.5 (Labconco, Kansas City, MO). The three lyophilized samples were used to ferment grape must of 12 °Be density, and the results are summarized in Table 1.

Fermentations. Repeated batch fermentations of glucose were carried out separately with (i) freeze-dried immobilized cells on DC material, (ii) free freeze-dried cells, and (iii) wet immobilized cells on DC material. The effects of temperature and initial glucose concentration (IGC) were examined.

To study the effect of IGC three 500 mL glass cylinders were used and each contained 200 mL of synthetic liquid medium containing glucose. This was made separately for each glucose concentration as follows: 11, 13, 18, 23, and 28% w/v. In each of the experiments 100 g of DC material supported biocatalyst, which held 4.9 g of wet weight cells, was freeze-dried, pretreated first with water and then with synthetic medium containing glucose, and added into the first cylinder. Furthermore, 4.9 g of wet weight free cells was freeze-dried and added into the second glass cylinder, and into the third cylinder was added 100 g of DC material supported biocatalyst having also 4.9 g of wet weight immobilized cells. For each IGC the three cylinders were incubated at 30 °C and the fermentations were carried out without agitation.

To study the effect of temperature, the same process was employed using an IGC of 23% w/v and batch fermentations were performed at temperatures of 5, 10, 15, 20, and 30 °C. All fermentations were carried out by using an initial wet weight cell concentration of 24.5 g/L to obtain a first indication for the operational stability of freeze-dried cells. A second batch was carried out by using the same DC material supported biocatalyst. In this case before the fermentation was completed, the liquid was filtered using a Büchner funnel and the DC material supported biocatalyst was washed twice, each time with 200 mL of liquid medium containing glucose. Fermentation kinetics were performed by measuring the °Be density at various time intervals. Before the end of the fermentation, samples were collected and analyzed for ethanol concentration and residual sugar by the HPLC method. All values were the mean of three replicates.

Electron Microscopy. The samples were coated with gold in a Balzers SCD 004 and then examined in a JEOL model JSM-5200 scanning electron microscope.

RESULTS AND DISCUSSION

The industrialization of a process except for the prerequisites is presented in the Introduction and needs to satisfy the criterion of productivity and other kinetic perameters. Therefore, experiments were organized to examine this possibility for freeze-dried immobilized cells. However, before this experimental work, factors of primary importance in the freeze-drying process would have to be studied. A cooling rate of 3 °C/min was found to be the best value, and the protective media and adaptation treatment at low temperatures of freeze-dried immobilized cells are also reported. In the cases studied, freeze-dried immobilized cells were compared with free freeze-dried cells and wet immobilized cells as alternatives for the possible use of cells in industry. The results are summarized in the Tables 1 and 2; kinetic parameters as related with IGC and temperature are presented in the Tables 3 and 4.

Table 1 shows that the protective agent does not affect significantly start-up and fermentation times as compared to those without use of a protective agent. After these results, it was decided to prepare all freeze-dried immobilized cells without protective media. Table 2 indicates the adaptation at low temperature, which was obtained by establishing gradually decreasing temperature conditions of 2 °C/day. This table shows freeze-dried immobilized cells had an improved start-up as compared with free freeze-dried cells, whereas wet

Table 3. Effect of Temperature on Kinetic Parameters Observed at the Fermentation of 23% Glucose by Free Freeze-Dried Cells, Freeze-Dried Immobilized Cells, and Wet Immobilized Cells[a]

biocatalyst	temp, °C	start-up, h	fermentation time, h	EtOH concn, g/L	EtOH productivity, g/L/h	residual sugar, g/L	sugar utilization, %
free freeze-dried cells (1st batch)	5	30	2160	8.5	0.04	15.7	93.1
	10	24	672	8.8	0.12	11.7	94.9
	15	12	105	9.5	0.94	15.1	93.4
	20	10	94	9.3	1.09	14.8	93.5
	30	6	85	9.5	1.19	12.2	94.7
freeze-dried immobilized cells (1st batch)	5	48	1872	8.6	0.04	31.4	86.3
	10	48	744		0.11	30.5	
	15	8	95	8.9	1.09	20.1	91.2
	20	8	105	8.3	0.85	29.4	87.2
	30	3	48	9.6	1.98	18.3	92.0
wet immobilized cells (1st batch)	5	12	744	8.6	0.11	1.8	99.2
	10	4	168	8.7	0.50	6.3	97.2
	15	0.2	45	9.9	2.46	1.7	99.2
	20	0.2	60	9.6	1.58	1.4	99.3
	30	0.2	32	9.6	2.96	1.2	99.4

[a] The standard deviations for ethanol concentration, ethanol productivity, residual sugar, and sugar utilization were less than 0.24, 0.5, 4, and 2 respectively.

Table 4. Effect of IGC on Kinetic Parameters Observed at the Fermentation of Glucose by Free Freeze-Dried Cells, Freeze-Dried Immobilized Cells, and Wet Immobilized Cells (Temperature = 30 °C)[a]

biocatalyst	IGC, g/L	start-up, h	fermentation time, h	EtOH concn, g/L	EtOH productivity, g/L/h	residual sugar, g/L	sugar utilization, %
free freeze-dried cells	110	4	32	4.8	1.46	3.8	96.5
	130	2	36	5.8	1.56	9.5	92.6
	180	4	46	7.2	1.53	20.0	88.8
	230	6	85	9.5	1.19	12.2	94.7
	280	8	108	9.8	0.78	32.1	88.5
freeze-dried immobilized cells	110	5	28	4.4	1.55	14.4	96.3
	130	4	26	5.3	2.00	12.4	98.4
	180	2	30	7.0	2.29	-	99.2
	230	3	48	9.6	1.98	2.1	99.1
	280	3	56	10.0	1.76	26.0	91.3
wet immobilized cells	110	0.2	10	5.3	5.21	0.7	99.3
	130	0.2	12	5.6	4.58	0.9	99.3
	180	0.2	17	7.4	4.29	1.2	99.3
	230	0.2	32	9.6	2.92	1.2	99.4
	280	0.2	54	10.3	1.83	13.7	95.1

[a] The standard deviations for ethanol concentration, ethanol productivity, residual sugar, and sugar utilization were less than 0.24, 0.5, 4, and 2 respectively.

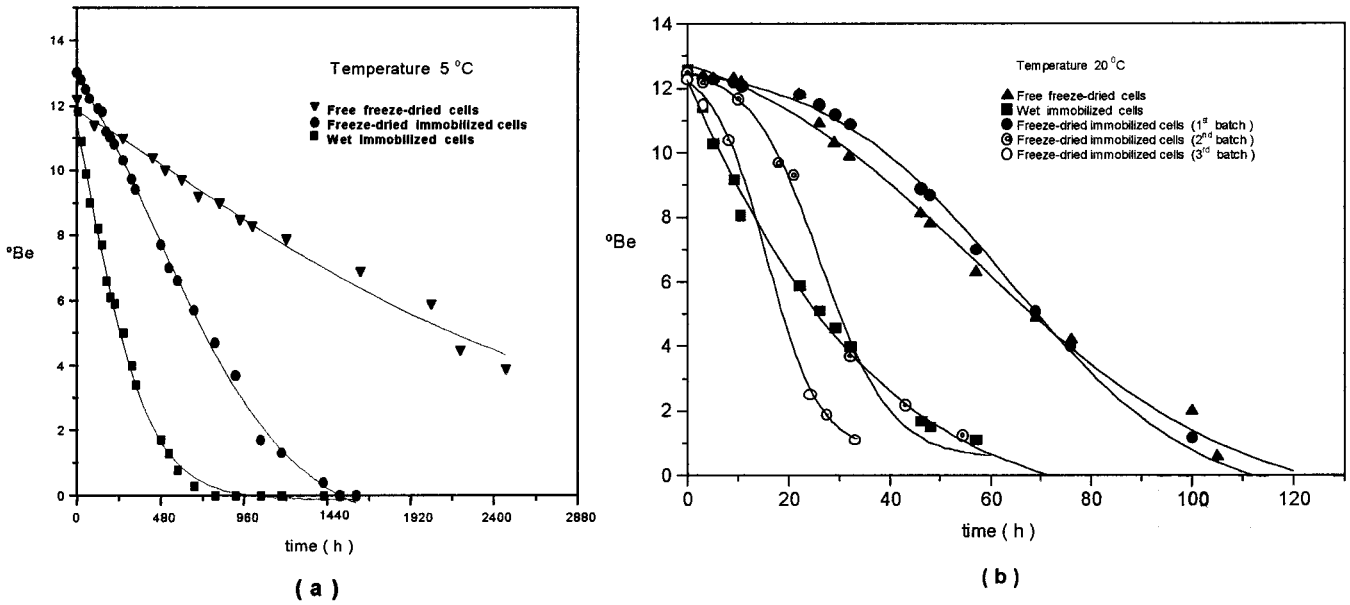

Figure 1. Fermentation kinetics observed using 23% glucose at (a) 5 and (b) 20 °C.

immobilized were better in all cases studied. Likewise, wet immobilized cells fermented more quickly below 10 °C as compared with free freeze-dried cells and freeze-dried immobilized cells. At 15 °C wet immobilized cells appeared to achieve less fermentation time.

Table 3 shows that a drop of temperature up to 15 °C does not affect significantly start-up. Temperatures below 10 °C increase many times the start-up of the process. Freeze-dried immobilized cells usually give better fermentation time, productivity, and sugar utilization as compared with free freeze-dried cells, whereas the best effect on results for wet immobilized cells were obtained as the temperature was decreased. Table 4 illustrates that the increase of IGC does not affect start-up in the case of wet immobilized cells. Freeze-dried immobilized cells had less start-up time as compared with free freeze-dried cells. Also, the fermentation time, residual sugar, and sugar utilization are better in the case of wet immobilized cells in all IGCs studied. However, freeze-dried immobilized cells gave improved results for the same parameters as compared with free freeze-dried cells in all IGCs. The fermentation time of freeze-dried immobilized cells became equal with that obtained by wet immobilized cells at an IGC of 280 g/L. Furthermore, one can observe in Figure 1a that the rate of fermentation of freeze-dried immobilized cells is higher than that of free freeze-dried cells and less than that of wet immobilized cells at all temperatures studied. It is obvious that the second and third batches obtained by freeze-dried immobilized cells improve further the fermentation rate (Figure 1b). Finally, it is obvious that the effect on fermentation rate at the low temperature of 5 °C obtained by freeze-dried immobilized cells is greater than that at 20 °C. The temperature dependence of the fermentation rate could be attributed to the reduction of the activation energy E_a and the presence of DC material as the temperature is decreased (Bardi and Koutinas, 1994). The avoidance of the use of protective agent is of primary importance due to practical and economical interests. However, the adaptation treatment at low temperatures improved significantly the start-up of the process. This improvement may be what was needed for the industrialization of freeze-dried immobilized cells. Below 10 °C the adaptation start-up of freeze-dried immobilized cells was about half that of free freeze-dried cells even though without

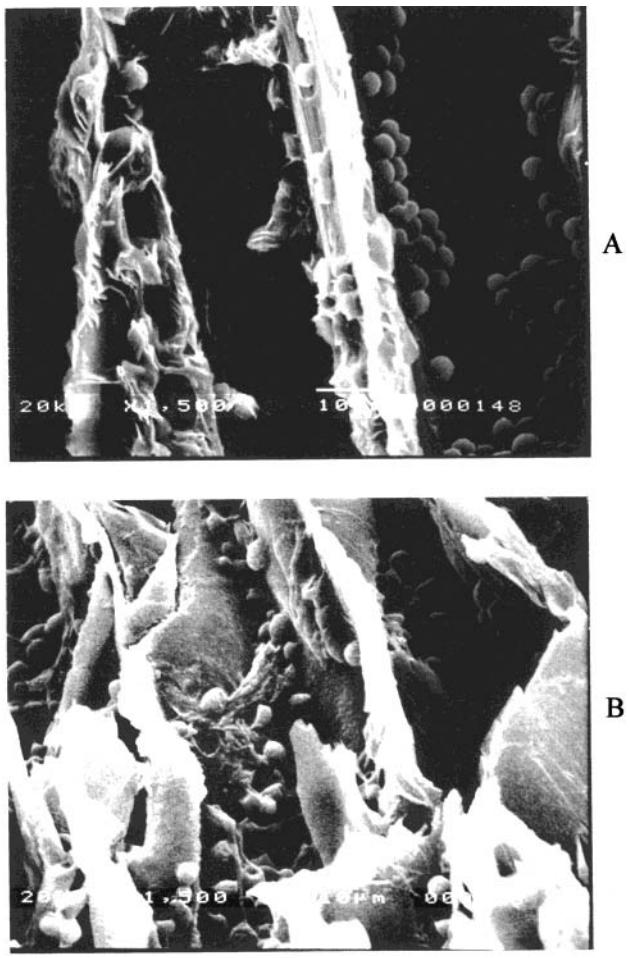

Figure 2. Electron micrographs showing (A) freeze-dried immobilized cells on DC material of the first batch without protective media at ×1500 and (B) wet immobilized cells on DC material of the first batch at ×1500. (The figure is reproduced here at 67% of the original.)

adaptation free freeze-dried cells gave better results. The adaptation treatment at low temperature is needed to avoid shock when the temperature drops.

At 30 °C freeze-dried immobilized cells resulted in a significant decrease of the fermentation time, mainly at the higher IGC, as compared with free freeze-dried cells. That increase of productivity and the improvement of the aroma and taste of wine and beer obtained by DC material supported biocatalyst (Bardi et al., 1996a,b) contribute to a promising form of the biocatalyst based on immobilization. Wet immobilized cells lead to further improvement of productivity and fermentation time. However, the use of wet immobilized cells requires for each wine-making and brewing facility additional investment and labor cost for immobilization because the biocatalyst cannot be preserved. The ability of freeze-dried immobilized cells to be more effective in higher IGC may make the biocatalyst suitable for potable alcohol production and wine-making for which higher alcohol concentrations are necessary.

It is obvious that the rate of fermentation of freeze-dried immobilized cells at the high IGC of 23% in the repeated batch fermentations is improved from batch to batch and the third batch showed a rate similar to that of wet immobilized cells (Figure 1b). This means that the system in practical conditions, which includes repeated batch fermentation processes, can yield results close to those of wet immobilized cells. Likewise, the system of freeze-dried immobilized cells is extremely more effective as compared with that of free freeze-dried cells (Figure 1a). Finally, freeze-dried immobilized cells continue to be attached on the support, as is shown by Figure 2.

In summary, one can conclude that the cells continue to be immobilized after freeze-drying and freeze-dried immobilized cells appear to be promising in the development of a new form of biocatalyst for wine-making and brewing that could be a marketable product. Further research is necessary to obtain this new biocatalyst.

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