Immobilization of Yeast on Delignified Cellulosic Material for Low Temperature Brewing

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A biocatalyst was prepared by immobilization of *Saccharomyces cerevisiae* strain AXAZ-1 on delignified cellulosic (D.C.) material and studied in the fermentation of wort for batch and continuous brewing. The immobilized yeast gave an important operational stability without decrease of its activity, even at low temperatures (0−5 °C), compared with free cells. Batch fermentations at various temperatures were faster than those of free cells and those usual in commercial brewing. A traditional bottom fermentation takes 8−10 days. Specifically, at 0 °C, the fermentation rate was 4−5 times higher than that of free cells. Diacetyl and polyphenol contents as well as bitterness and pH were lower than those when free cells were used for fermentations; at 0 °C, polyphenol content was ∼30% and bitterness 50% of the values noted when free cells were used for fermentations. The alcohol concentration at 0 °C was ∼20% higher than that of free cells. The continuous system was operated continuously for 3 months with relatively high productivity. However, polyphenols and bitterness were higher than those obtained in batch fermentations with immobilized cells. Preliminary taste tests indicated that the beer produced by the immobilized cell process had an acceptable clarity, aroma, and taste.

Keywords: *Cell immobilization; delignified cellulosic material; Saccharomyces cerevisiae; brewing; beer*

INTRODUCTION

Beer was known to early civilizations, but the brewing was an art and mystery. Although explanation for fermentation was not available until the nineteenth century, the steady improvement in manufacturing techniques was not impeded. The use of adsorbed yeast cells for the continuous production of beer was first attempted in 1899 by Barbet. Recently, a number of studies have been published on the production of beer by batch fermentation with immobilized cells on polyethylene film (Kolpakchi *et al.*, 1976), ceramic or polyethylene rings (Kolpakchi *et al.*, 1980), alginate gel (Pardonova *et al.*, 1982), and hollow PVA gel beads (Shindo *et al.*, 1990). Also, a number of studies have been published on brewing by continuous fermentation with immobilized yeast cells on PVC and porous bricks (Corrieu *et al.*, 1976; Navarro *et al.*, 1976), diatoms, PVC and plastic (Moll, 1977), pieces of brick (Moll and Duteurtre, 1979), Ca alginates (Godtfredsen *et al.*, 1981; Linko and Linko, 1981; Onaka *et al.*, 1985), and ceramics (Nakanishi *et al.*, 1989).

The use of an immobilized yeast cell system for alcoholic fermentation is an attractive and rapidly expanding research area because of its additional technical and economical advantages compared with the free cell system (Stewart and Russell, 1986; Margaritis and Merchant, 1984). However, for industrial application, further research is needed to obtain cell immobilization on a support that is more hygienic for food, cheap and abundant in nature, and suitable for low temperature fermentation in brewing. Manufacturers of beer know that the low temperature fermentation results in a product with improved aroma and taste. Finally, it has been reported that immobilized cells on delignified cellulose (D.C. material are suitable for low-temperature wine making (Bardi and Koutinas, 1994) and increased productivity. The aim of this investigation was to evaluate the use of D.C. material-supported biocatalyst in the brewing fermentation of brewing beer.

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MATERIALS AND METHODS

The biocatalyst was prepared by the immobilization of *Saccharomyces cerevisiae* strain AXAZ-1 on D.C. material as described previously (Bardi and Koutinas, 1994). Briefly, D.C. material was prepared after lignin removal from sawdust with sodium hydroxide solution. AXAZ-1, an alcohol-resistant and psychrophile *S. cerevisiae* strain isolated (Argiriou *et al.*, 1992) from the Greek agricultural area, was grown on the complete medium used in the previous study (Bardi and Koutinas, 1994). Pressed wet cells were prepared as in the aforementioned reference and employed directly in the fermentations. Wort was obtained from Athenian Brewery S.A., hopped, filtered, and sterilized. The pH of the wort was 5.1−5.2 and the °Be density was 6.9. The values of the percent original extract of the wort are shown in Table 3.

Alcoholic degrees were obtained, after distillation of samples, with a Gay-Lussac alcohol meter. The determination of ethanol enabled us to calculate the ethanol productivity, which is defined as the grams of ethanol per liter liquid volume produced per day. Beer productivity was calculated as grams of beer per liter total volume produced per day. Total carbohydrates were determined in all samples by the Lane-Eynon method (Egan *et al.*, 1981). Apparent extract percent, polyphenols and diacetyl content, as well as bitterness, color, and refractive index (20 °C) were determined by well-known methods (Hough *et al.*, 1982). Original and real extract percent were determined from a nomograph furnished by the Athenian Brewery S.A. Wet free cell concentrations were determined by the absorbance experimental procedure (Klein and Kressdorf, 1983; Bajpai and Margaritis, 1986) and are given in grams of wet weight per liter, as determined with standard curves.

Repeated Batch Fermentations at Room and Low Temperatures. For repeated batch fermentations, an amount

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Table 1. Kinetic Parameters Obtained in the Repeated Batch Fermentations at Various Temperatures (0−30 °C) with D.C. Material-Supported Biocatalyst

temp (°C)	repeated fermentn batche	fermentn time (h)	total carbohydrates (g/L)	EtOH concn (% v/v)	EtOH productivity (g/L/d)	beer productivity × 10^3 (g/L/d)
30	1	11	12.8	4.9	59.0	1.505
30	4	12	14.4	5.0	55.1	1.379
30	6	14	11.2	5.2	49.2	1.182
15	8	27	16.0	5.6	28.2	0.613
15	9	24	16.0	5.6	30.9	0.690
15	10	22	24.0	5.0	30.0	0.752
15	11	27	19.2	5.3	26.0	0.613
10	12	25	12.8	5.0	26.5	0.662
10	14	72	11.2	5.6	10.3	0.230
10	15	72	4.8	5.6	10.3	0.230
10	16	72	8.0	5.6	10.3	0.230
10	17	72	8.0	5.4	9.9	0.230
7	19	120	12.8	5.2	5.7	0.138
7	20	144	6.4	6.1	5.6	0.115
7	21	144	4.8	6.2	5.7	0.115
5	22	216	11.2	5.9	3.6	0.077
5	23	216	9.6	6.2	3.8	0.077
0	24	264	1.6	6.6	3.3	0.063
0	25	312	1.6	6.2	2.6	0.053
0	26	288	0.0	6.6	3.0	0.057
0	27	288	1.6	6.3	2.9	0.057

of D.C. material-supported biocatalyst (~170 g) was introduced into 400 mL of wort in a 1-L glass cylinder. The glass cylinder for each fermentation batch was incubated at the temperatures indicated in Table 1. The fermentations were carried out without agitation. Before the fermentation was completed, the liquid was filtered through a Büchner funnel, and the support was washed three times, each time with 400 mL of wort. The biocatalyst was pressed on the funnel to remove the liquid. After that, the biocatalyst was used for the next fermentation batch.

To compare the fermentation times and the other parameters obtained in the presence of the D.C. material-supported biocatalyst with those of free cells, similar runs were carried out simultaneously with the same cell concentration. Yeast cells immobilized on D.C. material were determined in a recent study (Bardi and Koutinas, 1994). All values were the mean of three repeats. The standard deviation for ethanol concentration was $<\pm 0.2$, for ethanol productivity was $<\pm 10$, and for residual sugar was $<\pm 2$.

Continuous Fermentation for Brewing at Low Temperatures. *Apparatus.* The reactor was a glass tower (2100 mL total working volume and 1450 mL liquid volume). D.C. material (620 g) was placed in the reactor with 1450 mL of wort as the immobilization support. The continuous fermentation medium (wort) was pumped in a upflow stream with the aid of a high accuracy peristaltic pump (Cole Parmer Instrument Company, Chicago, IL). An overflow exit line for the maintenance of constant volume was installed in the column. The total system was placed in a constant temperature chamber.

Experimental Procedure. Cells were immobilized on the D.C. material as follows: The glass tower fermentor was charged with 1450 mL of culture media containing glucose (120 g/L) and pressed AXAZ-1 (20 g/L). The pH was adjusted to 4.7. The mixed culture was allowed to ferment without feeding. After 8 h, the °Be density was 0.5 and feed (pH 5.6) and glucose (120 g/L) were pumped for 4 days at a dilution rate in the range 0.48−0.90 d^{-1} for biomass attachment. After biomass attachment, the reactor feed was started and operation continued for 90 days. Samples were collected at dilution rates of 0.48 and 0.90 d^{-1}. The results are presented in Table 5. Dilution rates were calculated by dividing the flow rate of liquid by the liquid volume of the fermentor. Furthermore, the ethanol productivity was expressed in g ethanol/L produced in 1 day and calculated on the basis of the liquid volume by multiplying the dilution rate by ethanol concentration. Beer productivity was expressed as grams of beer per liter total volume produced per day and was calculated by multiplying the flow rate (mL/d) by the density of beer (about 1 g/mL) and dividing by the total volume of the reactor (2.1 L).

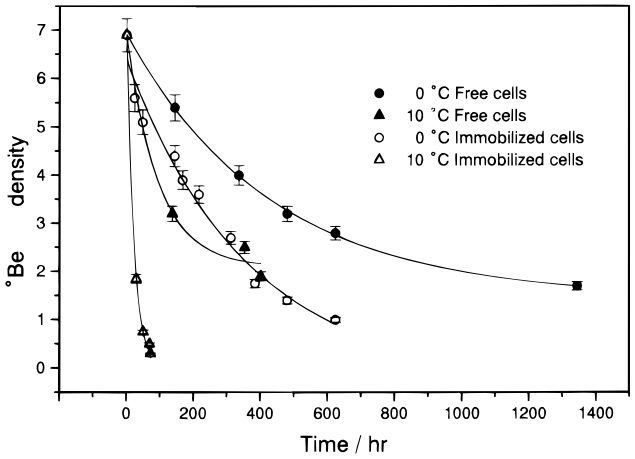

Figure 1. Fermentation kinetics observed in the fermentation of wort at 10 and 0 °C with D.C. material-supported biocatalyst compared with free cells.

Preliminary Taste Test. Samples of beer produced were kept at 4−5 °C for a month until tasted. People may differ from each other in understanding of the taste and aroma, so beers were tasted by 10 trained tasters. This taste test was made in comparison with commercial product, according to individual three-glass flavor testing forms (Hough *et al.*, 1982). Signed protocols are available for each taster.

RESULTS AND DISCUSSION

D.C. material-supported biocatalyst was used for repeated batch fermentations of wort at temperatures in the range 0−30 °C and for continuous ones at temperatures 3−15 °C. The temperature was subsequently reduced as indicated in Tables 1 and 5.

In the case of the 27 repeated batch fermentations, the duration was ~3 months without any loss of activity. From every batch, samples were collected for analyses and taste tests. Results are summarized in Tables 1 and 3. Likewise, results of the fermentations by free cells are presented in Tables 2 and 4 and kinetics of the fermentations performed in the presence of D.C. material-supported biocatalyst in contrast with that of free cells are illustrated in Figure 1.

The results in Table 1 show that the fermentation time with the immobilized cells was much shorter than

Table 2. Kinetic Parameters Obtained with Free Cells in the Fermentation of Wort

temp (°C)	fermentn time (h)	total carbohydrates (g/L)	EtOH concn (% v/v)	EtOH productivity (g/L/d)	beer productivity $\times 10^3$ (g/L/d)
30	142	16.0	5.4	5.0	0.116
30	145	16.2	5.4	4.9	0.114
10	624	16.0	5.3	1.1	0.027
10	615	15.5	5.3	1.1	0.027
0	320	24.0	5.4	0.6	0.013
0	1350	23.7	5.4	0.5	0.012

indicated for free cells, as shown in Table 2; that is, it was twelve, seven, and four or five times shorter than that of free cells at 30, 10, and 0 °C, respectively. This increase in the ethanol production rate is also shown in Figure 1. Total carbohydrates in the product were found in the range 0−26 g/L, whereas a typical commercial beer contains 9−60 g/L (Buckee and Hargitt, 1977). The pH lies in the range 3.6−4.4, which is the range of most commercial beers.

The alcohol content of beer is usually regarded as a measure of its strength and lies in the range 3.5−5.5% (v/v) for the most commercial products. Beer produced by these repeated batch fermentations contained higher alcohol content, especially at temperatures <7 °C. At 0 °C, the ethanol content with the immobilized cells was 6.2−6.6% (v/v) compared with 5.4% (v/v) for free cells. As shown in Table 3, the values of the original extract, real extract, apparent extract, and refractive index of the beers obtained by repeated batch fermentations with immobilized cells on D.C. material are in the ranges of most commercial beers. Bitterness was low, in the range 7.0−17.1 EBU. Bitterness in the commercial beers varies greatly within the range 10−40 EBU, and can be increased further by the addition of hop extracts. The concentrations of hop bittering substances of beers produced by D.C. material-supported biocatalyst (see Table 3) are lower than those from free cells (see Table 4). Probably, the cellulosic material adsorbs a part of the hop-bittering substances or they are converted by enzymes involved in the system. The beers obtained in our studies had a pale yellow color, which remained stable for a very long time (~10 months). The measurements of the color are quantitatively represented in Table 3 and are in the range 6−15 °EBC for most commercial beers.

At all temperatures studied, the diacetyl concentrations were at the level of commercial beers 0.06−0.5 ppm (Table 3). The optimum concentration is <0.15 ppm, and most products obtained by D.C. material-supported biocatalyst approach this value at all temperatures studied. In comparison, with fermentations by free cells (Table 4), the diacetyl concentration was lower for immobilized ones.

At all studied temperatures, the polyphenol concentrations were <170 ppm as compared with 190−250 ppm in commercial beers (Table 3). Furthermore, polyphenol contents in the products by D.C. material-supported biocatalyst were ~30−50% of those contained in beers brewed using free cells (Table 4). Probably, the D.C. material-supported biocatalyst adsorbs a part of polyphenols or destroys them biochemically, because the concentration of polyphenols in fermentations with free cells is larger, especially at low temperatures; from 10 to 0 °C, the values of the polyphenols are further decreased in the case of D.C. material. The beers produced in this study had very good clarity just after the end of the fermentation, so the low concentrations of polyphenols are justified. Therefore, no filtration was necessary for clarifying the beer. Generally, beers prepared by immobilized cells had a better clarity, and at temperatures <10 °C, the clarity was complete.

The preliminary taste test characterized the new beers as sweet, with a pleasant, soft aroma, fruity taste,

Table 3. Characteristics of Beer Obtained in Batch Fermentation with D.C. Material-Supported Biocatalyst

temp (°C)	% original extract	% real extract, E_r	% apparent extract E_a	refractive index, R_0	color (EBC)	polyphenol (ppm)	diacetyl (ppm)	bitterness (EBU)	final pH
30	11.25	3.58	1.78	35.50		153	0.09	10.3	4.0
30	11.42	3.54	1.68	35.50		148	0.09	9.8	3.8
30	12.20	3.35	1.67	35.60			0.08	10.3	3.9
15	11.87	3.45				157	0.05	11.0	3.7
15	11.60	3.17	1.19	34.55		155	0.09	12.1	3.6
15	11.42	4.00	2.32	35.60		162	0.10	10.5	3.8
15	11.46	3.98	2.24			154	0.11	11.2	3.8
10	12.00	3.54	2.32	35.60				12.5	4.0
10	12.06	3.54	1.72	36.80	13.8	163	0.12	17.1	4.2
10	11.87	3.45	1.48	35.65		161	0.08	12.1	4.0
10	12.07	3.56	1.57	36.20		152	0.09	13.2	4.0
10	11.97	3.63	1.67	36.30		157	0.10	12.8	4.0
7	11.84	3.94	2.08	37.15		105	0.09	11.2	4.0
7	13.40	4.25	1.52	36.20	11.5	99.4	0.11	10.4	3.9
7	13.20	3.80	1.48	35.70		100.1	0.14	12.0	3.9
5	13.40	4.25			14.5	116.0	0.10	10.1	3.9
5	13.20	3.80			9.8	112.0	0.12	11.5	4.0
0	14.40	4.27			8.8	102.0	0.12	10.3	3.8
0	13.20	3.80			9.2	108.0	0.09	10.5	3.8
0	14.40	4.27			9.8	84.7	0.11	10.8	3.9
0	14.20	4.45			11.5	83.0	0.08	7.0	3.8

Table 4. Characteristics of Beer Obtained in the Fermentation with Free Cell

temp (°C)	% original extract	% real extract, E_r	% apparent extract, E_a	refractive index, R_0	color (EBC)	polyphenol (ppm)	diacetyl (ppm)	bitterness (EBU)	final pH
30	12.20	3.86	1.92	37.25	12.8	295	0.12	20.2	4.4
30	12.39	4.04	2.08	37.95		320	0.15	22.0	4.4
10	12.22	4.05	2.14	37.85		335	0.15	18.1	4.4
10	12.38	4.02	2.07	37.90	11.6	340	0.24	21.2	4.4
0	12.90	4.00	2.08	37.89	11.0	287	0.24	17.2	4.3
0	12.19	3.87	1.90	37.21		325	0.17	19.3	4.4

Table 5. Kinetics of Continuous Brewing with D.C. Material-Supported Biocatalyst

temp (°C)	dilution rate (d^{-1})	test duration (h)	final density (°Be)	total carbohydrates (g/L)	EtOH concn (% v/v)	EtOH productivity (g/L/d)	beer productivity (g/L/d)
15	0.90	24	1.3	20.8			
15	0.90	24	1.0	16.0	5.5	39.1	619
15	0.90	24	0.8	12.8	6.1	43.4	619
15	0.90	24	1.2	19.2	5.3	37.7	619
15	0.90	24	0.9	14.4	6.3	44.8	619
15	0.90	24	1.0	16.0	6.5	46.2	619
10	0.90	24	1.0	16.0	6.8	48.3	619
10	0.90	24	1.0	16.0	6.5	46.2	619
10	0.90	24	1.0	16.0	6.5	46.2	619
10	0.90	24	1.0	16.0	6.3	44.8	619
5	0.90	24	0.3	4.8	6.0	42.7	619
5	0.90	24	0.5	8.0	6.5	46.2	619
5	0.90	24	0.5	8.0	6.8	48.3	619
3	0.90	24	2.0	32.0	4.8	34.1	619
3	0.90	24	1.5	24.0	6.3	44.8	619
3	0.48	24	1.8	28.8	6.1	23.1	333
3	0.48	24	1.5	24.0	5.3	20.1	333
3	0.48	24	1.5	24.0	4.7	17.8	333
3	0.48	24	1.5	24.0	4.6	17.4	333

Table 6. Characteristics of Beer Obtained in Continuous Fermentation with D.C. Material-Supported Biocatalyst

temp (°C)	% original extract	% real extract	apparent extract	refractive index, R_0	color (EBC)	polyphenol (ppm)	diacetyl (ppm)	bitterness (EBU)	final pH
15	11.60	3.17	1.19	34.55			0.08		
15	13.23	3.80	1.48		5.8	41	0.14	2.3	3.5
15	11.46	3.98	2.24			49	0.10	5.3	4.1
15	14.17	4.45				68	0.11	7.3	4.1
15	14.45	4.45			9.3		0.12	10.3	3.9
10	14.79	4.41			10.7	114	0.13	13.8	4.3
10	14.70	4.49				125	0.28	13.2	3.7
10	14.45	4.45			9.2	107	0.37	17.4	4.4
10	14.17	4.45				113	0.17	12.7	4.1
5	13.40	4.25	1.52	36.2		145	0.38	15.2	4.0
5	14.45	4.45				153	0.41	14.3	3.9
5	14.79	4.41				137	0.30	12.7	4.2
3	11.46	3.98	2.26			142	0.30	12.9	4.1
3	14.14	4.40			9.2	137	0.28	15.2	4.3
3	13.22	3.81	1.48			157	0.24	13.5	4.2
3	12.04	3.56			8.3	153	0.35	13.8	3.9
3	11.29	4.04					0.40	16.5	4.1
3	11.12	3.92	2.52	37.7		148			

body, after-taste, and different beers in comparison with the commercial beers. The taste and aroma of the beers produced by batch and continuous system were similar and more intense in beers produced at low temperatures.

The fermentation of wort in a continuous bioreactor was performed to study the operational stability of the immobilized strain AXAZ-1 on D.C. material and to compare repeated batch fermentations with continuous ones with regards to brewing. At every temperature, samples were collected when the steady state was attained. The bioreactor was operated continuously for 3 months. Results obtained by the analysis of the samples are presented in Tables 5 and 6. At temperatures <10 °C, beer productivities are much higher than those obtained at repeated batch fermentations (Table 5). At 3 °C, the ethanol productivity was about three- to four-fold higher in the continuous process than in those obtained at 5 °C in the repeated batch fermentations.

The values of the parameters original extract, real extract, apparent extract, refractive index, color, and pH in the continuous process are shown in Table 6. The values of these parameters lie in the ranges of the most commercial beers. Also, the ethanol concentration is greater than the usual values of beers but similar to that of the batch fermentations. The concentrations of polyphenols, diacetyl, and bitterness increased as the temperature is decreased, but they are in the ranges of the most commercial beers. In the batch system, the values of these parameters decreased as the temperature is decreased or remain constant in the case of diacetyl. The continuous bioreactor was continuously fed with hopped, aerated wort, and so more diacetyl was produced than in the batch system. Because of the losses of the hop-bittering substances, polyphenols are lower in the continuous system than in repeated batch fermentations. Probably, smaller amounts of these substances are adsorbed onto D.C. material than in the batch system because of the shorter time of their contact.

During the 3-month period of the continuous process of the reactor, no increase of free cell concentration was observed. Final cell concentration was <2 g/L. This value proves that the immobilized cells fermented the most sugars of the wort.

The beer we prepared had a very good clarity. It was held in package for a long time in the refrigerator (4−5 °C) and no haze was observed. Probably, this carrier of D.C. material adsorbs proteins, polyphenols, and/or haze precursors. At the end of the operation of the reactor, a small quantity of precipitate was observed on the bottom of the reactor.

The results and explanations just described lead to the conclusion that D.C. material-supported biocatalyst increases the rate of brewing. This rate increase makes

brewing at 0 °C as productive as industrial brewing processes at 12−15 °C. A traditional bottom fermentation takes 8−10 days. In the continuous process, the productivities become much higher than those of the repeated batch fermentations, especially at temperatures <10 °C. The positive taste test results and composition of beer compared with low temperature brewing obtained by the process, along with the increase of productivity observed, suggest possible utility of this process in commercial practice. The improvement of productivity and clarity of the product will lead to diminution of the installation and labor costs. Likewise, the improvement of the quality will increase the consumption and, therefore, result in larger profit.

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