Low-Temperature Brewing by Freeze-Dried Immobilized Cells on Gluten Pellets

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A biocatalyst, prepared by the immobilization of a cryotolerant strain of *Saccharomyces cerevisiae* on gluten pellets, was freeze-dried without any protecting medium and used for repeated batch fermentations of wort for each of the temperatures 15, 10, 5, and 0 °C. The fermentation time for freeze-dried immobilized cells was about 2-fold that of the corresponding time for wet immobilized cells on gluten pellets, and lower than the corresponding time for freeze-dried free cells, especially at 5 and 0 °C. Beers produced by freeze-dried immobilized cells contained alcohol levels in the range of 5.0−5.5% v/v, diacetyl concentrations lower than 0.5 mg/L, polyphenol concentrations lower than 145.5 mg/L, and free cell concentrations lower than 3 g/L. As a result, they had a very good clarity after the end of primary fermentation. The amounts of amyl alcohols were lower than 129.1 mg/L and reduced as the temperature was decreased. Ethyl acetate concentrations were found in the range of 22.1−29.2 mg/L, giving a very good aroma and taste in the produced beers.

Keywords: *Fermentation; immobilization; gluten pellets; freeze-drying; brewing; volatile byproducts*

INTRODUCTION

Although brewing has an old tradition, it is in the forefront of biotechnological development. Today, malting and brewing research is performed on many technical, biochemical, microbiological, and genetic topics. Some of the above possibilities, such as the genetic modification of malting barley or brewer's yeast, are not easily commercialized because of the uncertainty of consumers toward them (*1*). However, the immobilization of microbial cells by "active" entrapment within natural polymers or "passive" adsorption on solid supports has become a rapidly expanding research area (*2*).

It was known that an immobilized cell system has advantages over a free cell system (*3*). When such a technique is applied, the problem of supplying wineries and breweries with preserved and marketable ready-to-be-used immobilized cells must be solved. Freeze-drying is a valuable method for products of high added value. Loureiro (*4*) has performed freeze-drying experiments for cells immobilized by gel occlusion. More recently the viability and thermal stability of a strain of *Saccharomyces cerevisiae* freeze-dried in different sugar and polymer matrixes were studied (*5*). Baker's yeast cells immobilized on delignified cellulosic material were freeze-dried without cryoprotecting medium and used for low-temperature fermentation of glucose (*6*).

Immobilization, freeze-drying, and fermentation at low temperature are noteworthy methods to be applied in brewing, but the final decision depends on the quality and aroma of the product. Bardi et al. (*7*) have reported that in beers produced by fermentation of wort by cells immobilized on gluten pellets, higher alcohols were reduced as the temperature was decreased and ethyl acetate was higher for immobilized cells at low temperatures as compared to that of free ones. These two results led to better aroma and taste and less toxicity of beers, as regards higher alcohols and more specifically, fusel alcohols.

The aim of this study was to evaluate the use of freeze-dried immobilized cells on gluten pellets in low-temperature fermentation of wort in order to contribute to a scale-up for the industrialization of immobilized cells in brewing.

MATERIALS AND METHODS

Yeast Culture. AXAZ-1, an alcohol-resistant and psychrophillic *S. cerevisiae* strain isolated from grapes of the Greek agricultural area (*8*), was grown on a culture medium consisting of 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.5% $MgSO_4 \cdot 7H_2O$, and 4% glucose monohydrate, and used in the present study.

Preparation of the Immobilized Biocatalyst. The biocatalyst was prepared as described in a previous study (*9*) by the immobilization of *Saccharomyces cerevisiae* strain AXAZ-1 on gluten pellets. The gluten pellets were prepared by removing starch from flour dough by washing it exhaustively with water. The prepared wet gluten was shaped into small pellets of diameter 1.5−2 cm, dried at 105 °C for 5 h, and used for cell immobilization after mixing with AXAZ-1 in 12% glucose. A 100-g portion of the wet biocatalyst prepared by the above procedure will hold 1.4 g wet weight AXAZ-1 cells (or 4.2 g wet weight AXAZ-1 cells per 100 g of dry weight immobilized cells on gluten pellets).

Freeze-Drying Procedure. The wet immobilized cells on gluten pellets were cooled in a Biocool Freezer with a cooling rate of 3 °C/min and frozen down to −40 °C. The frozen sample was freeze-dried overnight at $15-5 \times 10^{-3}$ Bar and at −40 °C in a Labconco Freeze-Dry System, Freezone 4.5, without any protecting medium (*10*). The same procedure was followed for free *S. cerevisiae* cells.

Repeated Batch Fermentations at Low Temperatures. Wort was obtained from the Athenian Brewery S. A., hopped, filtered, and sterilized. The pH of the wort was 5.0 and the °Be density was fixed to 6.3−6.5. The values of the percent original extract of the wort are shown later in Table 2. For the first fermentation batch at each temperature (15, 10, 5, and 0 °C), 300 g of wet immobilized biocatalyst was freeze-dried. The immobilized cells on gluten pellets were freeze-dried

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Table 1. Kinetic Parameters Obtained in the Repeated Batch Fermentation of Wort, at Various Temperatures (0−15 °C), with Freeze-Dried Immobilized Biocatalyst, Wet Immobilized Biocatalyst, and Freeze-Dried Free Cells

temperature (°C)	fermentation time (h)	residual sugar (g/L)	ethanol concentration (% v/v)	ethanol productivity (g/L/day)	beer productivity (g/L/day)
			freeze-dried immobilized biocatalyst		
15	137	0.7	5.2	7.2	66.6
10	177	2.0	5.5	6.1	53.3
5	1667	0.8	5.2	0.6	5.5
0	1800	1.6	5.0	0.6	5.1
			wet immobilized biocatalyst		
15	62	0.3	5.6	20.8	177.5
10	110	1.9	5.4	9.4	83.4
5	750	0.3	5.6	1.4	12.2
0	1033	1.7	5.1	1.0	9.0
			freeze-dried free cells		
15	142	0.9	5.5	7.5	65.6
10	180	1.1	4.8	5.1	51.1
5	1899	3.6	5.2	0.6	5.9
0	3226	3.9	4.9	0.3	2.9

without any protecting medium. The absence of protecting medium has practical importance because we avoid the risk of a residue from the protecting medium in the final fermented product, which may affect beer quality. The freeze-dried immobilized cells on gluten pellets were hydrated for about 1 h, after which they were introduced into 250 mL of wort in a glass cylinder and used for fermentation. This is an amount equivalent to ~16.8 g of free cells/L. 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. After that, the biocatalyst was used for the second fermentation batch, and so on. In total, three repeated batch fermentations were performed at each temperature without agitation. The average values of all kinetic parameters at each temperature are presented later in the Results section in tabular form. The liquid was collected and immediately put under refrigeration (−20 °C) to avoid loss of volatiles and occurrence of side reactions until it was analyzed.

To compare the fermentation times and other fermentation parameters obtained in the presence of the freeze-dried immobilized cells on gluten pellets with those of wet immobilized cells and freeze-dried free cells, similar runs were carried out simultaneously with the same cell concentration (16.8 g pressed yeast cells per liter).

Methods of Analysis. Alcoholic degrees were obtained by means of gas chromatography (GC) and high-pressure liquid chromatography (HPLC). The alcoholic degree was determined from the average of these two values, which were very close. From the final ethanol concentration we were able 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 considering that beer density is equal to 1 g/L. Residual sugar was determined by the HPLC method using a SHIMADZU LC-9A liquid chromatograph. Column Shim-pack, SCR-101 N, mobile phase three times distilled and filtered water, and *n*-butanol as an internal standard were used. The temperature of column was 60 °C with a flow rate of 0.8 mL/min and refractive index detector was employed. Apparent extract (%w/w), polyphenols (mg/L), and diacetyl content (mg/L), as well as bitterness (EBU), color (EBC), and refractive index (at 20 °C) were determined in decarbonated and paper-filtered beer samples by the European Brewery Convention (EBC) methods of analysis (*11*). Original and real extract were determined from the nomogram furnished by the Athenian Brewery S. A. Wet free cell concentrations were determined by measurement of the optical density at 700 nm (*12*, *13*) and are given in grams of wet weight per liter, as determined with standard curves. Volatile byproducts were determined by means of gas chromatography using a Shimadzu GC-8A gas liquid chromatograph with a stainless steel column packed with Escarto-5905 consisting of Squalene 5%, Carbowax-300 90%, and di-2-ethyl-hexyl sebacate 5% v/v, with N_2 as the carrier gas (20 mL/min), and an FID detector (*14*). The injection port and detector temperatures were 210 °C, and the column temperature was 70 °C. The internal standard was butanol-1 at a concentration of 0.1% v/v. Samples of 4 μL of beer were injected directly in the column and the concentrations of the above compounds were determined using standard curves. All values were the mean of three repetitions.

Preliminary Taste Test. As we have written, the beers produced by freeze-dried immobilized cells, wet immobilized cells, and free freeze-dried cells were collected and immediately put under refrigeration (−20 °C). The beers had been brewed at temperatures 15, 10, 5, and 0 °C. They were not carbonated and were tasted by 5 trained testers after about a month of refrigeration. The evaluation was performed on a 0−10 scale. The average preferences of the 5 tasters vs the three types of fermentations are presented later in tabular form.

RESULTS AND DISCUSSION

Fermentation for Brewing by Freeze-Dried Immobilized Cells. The freeze-dried immobilized cells on gluten pellets can be recovered at each low temperature of 15, 10, 5 and 0 °C, adapted and ferment wort in a period of 5.7 days at 15 °C up to 75 days at 0 °C (Table 1). Traditional bottom fermentation takes 8−10 days. To compare the fermentation times and other parameters obtained in the presence of the freeze-dried immobilized cells on gluten pellets with those of wet immobilized cells on gluten pellets and freeze-dried free cells, similar runs were carried out simultaneously with the same initial cell concentration (16.8 g/L). Comparing the fermentation times for freeze-dried immobilized cells with those of freeze-dried free cells it is obvious that the fermentation time in the first case is lower mainly at temperatures of 5 and 0 °C. The freeze-dried immobilized cells on gluten pellets retained their shape without any shrinking after freeze-drying as we noted in a previous study (*10*). Beers produced by freeze-dried immobilized cells contained comparable alcohol to those produced by wet immobilized cells and freeze-dried free cells, but ethanol productivity and beer productivity were decreased: immobilized cells>freeze-dried immobilized cells>freeze-dried free cells. Just after their production, the beers were preserved at −20 °C until they were analyzed, so no biochemical or chemical change could take place. The residual sugar of beers produced by freeze-dried immobilized cells was in the range of 0.7 to 2.0 g/L and this shows that the freeze-dried biocatalyst is very active and suitable for brewing.

At all studied temperatures the diacetyl concentrations in these beers were lower than 1 mg/L, and the

Table 2. Characteristics of Beer Obtained in the Repeated Batch Fermentation of Wort, at Various Temperatures (0−15 °C), with Freeze-Dried Immobilized Biocatalyst, Wet Immobilized Biocatalyst, and Freeze-Dried Free Cells

temp. (°C)	% original extract	% real extract E_r	% apparent extract E_a	refractive index R_o	color (EBC)	polyphenols (mg/L)	diacetyl (mg/L)	bitterness (EBU)
				freeze-dried immobilized biocatalyst				
15	10.3	3.4	1.7	1.3418	10.0	145.5	0.4	6.1
10	10.7	3.3	1.6	1.3409	9.8	131.4	0.3	6.3
5	11.2	3.1	1.8	1.3408	9.2	124.4	0.4	3.5
0	11.2	3.3	2.1	1.3433	7.7	83.7	0.5	2.3
				wet immobilized biocatalyst				
15	11.3	3.2	1.2	1.3398	9.3	145.0	0.3	5.4
10	11.0	3.3	1.4	1.3410	9.3	141.2	0.3	6.3
5	14.0	3.3	1.3	1.3413	8.3	99.5	0.6	3.8
0	10.5	3.4	2.1	1.3420	7.8	55.9	0.6	2.5
				freeze-dried free cells				
15	10.3	3.5	1.9	1.3447	17.7	236.2	0.5	27.5
10	11.1	3.4	1.6	1.3483	19.7	229.4	1.0	18.4
5	9.2	3.8	2.5	1.3436	22.6	218.6	0.6	22.2
0	9.4	3.8	2.5	1.3460	20.4	219.6	0.9	19.5

Table 3. Volatile Byproducts in Beer Produced by Repeated Batch Fermentations of Wort at Various Temperatures with Freeze-Dried Immobilized Biocatalyst

temperature (°C)	acetaldehyde (mg/L)	ethyl acetate (mg/L)	propanol-1 (mg/L)	isobutanol (mg/L)	amyl alcohols (mg/L)	total volatiles (mg/L)	ethanol (% v/v)
15	17.1	29.2	26.0	43.6	129.1	245.0	5.2
10	21.0	28.4	21.4	31.1	111.0	212.9	5.5
5	24.0	26.4	17.8	25.3	72.3	165.8	5.2
0	14.2	22.1	9.8	21.8	67.1	135.0	5.0

polyphenol concentrations were less than 145.5 mg/L (Table 2) as compared to 190−250 mg/L in commercial beers. The polyphenol concentrations were decreased as the temperature was decreased, as happened in beers brewed using wet immobilized cells. The polyphenol concentrations in these beers were ∼38−61% of those contained in beers brewed using freeze-dried free cells. The free-cell concentration in beers produced by immobilized cells was very low: less than 3 g/L in some cases and less than 2 g/L in most cases. High concentration of immobilized yeast has been attained, implying low cell growth. The beers had a very good clarity after the end of the primary fermentation, with low concentrations of polyphenols and little cell growth. Therefore, no filtration was necessary for beer clarification. Beers prepared with cells immobilized on gluten pellets were characterized by good clarity, especially at temperatures below 10 °C. The removal of some proteins and polymerized polyphenols is likely to improve beer stability. The bitterness of the beers produced by freeze-dried immobilized cells on gluten pellets was low (in the range of 2.3−6.3 EBU) and in the same range as those in beers produced by wet immobilized cells on gluten pellets. Bitterness of the beers produced by freeze-dried free cells is much higher than that of freeze-dried immobilized cells. In all cases bitterness is reduced by the drop of temperature. For freeze-dried immobilized cells, bitterness at 0 °C was only 40% of that at 15 °C. For commercial beers, bitterness varies from 10 to 40 EBU, and can be increased further by the addition of hop extracts. Beers produced by freeze-dried immobilized cells on gluten pellets had a yellow color in the range 7.7−10.0 EBC. The level was the same as in beers produced by wet immobilized cells on gluten pellets and lower than those in beers produced by freeze-dried free cells.

Volatile Byproducts. *Higher Alcohols.* Table 3 shows that the amounts of higher alcohols in beers produced by freeze-dried immobilized cells on gluten pellets were reduced as the temperature was decreased. This result is in agreement with previous study (*15*) . Amyl alcohols, propanol-1, and isobutanol in fermentations conducted at 0 °C were about half of those at 15 °C. This reduction of higher alcohols leads to improvement of quality and nutritional value because these byproducts contribute to off-flavor and have toxicity. Likewise, percentages of amyl-alcohols on total volatiles determined were reduced by the drop of temperature. This reduction is also an indicator for the improvement of quality and nutritional value by the reduction of temperature.

Table 4. Taste Test Results of Beers Obtained in the Batch Fermentation of Wort with Freeze-Dried Immobilized Biocatalyst (+) as Compared with that of Free Freeze-Dried Cells (Δ) and Wet Immobilized Biocatalyst (□)

scale and characteristics		testers 1	2	3	4	5
fine	10					
excellent	9					
very good	8	+□	+□	+□	+□	+□
good	7					
might be good	6					
medium	5	Δ	Δ		Δ	
acceptable	4			Δ		Δ
might be bad	3					
bad	2					
very bad	1					
unsuitable	0					

Acetaldehyde. Acetaldehyde concentrations in beers produced from batch fermentations using freeze-dried immobilized cells were in the range 14.2−24.0 mg/L.

Ethyl Acetate. Ethyl acetate is the major ester in beers but other esters also contribute to the overall flavor of beer. The ethyl acetate concentration in beers produced by freeze-dried immobilized cells was in the range 22.1−29.2 mg/L. These concentrations obtained by freeze-dried immobilized cells are about in the same

Table 5. Kinetic Parameters Obtained in the Repeated Batch Fermentation of Wort, at 15 °C, with Freeze-Dried Immobilized Biocatalyst and Some Characteristics of the Obtained Beers

month	repeated batch fermentations	fermentation time (h)	residual sugar (g/L)	ethanol concentration (% v/v)	ethanol productivity (g/L/day)	beer productivity (g/L/day)	% original extract	% real extrac E_r	% apparent extract E_a	refractive index R_o	color (EBC)	polyphenols (mg/L)	diacetyl (mg/L)	bitterness (EBU)
1	1	130	0.1	5.3	7.7	69.9	10.7	3.4	1.6	1.3426	9.8	138.4	0.6	6.2
1	2	150	1.9	4.9	6.1	60.6	9.6	3.6	2.2	1.3412	10.1	145.3	0.3	6.0
1	3	130	0.1	5.4	7.9	69.9	10.7	3.3	1.6	1.3416	10.0	152.8	0.3	6.0
2	8	120	0.9	5.4	8.6	75.8	10.9	3.3	1.5	1.3420	10.0	149.6	0.2	6.0
3	15	105	1.1	5.1	9.2	86.6	10.5	3.4	1.7	1.3425	9.9	158.7	0.2	6.0
8	32	130	1.6	5.1	7.5	69.9	10.7	3.4	1.7	1.3430	9.8	150.5	0.4	6.1
12	45	150	2.2	5.2	6.6	60.6	9.9	3.6	2.1	1.3428	9.8	146.7	0.4	5.8
14	56	150	2.6	4.8	6.0	60.6	9.8	3.8	2.2	1.3440	9.6	132.1	0.5	5.8

levels as those obtained by wet immobilized cells (*7*). Furthermore, ethyl acetate concentrations in beers from free freeze-dried cells were in much lower levels.

Percentages of ethyl acetate on total volatiles determined were increased as the temperature was decreased. This happened for freeze-dried immobilized cells, wet immobilized cells, and free freeze-dried cells. This increase improves the flavor of beers as the temperature is reduced.

Organoleptic Quality of Beer. Beer produced by freeze-dried immobilized cells and wet immobilized cells had a better organoleptic quality than those produced by freeze-dried free cells (Table 4). Beers from freeze-dried immobilized cells had a fine clarity, whereas those of free cells had bad clarity. This gives the possibility of avoiding or reducing time of maturation. Beers produced by freeze-dried immobilized cells at temperatures 0−10 °C had a pleasant bread flavor and fruity aroma, but those produced at the traditional temperature of 15 °C did not. These results at low temperatures comply with the increase of percentages of ethyl acetate and decrease of amyl alcohols as the temperature was reduced (Table 3).

The preliminary taste test characterized the young beers produced by freeze-dried immobilized cells as sweet, with a pleasant, soft, banana aroma and taste, with body and after taste. The same organoleptic characteristics have been noticed for beers produced by wet immobilized cells on gluten pellets (*16*). The aroma and taste of young beers were more intense in beers produced at low temperatures. The aroma and taste remained stable for about 1 year of storage, but after this time began to weaken.

Operational Stability of the Freeze-Dried Immobilized Biocatalyst in a Fermentation System. We also examined the operational stability of the freeze-dried immobilized cells on gluten pellets for 56 repeated batch fermentations of wort performed in a period of 14 months (Table 5). The fermentation time was decreased up to the fifteenth fermentation batch and after that it started to be increased until it was stabilized to 150 h. Ethanol concentrations in the produced beers were within commercial levels (3.5−5.5% v/v) and residual sugar concentrations were very low. As regards diacetyl concentrations, they were less than 0.6 mg/L, a noteworthy result for primary fermentation. As we can see in Table 5, diacetyl concentration was decreased up to the fifteenth fermentation batch and after that it was increased. This result in combination to the change of the fermentation time and residual sugar shows a little loss of activity of the biocatalyst but remains clearly acceptable for application. Polyphenol concentrations were fluctuated in low levels, and original, real, and apparent extract were within commercial levels (7.5−12% w/w, 1.3−5% w/w, and 1.5−3.3% w/w, respectively).

Generally, we can say that the freeze-dried immobilized biocatalyst retains its operational stability for a long fermentation period with noteworthy results. This result, in addition to the relatively constant chemical composition of beer during this long period, makes the new biocatalyst attractive for a decision to scale-up the process. Likewise, the possibility to produce beer at low temperatures, improving the organoleptic quality, nutritional value, and clarity just after fermentation, is an additional factor that contributes to a scale-up of brewing by freeze-dried immobilized cells. This is strengthened further by the abundance of gluten in nature, its low cost, and its food-grade purity.

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