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0273-2289/02/97/0105/$14.25

Low-Temperature Brewing by Freeze-Dried Immobilized Cells

Argyro Bekatorou, Magda J. Soupioni, Athanasios A. Koutinas, and Maria E. Kanellaki*

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

Received December 29, 2000; Revised May 23, 2001; Accepted May 25, 2001

Abstract

We propose a novel biocatalyst in brewing. A cryotolerant strain of *Saccharomyces cerevisiae* was immobilized on delignified cellulosic material followed by freeze-drying of the immobilized cells without the use of any cryoprotectant. The freeze-dried immobilized biocatalyst was used in repeated-batch fermentation of wort and showed reduced fermentation time and increased productivities as compared with free freeze-dried cells (FFDCs). It also demonstrated suitability for low-temperature brewing (5 and 0°C). The fermentation time in repeated-batch fermentations at 15°C was 1.5–2 d for a period of 13 mo, showing a high operational stability of the system. At 0°C the freeze-dried immobilized biocatalyst showed a 2- to 3.5-fold decrease in fermentation time in comparison with FFDCs. Polyphenol contents, bitterness, and diacetyl concentration were lower in beers produced by freeze-dried immobilized cells as compared with FFDCs. At 0°C polyphenols were 40% lower than at 15°C. Higher alcohols were reduced and ethyl acetate increased in comparison with FFDCs. Amyl alcohols at 0°C were lower than half of their content at 15°C, while ethyl acetate was 31 mg/L at 0°C and 18 mg/L at 15°C. These data justify the improved aroma and taste of beers produced by freeze-dried immobilized biocatalyst mainly at low temperatures.

Index Entries: Brewing; freeze drying; immobilized cells; low temperature; fermentation.

Introduction

Freeze-drying (or lyophilization) is a good preservation method for foods as well as for microorganisms. Cryotolerant and ethanol-resistant

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

yeast cells, immobilized on food-grade purity supports, such as gluten pellets and delignified cellulosic (DC) material, produced wine and beer of improved taste and aroma with low-temperature fermentation and resulted in relatively higher productivities *(1–4)*. Although researchers have made efforts in the past to utilize immobilized cells in brewing *(2,4,5–18)*, the industrialization of the process has not yet been achieved. Even if such a process is developed, small enterprises would have problems with the handling of cell immobilization in the factory. However, these problems could be resolved by the development of new enterprises that would produce the biocatalyst through freeze-drying of immobilized cells. To industrialize cell immobilization in brewing, the support must satisfy prerequisites such as food-grade purity, abundance in nature, low cost, and ease of handling in industry. In addition, the support must be a heavy material and not a gel to avoid easy destruction of the biocatalyst. The DC material satisfies these requirements and wet immobilized cells on this material have been used in low-temperature brewing, producing beer of fine quality *(2–4)*. For the use of such an immobilized yeast biocatalyst, or different immobilization techniques for successful industrial applications *(16,17,19,20)*, it is estimated that freeze-drying is a valuable method of supplying preserved and marketable ready-to-use immobilized cells. Iconomopoulou et al. *(21)* has freeze-dried cells immobilized on DC material without using protecting media and by using a proper cooling rate of the cells during the cooling phase of the process. Using the freeze-dried immobilized biocatalyst, they have performed successful experiments for glucose anaerobic fermentation to alcohol production. However, for industrial application, further research is needed in order to prove the suitability of this method.

Therefore, the aims of the present study were to perform low-temperature brewing using freeze-dried immobilized cells and to examine the parameters that lead to industrialization of a process using biocatalyst supported by freeze-dried DC material.

Materials and Methods

The biocatalyst was prepared by immobilizing the AXAZ-1 strain of *Saccharomyces cerevisiae* species on DC material as described previously *(1)* and then freeze-drying the immobilized biocatalyst.

AXAZ-1, an alcohol-resistant and cryotolerant *S. cerevisiae* strain isolated from the Greek agricultural area *(22)*, was grown on culture medium containing 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 *(1)*.

In brief, the DC material was prepared with lignin removal from sawdust after treatment with 1% sodium hydroxide solution.

The fermentations were performed with wort obtained by the Athenian Brewery S.A., hopped, filtered, and sterilized. The pH of the wort was 5.0–5.1 and the °Be density was 7.5–7.9. The percentages of the original extract of the wort are shown in Table 1.

Table 1
Characteristics of Beer Obtained in Repeated-Batch Fermentation of Wort (14 °Plato), at Various Temperatures, with Freeze-Dried Immobilized Biocatalyst

Temperature (°C)	Original extract (%)	Real extract, E_r (%)	Apparent extract, E_a (%)	Refractive index, R_o	Color (EBC)	Polyphenol (ppm)	Diacetyl (ppm)	Bitterness (EBU)
15	10.8	3.4	1.6	1.3435	11.9	167	0.3	8.3
15	10.7	3.3	1.6	1.3428	19.5	162	0.4	10.2
15	11.6	3.1	1.1	1.3434	15.6	190	0.2	13.0
10	8.1	4.1	3.1	1.3420	11.3	122	0.3	7.1
10	10.8	3.3	1.5	1.3415	8.2	111	0.3	5.5
10	9.8	3.6	2.1	1.3416	9.9	127	0.3	7.7
10	10.8	3.3	1.5	1.3415	11.3	127	0.3	9.4
10	10.8	3.3	1.5	1.3406	10.2	119	0.2	8.3
5	8.7	3.9	2.7	1.3406	9.2	92	0.8	10.8
5	9.0	3.8	2.5	1.3400	11.6	103	0.4	11.1
5	9.7	3.6	2.2	1.3412	10.5	105	0.4	11.4
0	8.6	3.9	2.8	1.3420	8.5	98	1.2	7.8
0	8.9	3.8	2.6	1.3405	7.7	93	0.4	10.2
0	9.7	3.6	2.2	1.3415	10.2	100	0.4	11.4

Freeze-Drying

After immobilizing the AXAZ-1, the biocatalyst was cooled in a Biocool Freezer, with methanol as freezing liquid, at a cooling rate of 3°C/min and frozen down to –40°C. The frozen sample was freeze-dried overnight at 15–5.10^{-3} bar in a Labconco Freeze Dry System, Freezone 4.5. No protecting medium was used during the freeze-drying *(21)*. The same procedure was followed for free cells of the strain AXAZ-1.

Fermentations

For the first fermentation batch at each temperature (15, 10, 5, and 0°C), 170 g of wet immobilized biocatalyst was freeze-dried. The immobilized cells on DC material were freeze-dried without any protecting medium. The absence of protecting medium has practical importance because it avoids the risk of a residue from the protecting medium in the final fermented product, which may affect its quality. The freeze-dried immobilized cells were introduced into 400 mL of wort contained in a 1-L glass cylinder. This is an amount equivalent to ~20.83 g/L of free cells, considering that 100 g of the DC material carries 4.9 g of immobilized cells as determined in a previous study *(1)*. 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 then used for the second fermentation batch and so on. In total three (five at 10°C) repeated-batch fermentations were performed at each temperature without agitation. Table 2 presents some kinetic parameters at each temperature. The liquid was collected and immediately refrigerated (–20°C) to avoid loss of volatiles and occurrence of side reactions until it was analyzed.

To compare kinetic parameters obtained by freeze-dried immobilized biocatalyst with those of free freeze-dried cells (FFDCs), similar runs were carried out simultaneously with the same cell concentration.

Operational Stability of System

Fifty-eight repeated-batch fermentations of wort at 15°C were carried out within a period of 13 mo, using in the first batch an amount of freeze-dried immobilized biocatalyst in order to study the operational stability of freeze-dried immobilized cells in brewing. In particular, 106.3 g of freeze-dried immobilized biocatalyst was introduced into 250 mL of wort (14 °Plato) contained in a 1-L glass cylinder. This is an amount of immobilized cells equivalent to ~20.83 g/L of free cells. The fermentations were carried out without agitation, as described in the previous paragraph. The average values of all kinetic parameters obtained in the 58 repeated batch fermentations are presented in Table 3.

Table 2
Kinetic Parameters Obtained in Repeated-Batch Fermentation of Wort (14 °Plato) at Various Temperatures with Freeze-Dried Immobilized Biocatalyst

Temperature (°C)	Repeated fermentation batches	Fermentation time (h)	Total carbohydrates (g/L)	EtOH concentration (% [v/v])	EtOH productivity (g/[L·d])	Beer productivity (g/[L·d])
15	1	130	0.1	6.2	9.0	140.7
15	2	100	0.1	6.7	12.7	182.9
15	3	95	0.1	6.0	12.0	192.6
10	1	216	4.4	5.3	4.7	84.7
10	2	166	1.4	6.0	6.9	110.2
10	3	116	2.1	5.8	9.5	157.7
10	4	120	2.3	6.3	10.0	152.4
10	5	98	2.3	5.2	10.1	186.7
5	1	1894	4.5	6.0	0.6	9.7
5	2	832	3.4	6.1	1.4	22.0
5	3	780	4.0	6.3	1.5	23.5
0	1	1824	5.0	4.6	0.5	10.0
0	2	1584	2.9	4.9	0.6	11.5
0	3	720	0.3	5.1	1.3	25.4

Table 3
Kinetic Parameters Obtained in Repeated-Batch Fermentation of Wort (14 °Plato), at 15°C, with Biocatalyst Supported by Freeze-Dried DC Material

Month	Repeated fermentation batches	Fermentation time (h)	Total carbohydrates (g/L)	EtOH concentration (% [v/v])	EtOH productivity (g/[L·d])	Beer productivity (g/[L·d])
1	1	80	0.8	5.9	17.9	230.8
3	11	45	1.5	5.8	24.5	410.3
4	19	55	0.6	5.2	18.0	335.7
5	26	35	1.0	6.7	36.4	527.5
6	32	35	1.0	7.0	38.1	527.5
7	39	40	0.3	5.1	24.4	461.5
12	52	50	0.5	5.0	18.8	369.2
13	58	40	0.8	5.1	24.3	461.5

Analysis of Residual Sugar, Ethanol, and Characteristic Parameters of Beer

Degrees of alcohol were obtained by means of gas chromatography and high-pressure liquid chromatography (HPLC). The degree of alcohol 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 of 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. A 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 the column was 60°C with a flow rate of 0.8 mL/min, and a refractive index detector was employed. Apparent extract (% [w/w]), polyphenols (milligrams/liter), and diacetyl content (milligrams/liter), as well as bitterness (EBU), color (EBC), and refractive index (at 20°C) were determined in decarbonated and paper-filtered beer samples by the EBC methods of analysis *(23)*. Original and real extract percentages were determined from the nomogram furnished by the Athenian Brewery S.A. Wet free-cell concentrations were determined by the absorbance experimental procedure *(24,25)* and are given in grams of wet weight per liter, as determined with standard curves.

Determination of Volatile Byproducts

The effect of temperature on volatile byproducts in beers produced by freeze-dried immobilized cells, wet immobilized cells, FFDCs, and wet free cells was determined in all performed fermentations.

Acetaldehyde, ethyl acetate, propanol-1, isobutyl alcohol, and amyl alcohols 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 5% Squalene, 90% Carbowax-300, and 5% (v/v) di-2-ethyl-hexyl sebacate, with N_2 as the carrier gas (20 mL/min) and a flame ionization detector (FID) *(26)*. The injection port and detector temperatures were 210°C and the column temperature was 70°C. The internal standard was *n*-butanol at a concentration of 0.1% (v/v). Samples of 4 µL of beer were injected directly into the column, and the concentrations of the aforementioned compounds were determined using standard curves. All values were the mean of three repetitions.

Methanol was also determined by a Shimadzu GC-8A Gas Liquid Chromatograph using Porapac-S as column material, N_2 as the carrier gas (20 mL/min), and an FID. The injection port and detector temperatures were 210°C and the column temperature was programmed between 140 and 180°C. *n*-Butanol was used as the internal standard, and 2-µL samples of beer were injected directly into the column.

Results

Taking into account that the industrialization of immobilized cells, except the criteria presented in the Introduction, requires the development of a marketable biocatalyst, freeze-dried immobilized cells were examined regarding their productivity and other kinetic parameters in low-temperature brewing. To examine the quality of beer produced by freeze-dried immobilized cells and the effect of temperature on it, characteristics of beer produced at various temperatures were also analyzed. To compare the beer produced by freeze-dried immobilized cells with that by FFDCs, experiments were performed in parallel. The results are summarized in Tables 1, 2, 4, and 5 and Fig. 1.

Table 2 shows the kinetic parameters in wort fermentations performed by freeze-dried immobilized cells at various temperatures. The first fermentation batch was performed by freeze-dried immobilized biocatalyst at each temperature (15, 10, 5, 0°C) and repeated-batch fermentations followed at each temperature. At 15°C, which is the fermentation temperature of traditional brewing, the fermentation time was in the range of 4–5.5 d. This is considered to be lower than that obtained in industrial brewing, when taking into account the relatively high ethanol concentrations obtained in these experiments. At 10°C, which is considered a low temperature in brewing, the fermentation time, productivities, and ethanol contents were about the same as those at 15°C. At 5 and 0°C, the fermentation time became less than 79 and 76 d, respectively, which can be considered acceptable by the industry for the production of beers at very low temperatures.

The effect of temperature on fermentation kinetics for freeze-dried immobilized biocatalyst and FFDCs are presented in Fig. 1. As the temperature decreased, the freeze-dried immobilized biocatalyst gave better results as compared with FFDCs. Fermentation times and productivities for the freeze-dried immobilized biocatalyst were improved as compared with those obtained by FFDCs (Table 4). For both immobilized and free cells, the ethanol and beer productivities were improved in the successive batches at each temperature, mainly owing to the reduction in the fermentation time and the about constant or, in many cases, higher alcohol contents (Tables 2 and 4).

Total carbohydrates (residual sugars) in beers were found in the range of 0.1–5.0 g/L, and the alcohol content in most cases was 5.1–6.3% (v/v). The alcohol content is usually regarded as a measure of beer strength and lies in the range of 3.5–5.5% (v/v) for most commercial products. The alcohol content at temperatures higher than 5°C was increased by 20% as compared with that at 0°C for the freeze-dried immobilized biocatalyst. As shown in Table 1, the original extract, real extract, and apparent extract of beers obtained by the freeze-dried immobilized biocatalyst were in the ranges of most commercial beers. Bitterness in beers obtained by freeze-dried immobilized biocatalyst was low, in the range of 5.5–13.0 EBU while that for FFDCs was in the range of 13.2–39.8. Probably the cellulosic mate-

Table 4
Kinetic Parameters Obtained in Repeated-Batch Fermentation of Wort (14 °Plato) at Various Temperatures with FFDCs

Temperature (°C)	Repeated fermentation batches	Fermentation time (h)	Total carbohydrates (g/L)	EtOH concentration (% [v/v])	EtOH productivity (g/[L·d])	Beer productivity (g/[L·d])
15	1	166	2.3	5.5	6.3	141.7
15	2	116	0.2	5.4	8.8	202.8
15	3	143	0.2	5.7	7.6	165.8
10	1	208	1.1	5.0	4.5	114.0
10	2	168	1.0	4.6	5.2	141.2
10	3	164	1.3	4.7	5.5	144.6
5	1	2900	4.1	5.0	0.3	8.2
5	2	1856	3.6	5.1	0.5	12.8
5	3	940	3.2	5.5	1.1	25.2
0	1	4098	6.0	5.2	0.2	5.8
0	2	3016	3.2	4.7	0.3	7.9
0	3	2564	2.6	4.7	0.3	9.2

Table 5
Characteristics of Beer Obtained with FFDCs, in Fermentation of Wort (14 °Plato), at Various Temperatures

Temperature (°C)	Original extract (%)	Real extract, E_r (%)	Apparent extract, E_a (%)	Refractive index, R_o	Color (EBC)	Polyphenol (ppm)	Diacetyl (ppm)	Bitterness (EBU)
15	9.2	3.9	2.6	1.3465	16.1	195	0.3	39.8
15	10.8	3.4	1.6	1.3435	14.9	250	0.4	21.7
15	11.0	3.3	1.5	1.3440	22.2	263	0.8	21.1
10	11.1	3.5	1.7	1.3530	17.3	226	0.9	14.0
10	11.2	3.4	1.6	1.3495	21.0	230	1.0	22.0
10	10.9	3.3	1.5	1.3425	20.7	232	1.0	19.3
5	8.6	4.0	2.9	1.3430	21.9	227	0.7	19.2
5	9.3	3.8	2.5	1.3440	22.2	217	0.5	23.8
5	9.8	3.7	2.2	1.3438	23.6	212	0.4	23.6
0	9.1	4.1	2.9	1.3507	23.3	229	1.1	13.2
0	9.3	3.8	2.5	1.3444	18.8	199	0.9	25.7
0	9.9	3.6	2.1	1.3429	19.1	231	0.6	19.6

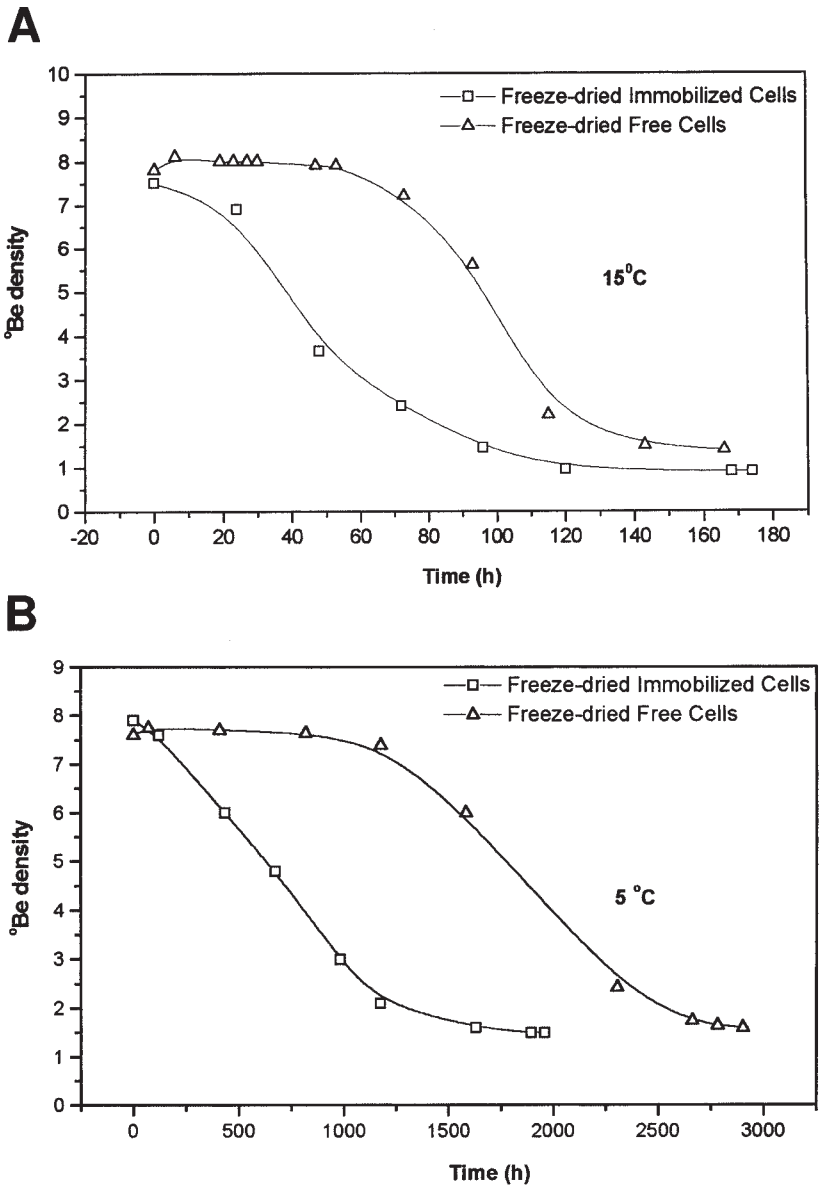

Fig. 1. Fermentation kinetics observed in the fermentation of wort at **(A)** 15 and **(B)** 5°C with freeze-dried immobilized cells on DC material compared with FFDCs.

rial adsorbed a part of the hop-bittering substances. This reduction in bitterness was also found in the case of wet immobilized cells *(2)*. The values of color are quantitatively represented in Table 1 and range between 7.7 and 19.5 EBC as for most commercial beers.

At all temperatures studied, the diacetyl concentrations were mainly in the range of 0.2–0.4 ppm (Table 1). In comparison with fermentations

performed by FFDCs (Table 5), the diacetyl contents for freeze-dried immobilized biocatalyst were lower. At all temperatures, the polyphenol concentrations were <167 ppm (Table 1), as compared with 190–250 ppm in commercial beers. Polyphenol contents were continuously decreased as the temperature was reduced. The polyphenol concentration at 0°C was 40% lower than that at 15°C. No significant reduction was observed for FFDCs. Polyphenol contents in beers obtained by freeze-dried immobilized biocatalyst were about half the values obtained by FFDCs (Table 5). Such a reduction was also observed in beers produced by wet immobilized cells compared with beers produced by wet free cells *(2)*. Probably the DC material adsorbs a part of polyphenols since their concentration in beers produced by free cells is higher, especially at low temperatures, than that in beers produced by wet immobilized cells or freeze-dried immobilized ones. The beers produced by freeze-dried immobilized cells acquired an important clarity just after the end of fermentation avoiding further filtration to clarify the beer. The clarity was improved as the temperature was reduced and resembled the clarity of beers obtained by employing wet immobilized cells *(2)*. The clarity of beers produced by freeze-dried immobilized cells justifies the low contents of polyphenols and their reduction with the drop in temperature.

An important parameter for the industrialization of immobilized cells is the high operational stability of the biocatalyst, which was examined for the freeze-dried immobilized cells. Fifty-eight repeated-batch fermentations were performed using in the first fermentation batch freeze-dried immobilized cells, and the results are presented in Tables 3 and 6. Table 3 shows that 58 repeated batch fermentations were performed within a period of 13 mo. The fermentation time was decreased up to the thirty-second fermentation batch for a fermentation period of 6 mo, and then it was increased and stabilized to ~45 h. Because the fermentation time is a main factor for the determination of ethanol and beer productivity, these change reversibly to the fermentation time change, but an alcohol concentration higher than 5% (v/v) and important ethanol and beer productivities were obtained. Polyphenol contents were low and lower than that for FFDCs for the long period of 13 mo. Diacetyl concentration and bitterness were also low.

The preliminary taste test characterized the new beers as having a pleasant aroma, fruity taste, body, and aftertaste, and in general were about similar to beers produced by wet immobilized cells on DC material. The aroma and taste of the beers were improved as the temperature was decreased. This improvement can be identified by the low concentrations of off-flavor compounds such as amyl alcohols and other higher alcohols (isobutanol and propanol-1) and by the increase in ethyl acetate (Table 7). The percentages of ethyl acetate and amyl alcohols on total volatile byproducts, for freeze-dried and wet immobilized cells immobilized cells were similar (Fig. 2). The percentages of amyl alcohols on total volatile byproducts for freeze-dried and wet immobilized cells were lower than

Table 6
Characteristics of Beer Obtained in Repeated-Batch Fermentation of Wort (14 °Plato), at 15°C, with Biocatalyst Supported by Freeze-Dried DC Material

Month	Repeated fermentation batches	Original extract (%)	Real extract, E_r (%)	Apparent extract, E_a (%)	Refractive index, R_o	Color (EBC)	Polyphenol (ppm)	Diacetyl (ppm)	Bitterness (EBU)
1	1	9.9	3.5	2.0	1.3408	11.9	148	0.3	12.3
3	11	10.4	3.3	1.7	1.3400	12.1	136	0.3	13.7
4	19	9.9	3.5	2.0	1.3406	10.3	158	0.3	13.5
5	26	10.8	3.3	1.5	1.3410	11.6	170	0.3	14.6
6	32	10.7	3.4	1.6	1.3421	9.9	157	0.3	14.5
7	39	10.8	3.3	1.5	1.3410	9.6	169	0.3	12.3
12	52	11.1	3.2	1.3	1.3408	10.8	133	0.5	11.8
13	58	10.5	3.4	1.7	1.3418	13.1	146	0.7	14.2

Table 7
Volatile Byproducts in Beer Produced by Repeated-Batch Fermentation of Wort (14 °Plato) at Various Temperatures with Freeze-Dried Immobilized Biocatalyst

Temperature (°C)	Repeated fermentation batches	Acetaldehyde (mg/L)	Ethyl acetate (mg/L)	Propanol-1 (mg/L)	Isobutanol (mg/L)	Amyl alcohols (mg/L)	Methanol (mg/L)	Total volatiles (methanol excluded) (mg/L)	Ethanol (% [v/v])
15	1	18	20	14	18	76	21	146	6.2
15	2	15	17	18	15	72	22	137	6.7
15	3	14	17	22	15	69	50	137	6.0
10	1	Traces	24	14	9	56	41	103	5.3
10	2	7	26	14	12	50	39	109	6.0
10	3	14	23	15	11	61	45	124	5.8
10	4	13	27	16	12	55	33	123	6.3
10	5	8	37	13	8	56	35	122	5.2
5	1	11	26	6	7	41	39	91	6.0
5	2	10	29	6	7	43	42	95	6.1
5	3	11	29	10	8	44	41	102	6.3
0	1	10	31	8	6	28	45	83	4.6
0	2	10	31	9	5	32	45	87	4.9
0	3	8	32	9	5	34	43	88	5.1

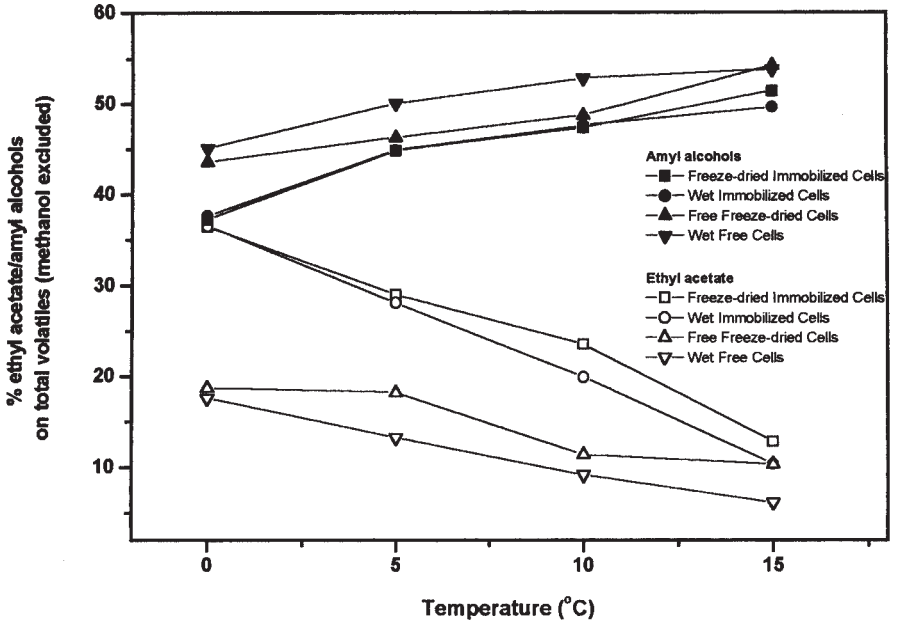

Fig. 2. Percentages of amyl alcohols and ethyl acetate on total volatiles determined in beer produced by freeze-dried immobilized cells, wet immobilized cells, FFDCs, and wet free cells.

those for freeze-dried and wet free cells, whereas the percentages of ethyl acetate on total volatile byproducts were higher for the immobilized cells (Fig. 2). This also justifies the improvement of quality in the cases of freeze-dried immobilized cells by the decrease in temperature.

Discussion

The stability of high productivity, low fermentation time, and high alcohol concentration for a long fermentation period contributes to the industrialization of the freeze-dried immobilized biocatalyst in brewing owing to the reduction in cost (Table 3). The production of beer with improved clarity and a fermentation time of 1.5–2 d, stable for a long time, is attractive. In addition, the stability of beer characteristics leads to constant quality of beer and standardization of the product.

The presented results lead to the conclusion that freeze-dried immobilized cells on DC material can successfully produce beer. This biocatalyst increases the rate of brewing as compared with FFDCs. Even though the fermentation time and productivities differ from those of wet immobilized cells, these parameters still remain high. The freeze-dried immobilized biocatalyst performs low-temperature brewing in the range of 0–10°C with fermentation times acceptable by the industry. This fact in relation to the increased clarity at low temperatures and the improvement in taste and aroma may contribute to the diminution of the maturation time or even

lead to avoidance of maturation during the industrial process. Low-temperature brewing leads to a reduction in the off-flavor higher alcohols as well as polyphenols and diacetyl.

The freeze-dried immobilized biocatalyst is suitable for brewing, but further industrial research is needed through pilot-plant operations to validate this perspective. If this final attempt is successful, the new biocatalyst that will be created will contribute to the industrialization of immobilized cells.

Acknowledgments

We wish to thank the Foundation of State Scholarships (I.K.Y. Athens, Greece) for financial support. We also acknowledge the financial support of this work by the Greek government (General Secretariat of Research and Technology) through the EPET program.

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