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Low temperature brewing using cells immobilized on brewer's spent grains

Nikolaos Kopsahelis, Maria Kanellaki, Argyro Bekatorou *

Food Biotechnology Group, Department of Chemistry, University of Patras, GR-26500 Patras, Greece

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Abstract

A biocatalyst, prepared by immobilization of the psychrotolerant yeast strain *Saccharomyces cerevisiae* AXAZ-1 on delignified brewer's spent grains, was used for brewing at very low temperatures, resulting in beers with fine clarity, excellent quality and mature character after the end of primary fermentation. Fermentation times were low (only 20 days at 0 °C), while ethanol and beer productivities were high, showing suitability of the biocatalyst for very low temperature brewing. Diacetyl (61–167 ppb), 2,3-pentadione (32–109 ppb) and DMS (11–37 ppb) concentrations in all the green beers were low (the lowest values being those found in beers produced at 0–5 °C). GC/GC–MS analysis showed significant quantitative differences in the composition of aroma volatiles, revealing an impact of fermentation temperature on organoleptic qualities. The increased productivities and fine quality of the products, with low vicinal diketone, DMS and amyl alcohol concentrations, may allow elimination of maturation time, resulting in reduction of production and investment costs.

Keywords: Brewing; Brewer's spent grains; Immobilized cells; Volatiles; SPME GC–MS

1. Introduction

The use of immobilized cells in alcoholic beverage production and their potential advantages over free cell systems have been widely studied and reviewed (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004; Margaritis & Merchant, 1984). Research in brewing over the past three decades has focussed on the application of immobilized cells, mainly to facilitate continuous processing, shorten maturation time and consequently reduce production costs. At commercial level, immobilized cell systems have been applied for low-alcohol beer production and in a few cases for secondary beer fermentation (Linko, Haikara, Ritala, & Penttilä, 1998; Margaritis & Merchant, 1984; Moll & Duteurtre, 1979; Nedovic et al., 2001; Ryder & Masschelein, 1985; Yamauchi et al., 1995). Decisive parameters for the application of immobilized cell systems in brewing are the quality, carrier cost, ease of handling and regeneration, operational stability, contamination aspects, as well as the uncertainty of consumers about new technologies and the desire to maintain the traditional character of the product.

Low temperature brewing, using psychrotolerant and ethanol resistant yeasts immobilized on food grade supports, such as gluten pellets, delignified cellulosic materials and pieces of fruits, led to the production of beers with excellent taste and aroma, while ethanol and beer productivities were high, even at extremely low temperatures (0–5 °C). The combination of immobilization and freeze-drying techniques for the preparation of such biocatalysts, was applied successfully, giving a solution to the problem of supplying breweries or wineries with preserved and marketable ready-to-use immobilized cells (Bardi, Koutinas, Soupioni, & Kanellaki, 1996; Bekatorou, Koutinas, Kaliafas, & Kanellaki, 2001; Bekatorou, Koutinas, Psarianos, & Kanellaki, 2001; Bekatorou et al., 2002; Bekatorou, Soup-

* Corresponding author. Tel.: +30 2610 997123; fax: +30 2610 997105.
E-mail address: ampe@chemistry.upatras.gr (A. Bekatorou).

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ioni, Koutinas, & Kanellaki, 2002; Iconomou, Psarianos, & Koutinas, 1995).

Brewer's spent grains (BSG) constitute a low cost, food grade by-product of the brewing industry, produced after the mashing stage. They consist of (% dry weight): dry matter 26, crude fibre 18, digestible fibre 8, crude protein 23, digestible protein 19, pentosans 19, lignin 16, starch and β-glucans 11–12, cellulose 9, lipids 8–9 and total ash 4. They also contain glutamine-rich protein of high biological value (Hough, 1985; Kanauchi & Agata, 1997; Prentice & Refsguard, 1978; Sabbioni, Superchi, & Bonomi, 1995). Therefore, they can be used as an animal feed, rich in protein and fibre. The high moisture, protein and lignocellulosic content have also made them excellent substrates for the cultivation of mushrooms, leading to increase of their nutritional value (Wang, Sakoda, & Suzuki, 2001). During the past 2 years, efforts funded by the European Social Fund (ESF) are being made to upgrade BSG, including the production of biocatalysts based on immobilized cells for alcoholic beverage production, and the hydrolysis of BSG residual starch, using starch-converting fungi. In the second case, the aim is to evaluate the potential use of the treated BSG as carbohydrate substrates for brewer's yeast production or, alternatively, as protein-enriched animal feed. The production of beer using cells immobilized on BSG is a very attractive perspective, because BSG are a natural, abundant and easily available natural cellulosic material, fully compatible with beer. Cells immobilized on BSG have been applied successfully in various brewing processes (Branyik, Vicente, Dostalek, & Teixeira, 2005). The use of delignified BSG as a support for psychrotolerant yeast immobilization, for use in extremely low temperature brewing, and its influence on the volatile composition of the product, have not been reported, and constitute the aim of the present study.

2. Materials and methods

2.1. Yeast strains and media

Saccharomyces cerevisiae AXAZ-1 is an alcohol-resistant and psychrotolerant strain isolated from Greek grapes (Argiriou et al., 1996). It was grown on culture medium consisting of 4 g yeast extract/l, 1 g $(NH_4)_2SO_4$/l, 1 g KH_2PO_4/l, 5 g $MgSO_4 \cdot 7H_2O$/l and 40 g glucose monohydrate/l and harvested at 4000 rpm for 10 min. Media containing the above composition of nutrient salts plus 12% w/v glucose, were used as nutrient source during the cell immobilization procedure. All media were sterilized at 130 °C and at 1–1.5 atm for 10 min. Wort was obtained from the Athenian Brewery S.A., hopped, filtered and pasteurized. The pH of the wort was 5.0 and the density was fixed at 12 °P. BSG were obtained from the Athenian Brewery S.A. They had 80% moisture, 20% dry matter, 2.5 °P remaining extract (washable) and pH 6.0.

2.2. Preparation of the support and immobilization of cells

BSG were used after delignification, which was done according to Bardi and Koutinas (1994). Specifically, 600 g of BSG were mixed with 1600 ml solution of 1% w/v NaOH, and were boiled with continuous stirring. After about 3 h, the delignified BSG (DBSG) were well washed with water, drained and sterilized by autoclaving at 120 °C for 15 min. Cell immobilization on DBSG was carried out by suspending 16 g of *S. cerevisiae* AXAZ-1 cells in 800 ml of glucose synthetic medium (12% w/v), and mixing with 200 g of DBSG. DBSG were kept below the surface of the fermenting liquid, using a suitable net construction. Soft mixing was achieved by the produced CO_2 during fermentation. The system was allowed to ferment for 6–8 h until all sugar was utilized (density < 0.9 °P). The liquid was then decanted and the biocatalyst was washed twice with 400 ml of fresh glucose medium and was used for the following brewing experiments.

2.3. Brewing

An amount of 200 g of the immobilized biocatalyst (*S. cerevisiae* AXAZ-1 cells immobilized on DBSG) was introduced into 400 ml pasteurized wort, with an initial density of 12 °P, in a 1 l glass cylinder. The total bioreactor volume was 650 ml. Repeated fermentation batches were carried out, initially at ambient brewing temperature (15 °C), for the adaptation of cells in wort, and fermentations followed, reducing the temperature of the system successively to 10, 5 and 0 °C. After the end of each batch, the biocatalyst was washed with 200 ml of fresh wort and was used for the next fermentation batch. The produced *green* beers were collected and immediately analyzed for ethanol, residual sugar, free cells, diketones (diacetyl and 2,3-pentadione), dimethyl sulfide (DMS), bitterness, colour and volatile by-products by GC and SPME GC–MS. Samples of the beers produced at all the studied temperatures were tested immediately after preparation (green beers) by 10 non-trained testers (consumers), according to a taste test protocol (Bekatorou et al., 2002; Tsakiris, Sipsas, Bekatorou, Mallouchos, & Koutinas, 2004) and sensory evaluation was also carried out by a trained tester of the brewing industry (Athenian Brewery S.A.).

2.4. Assays

2.4.1. Fermentation analyses

Fermentation kinetics were determined by measuring the °P density at various time intervals. Ethanol and residual sugar were determined on a Shimadzu LC-9A HPLC system. A Shim-pack (SCR-101 N) column, a refractive index detector, three times distilled and filtered water as mobile phase (0.8 ml/min), and 1-butanol (0.05% v/v) as internal standard, were used. Column temperature was 60 °C. Sample dilution was 1% v/v and injection volume was 40 μl. Ethanol productivity was calculated as grammes

of ethanol per litre of liquid volume produced per day. Beer productivity was calculated as grammes of beer per litre of total working volume produced per day. Free cell concentrations (g/l) were determined by measurement of the optical density at 700 nm, using calibration curves. Original, apparent, and real extracts (% w/w), diacetyl (μg/l or ppb), 2,3-pentadione (ppb), DMS (ppb), bitterness (EBU), colour (EBC) and refractive index, were determined in decarbonated and paper-filtered beer samples, according to the EBC methods of analysis (EBC, 1987). The standard deviations were diacetyl ⩽10.2; 2,3-pentadione ⩽9.8; DMS ⩽5.1; bitterness ⩽3.6; colour ⩽ 2.1.

2.4.2. Determination of volatiles

Volatiles were determined by means of gas chromatography on a Shimadzu GC-8A Gas Liquid Chromatograph, with a stainless steel column packed with Escarto-5905, consisting of 5% squalene, 90% Cabowax-300 and 5% bis(2-ethylhexyl) sebacate, with N_2 as the carrier gas (20 ml/min) and a FID detector. The injection port and detector temperatures were 210 °C and the column temperature was 70 °C. The internal standard was 1-butanol (0.1% v/v). Samples of 4 μl of beer were injected directly into the column and the concentrations of the above compounds were determined using both calibration curves and the internal standard method. Ethanol was determined on a Shimadzu GC-8A system, consisting of a column packed with Porapac-S, N_2 as the carrier gas (20 ml/min), and a FID detector. The injection port and detector temperatures were 210 °C and the column temperature was programmed at 140–180 °C, rising by 10 °C/min. 1-Butanol (0.1% v/v) was used as the internal standard and samples of 2 μl of beer were injected directly into the column. The standard deviations were acetaldehyde ⩽0.48; ethyl acetate ⩽4.9; iso-amyl acetate ⩽0.1; ethyl capronate ⩽0.09; 1-propanol ⩽3.5; isobutyl alcohol ⩽3.1; amyl alcohols ⩽9.9.

2.4.3. GC–MS analysis

The volatiles in samples of beers produced by cells immobilized on DBSG, at 15 and 0 °C, were analyzed by means of gas chromatography–mass spectroscopy (GC–MS). Volatiles were isolated by the solid-phase micro-extraction method (SPME). The fibre used for the absorption of volatiles was a 50/30 μm DVB/Carboxen/PDMS StableFlex for manual holder (Supelco, USA). The conditions of headspace-SPME sampling used were as follows: 20 ml sample and 6 g NaCl (saturated solution ~30%) were transferred into a 40 ml screw-capped glass vial with a rubber septum. The contents were stirred for 5 min at 30 °C, and the fibre was then exposed to the headspace for 30 min. The length of the fibre in the headspace was kept constant. Desorption of volatiles took place in the injector of the gas chromatograph in splitless mode, at 250 °C for 5 s. Before each analysis, the fibre was exposed to the injection port for 5 min to remove any volatile contaminants.

GC/MS analysis was performed on a Fisons 8000 series gas chromatograph (Model 8060) coupled to a Fisons MD-800 quadrupole mass spectrometer. Helium was used as carrier gas (1.0 ml/min). Separation of compounds was performed on a DB-WAX column (30 m × 0.25 mm; DF = 0.25 μm). Oven temperature was programmed to rise from 40 to 250 °C with a rate of 4.0 °C/min. It was held at 250 °C for 5 min. The injector, ion source and interface temperatures were set at 250, 200 and 250 °C, respectively. Electron impact mass spectra were recorded at 70 eV ionization energy and in the 30–400m/z mass range. Identification of compounds was done by comparing the retention times and MS data with those of standard compounds and by MS data obtained from Wiley and NIST libraries. Quantitative determination was carried out, based on relative areas, and 2-pentanol was used as internal standard.

2.5. Electron microscopy

Pieces of the immobilized biocatalyst (*S. cerevisiae* AXAZ-1 cells immobilized on DBSG) were washed with deionized water and dried overnight at 30 °C. The samples were coated with gold in a Balzers SCD 004 Sputter Coater

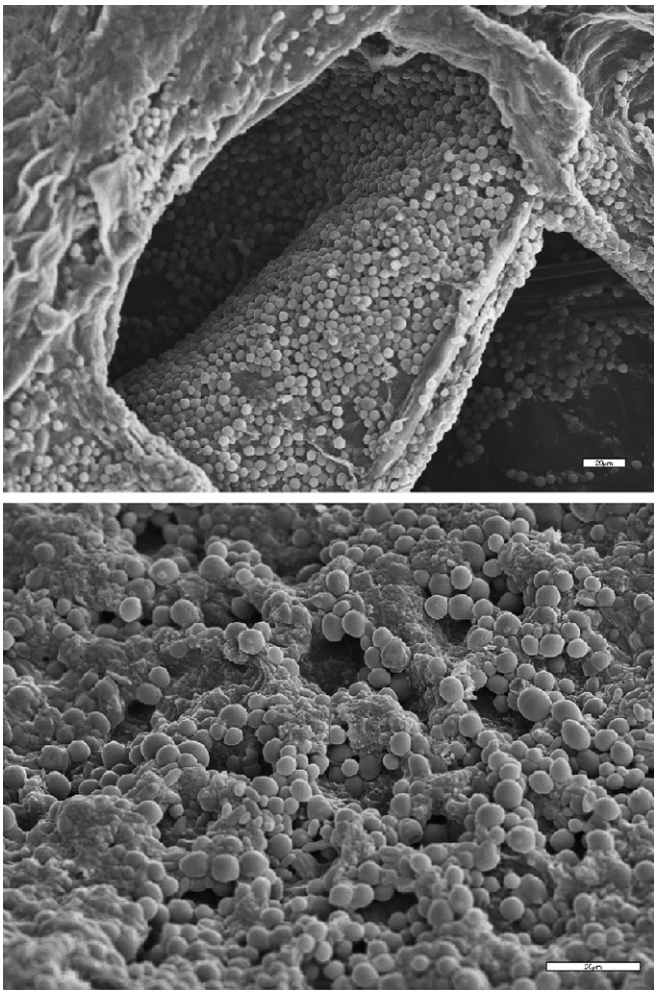

Fig. 1. Electron micrograph showing the morphology of the DBSG surface after the immobilization (top: ×600; bottom: ×1200).

for 3 min and examined in a Jeol model JSM-6300 scanning electron microscope.

3. Results and discussion

3.1. Brewing

Brewing, using psychrotolerant yeast immobilized on delignified brewer's spent grains (DBSG) at very low temperatures, is reported. The suitability of the immobilized biocatalyst for low temperature brewing was evaluated in terms of fermentation productivity (kinetics), and organoleptic quality, with emphasis on volatile composition. The immobilization of cells on DBSG, as well, as the suitability of the immobilized biocatalyst for alcoholic fermentation, was confirmed by its good operational stability during repeated batch fermentations of wort, even at very low temperatures (15–0 °C), and by electron microscopy, showing yeast cells attached on the porous surface of DBSG (Fig. 1).

Repeated fermentation batches of wort (original gravity 11.9–12.1% w/w) were obtained, using the psychrotolerant strain *S. cerevisiae* AXAZ-1 immobilized on DBSG at extremely low temperatures (5 and 0 °C). Fermentation kinetic parameters and analytical characteristics of the produced beers (used in industrial routine analysis), are shown in Tables 1 and 2. It is obvious that the immobilized biocatalyst retained its operational stability, even at very low temperatures, and fermented wort in times that ranged from 1 day at 15 °C, to only 20 days at 0 °C. In all cases, residual sugar was low (reducing sugars 1.8–4.4% w/v) and in the range of most commercial products. Ethanol concentrations were at the same high levels (4.4–4.9% v/v) in all the produced beers, showing the possibility of efficient fermentation over a wide range of temperatures (0–15 °C). Beer productivities (29–615 g/l/d), and ethanol productivities (1.8–38.7 g/l/d) were high, and were reduced as the temperature decreased. The final free cell concentrations were low (0.4–1.6 g/l) and the green beers had a fine clarity after the end of primary fermentation.

Vicinal diketones (diacetyl and 2,3-pentadione) are fermentation by-products with extremely low threshold values, responsible for strong *toffee*, *butterscotch*, *honey* and *vanilla-like* off-flavours and aromas in beers at concentrations higher than 0.5 mg/l (Branyik et al., 2005; Hough, Briggs, Stevens, & Young, 1982; Willaert & Nedovic, 2006). Therefore, maturation of beer at low temperatures is necessary for their reduction to the corresponding flavourless diols. This process (*diacetyl rest*) demands high storage capacity, cooling equipment and energy cost (Hough, 1985). At all the studied temperatures, the diacetyl and 2,3-pentadione concentrations in the green beers were found to be low (61–167 ppb and 32–109 ppb, respectively), and they were reduced as the fermentation temperature was decreased (Table 2 and Fig. 2). Specifically, at 0–5 °C, the 2,3-pentadione content was about threefold lower than that at 15 °C, and at levels similar to those found in mature commercial products (about 30 ppb). Diacetyl in beers produced at 0–5 °C was about half that at 15 °C, and was about double that found in commercial products (about 30 ppb), but within acceptable levels (<1 ppm). Dimethyl sulfide (DMS) is a compound derived from malt, extracted to wort and affected by yeast metabolism (Hough, 1985; Hough et al., 1982). It is usually present

Table 1
Kinetic parameters of the repeated batch fermentations of wort using *S. cerevisiae* AXAZ-1 cells immobilized on DBSG, at successively reduced temperatures

Temperature (°C)	Batch	Initial density (°P)	Fermentation time (h)	Ethanol (% v/v)	Ethanol productivity (g/l/d)	Beer productivity (g/l/d)
15	1	11.7	29	4.9	32.3	513
15	2	11.7	25	4.8	37.9	615
15	3	11.7	26	4.4	31.6	559
15	4	11.7	25	4.9	38.7	615
15	5	11.9	26	4.8	34.5	559
15	6	11.7	27	4.6	33.0	559
15	7	11.9	27	4.9	35.2	559
15	8	11.9	27	4.9	35.2	559
15	9	11.9	28	4.9	32.3	513
15	10	11.7	27	4.7	33.8	559
15	11	11.5	28	4.8	31.6	513
10	12	11.5	52	4.8	17.2	280
10	13	12.0	50	4.8	18.1	293
10	14	11.5	50	4.7	17.7	293
10	15	11.5	51	4.9	18.4	293
10	16	11.5	50	4.9	18.4	293
5	17	11.7	216	4.8	4.2	68
5	18	11.5	201	4.4	4.1	73
0	19	11.5	503	4.9	1.8	29
0	20	11.7	482	4.8	1.9	31

Table 2
Characteristics of the beers produced by the repeated batch fermentations of wort using *S. cerevisiae* AXAZ-1 cells immobilized on DBSG, at successively reduced temperatures

Temperature (°C)	Batch	Original gravity (% w/w)	Real extract (% w/w)	Apparent extract (% w/w)	Bitterness (EBU)	Colour (EBC)	2,3-Pentadione (ppb)	DMS (ppb)	Diacetyl (ppb)
15	1	12.1	4.9	3.2	17.7	8.8	98	37	140
15	2	12.1	4.7	2.9	18.3	8.9	109	32	113
15	3	11.9	5.1	3.5	16.2	8.7	84	35	99
15	4	12.1	4.7	2.8	19.0	8.7	81	34	122
15	5	12.0	4.5	2.7	18.4	8.6	81	35	131
15	6	12.1	4.9	3.4	17.2	9.0	75	34	167
15	7	12.0	4.5	2.7	17.9	8.9	67	30	100
15	8	12.1	4.7	2.9	18.5	9.1	89	28	112
15	9	12.1	4.9	3.4	16.3	8.5	95	24	96
15	10	12.0	4.8	3.0	16.8	8.8	98	29	92
15	11	12.0	5.0	3.4	18.4	8.6	101	30	101
10	12	12.0	4.9	3.2	19.5	9.2	82	25	102
10	13	12.1	5.0	3.4	19.1	9.1	77	27	94
10	14	12.0	4.6	2.7	17.2	9.0	80	27	105
10	15	12.0	4.7	2.8	16.6	8.9	69	25	92
10	16	12.1	5.0	3.5	18.3	9.2	64	28	99
5	17	12.0	4.5	2.7	18.2	9.1	33	14	62
5	18	12.0	4.9	3.3	18.8	8.6	38	18	79
0	19	12.0	4.8	3.0	19.0	8.5	36	11	89
0	20	12.1	5.1	3.5	18.5	8.8	32	16	61

in most beers above its threshold (about 33 ppb), and it is a primary flavour compound, contributing significantly to the *lager* beer character. At concentrations above 100 μg/l, DMS may be responsible for undesired *cooked sweet-corn* flavours. In the green beers produced by immobilized cells, the DMS concentrations were found to be 11–37 ppb, and lower than the amount found in the specific mature commercial product (<40 ppb), as well as in most commercial beers (14–205 ppb). Also, the DMS content was reduced with the reduction of fermentation temperature (at 0 °C it was threefold lower than that at 15 °C) (Table 2 and Fig. 2). Bitterness values were stable (16.2–19.5 EBU), in the levels of most commercial products, and did not seem to be affected by the fermentation temperature (Table 2). In commercial beers, bitterness varies in the range 10–40 EBU, depending on the type of the product, and can be further increased by the addition of hop extracts. Finally, the green beers had a fine clarity after the end of the main fermentation, and their colour values were 8.5–9.2 EBC, as in most commercial products (Table 2).

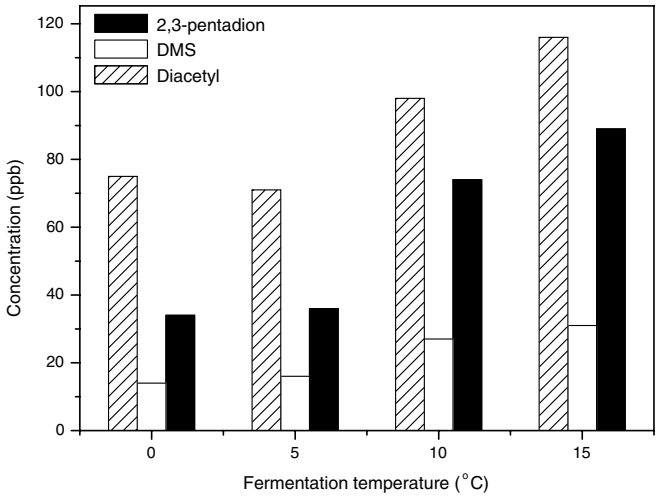

Fig. 2. Variation of 2,3-pentadione, diacetyl and DMS concentrations in beers produced by *S. cerevisiae* AXAZ-1 immobilized on DBSG at various temperatures (15–0 °C).

3.2. Volatiles

Analysis of the major volatiles by gas chromatography (Table 3) showed that the total higher alcohol content (81–119 mg/l) in beers produced using cells immobilized on DBSG, was lower than that found in the corresponding mature commercial product (about 130 mg/l), and it was reduced as the fermentation temperature decreased. Specifically, 1-propanol, isobutyl alcohol and amyl alcohols at 0 °C were reduced by about 41%, 33% and 30%, respectively, compared to those in beers produced at 15 °C (Fig. 3). The ethyl acetate concentrations (13.0–27.9 mg/l) were also reduced by about 30% at 0 °C compared to those at 15 °C, but the ratio of alcohols to esters was the same at all the studied fermentation temperatures. The iso-amyl acetate (*banana-like*) concentrations (0.12–0.67 mg/l) were within desired limits (much below 3 mg/l) and did not seem

Table 3
Volatile by-products in beers produced by the repeated batch fermentations of wort using *S. cerevisiae* AXAZ-1 cells immobilized on DBSG, at successively reduced temperatures

Temperature (°C)	Batch	Acetaldehyde (mg/l)	Ethyl acetate (mg/l)	1-Propanol (mg/l)	Isobutyl alcohol (mg/l)	Amyl alcohols (mg/l)	Iso-amyl acetate (mg/l)	Ethyl caproate (mg/l)
15	1	18.2	20.2	19.5	20.2	79.4	0.57	0.38
15	2	15.6	25.3	21.2	20.0	80.6	0.63	0.37
15	3	12.7	27.9	20.4	19.8	80.3	0.67	0.42
15	4	14.7	21.4	21.2	21.3	78.3	0.66	0.41
15	5	16.3	19.2	19.8	19.4	78.1	0.59	0.40
15	6	12.1	19.6	19.4	18.8	79.9	0.45	0.29
15	7	15.5	25.4	18.6	19.7	79.2	0.49	0.34
15	8	14.7	22.2	18.7	20.1	77.8	0.55	0.36
15	9	13.0	20.8	20.5	19.1	79.0	0.47	0.38
15	10	12.9	19.1	21.1	19.3	81.5	0.52	0.30
15	11	13.9	18.5	19.3	19.6	80.2	0.54	0.34
10	12	17.9	17.3	17.8	18.8	77.4	0.62	0.33
10	13	17.2	18.9	17.2	18.4	76.2	0.59	0.36
10	14	16.8	22.7	17.0	18.6	76.6	0.63	0.31
10	15	16.6	23.4	18.3	18.3	77.1	0.46	0.28
10	16	17.1	22.5	16.9	18.9	75.1	0.56	0.29
5	17	18.0	16.5	11.8	17.8	68.3	0.45	0.12
5	18	16.9	18.8	12.7	17.1	66.9	0.50	0.21
0	19	14.6	13.0	12.5	13.1	55.3	0.12	0.07
0	20	15.1	17.4	10.8	13.7	55.7	0.34	0.15

to be affected by the fermentation temperature in the range 15–5 °C, but were reduced to one half at 0 °C. The ethyl caproate (*wine-like/fruity*) concentrations (0.07–0.42 mg/l) were also reduced by more than 50% at 5–0 °C. Acetaldehyde concentrations in the green beers were stable at all the studied temperatures (12.1–18.2 mg/l) and at higher levels than those found in the corresponding mature commercial product (about 5 mg/l).

3.3. GC–MS analysis

The identification and quantitative analysis of volatiles by GC–MS was done to evaluate the differences in volatile composition between beers produced using cells immobilized on DBSG at distantly different fermentation temperatures (15 and 0 °C). A headspace, solid-phase microextraction sampling technique (SPME) was employed. In both types of samples, 80 compounds were identified, 64 of which with reliability (Table 4). Most of the identified compounds were esters (mainly ethyl esters of fatty acids and acetic esters of higher alcohols), which are usually found in most beers, contributing to flavour due to their low threshold values (Etievant, 1991). Various organic acids, alcohols, and carbonyl compounds were also identified. The quantitative determination, based on relative areas, with 2-pentanol as internal standard, showed that the amount of total volatiles was higher in beers produced at 15 °C. Specifically, the amounts of esters and alcohols at 15 °C were higher, indicating higher metabolic activity of the yeast and/or increased chemical transformations at that temperature. Miscellaneous compounds, that have relatively low threshold values and usually affect beer flavour with either their *fruity*, *floral* or *caramel* flavours (Etievant, 1991), such as b-myrcene, limonene, linalool, β-damascenone, α-terpineol, anethole, geraniol, phenylethyl alcohol, 3-furaldehyde, 2-furanmethanol, dihydro-5-pentyl-2(3*H*)-furanone and 4-hydroxy-2-methyl-acetophenone, were also identified. Their amounts were about the same or slightly higher in beers produced at 15 °C, except phenylethyl alco-

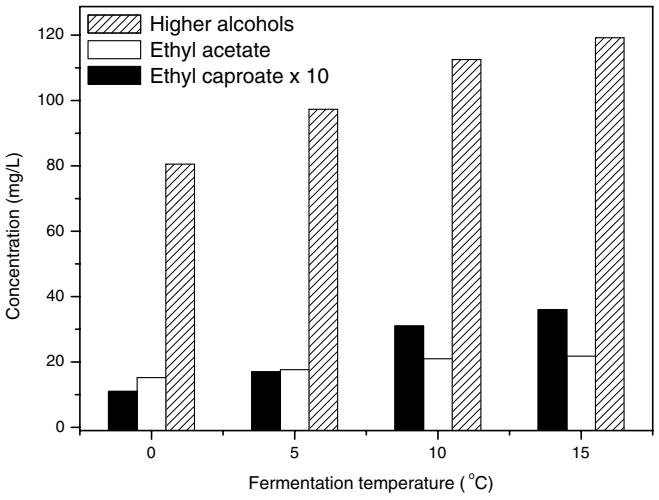

Fig. 3. Variation of higher alcohols, ethyl acetate and ethyl caproate concentrations in beers produced by *S. cerevisiae* AXAZ-1 immobilized on DBSG at various temperatures (15–0 °C).

Table 4
Identification and quantitative analysis of volatiles (based on standards of volatile compounds) in beers produced by *S. cerevisiae* AXAZ-1 immobilized on DBSG, at 15 and 0 °C

Compound	Rt (min)	Ri		Conc. (mg/l)	
				0 °C	15 °C
Acetaldehyde	1.89	152	*a*	0.43	0.52
Dimethyl sulfide	2.03	163	*b*	0.03	0.15
Acetone	2.33	188	*a*	–	–
Ethyl acetate	2.85	229	*a*	5.52	13.19
Propanoic acid, ethyl ester	3.68	296	*a*	0.09	0.24
Propanoic acid, 2-methyl-, ethyl ester	3.79	305	*a*	0.02	0.06
2,3-Butanedione	3.94	317	*b*	0.06	0.20
Acetic acid, 2-methylpropyl ester	4.53	364	*a*	0.27	0.66
Butanoic acid, ethyl ester	4.97	400	*a*	0.87	1.21
1-Propanol	5.12	406	*a*	0.27	0.79
2-Methyl-butanoic acid, ethyl ester	5.30	413	*a*	–	–
3-Methyl-butanoic acid, ethyl ester	5.62	425	*b*	–	–
Hexanal	5.88	435	*c*	–	–
2-Methyl-1-propanol	6.45	457	*a*	2.38	3.25
3-Methyl-1-butanol, acetate	6.89	474	*a*	14.2	50.6
2-Pentanol	7.12	482	*a*	–	–
b-Myrcene	7.81	506	*a*	–	–
4-Methyl-2-pentanol	8.33	530	*c*	–	–
Limonene	8.83	548	*a*	0.12	0.13
3-Methyl- + 2-methyl-1-butanol (amyl alcohols)	9.60	577	*a*	47.56	76.16
Hexanoic acid, ethyl ester	10.19	600	*a*	41.64	110.53
Styrene	10.74	616	*b*	–	–
2-Methyl-propanoic acid, 3-methylbutyl ester	11.11	628	*c*	–	–
Acetic acid, hexyl ester	11.35	635	*a*	0.16	0.23
cis- or *trans*-3-Hexenoic acid, ethyl ester	11.55	642	*c*		
1-Hydroxy-2-propanone	12.25	662	*b*	0.13	0.14
cis- or *trans*-3-Hexenoic acid, ethyl ester	12.28	664	*c*	–	–
Hydroxy-acetaldehyde	12.75	677	*c*	0.13	0.24
Heptanoic acid, ethyl ester	13.33	694	*b*	2.54	0.78
6-Methyl-5-hepten-2-one	13.42	697	*b*	–	–
1-Hexanol	14.17	719	*a*	0.07	0.11
Acetic acid, heptyl ester	14.65	734	*b*	0.37	0.18
Acetic acid, 2-ethylhexyl ester	14.96	745	*b*	–	–
Nonanal	15.24	751	*c*	0.22	0.09
Octanoic acid, ethyl ester	16.87	800	*a*	152	349
Acetic acid	17.18	810	*a*	6.50	3.93
3-Methylbutyl hexanoate	17.48	819	*c*	0.17	0.78
3-Furaldehyde	17.51	820	*c*	–	–
Acetic acid, octyl ester	18.02	836	*b*	0.15	0.30
7-Octenoic acid, ethyl ester	18.31	847	*c*	–	–
2-Ethyl-1-hexanol	18.58	855	*b*	–	–
Decanal	18.70	857	*c*	0.17	0.09
Benzaldehyde	19.37	878	*c*	–	–
Nonanoic acid, ethyl ester	19.96	897	*b*	0.51	0.47
2,3-Butanediol	20.27	906	*b*	2.64	3.22
3,7-Dimethyl-1,6-octadien-3-ol (linalool)	20.41	911	*a*	0.43	0.20
1-Octanol	20.79	923	*a*	0.16	0.67
1,3-Butanediol	21.44	943	*b*	0.44	0.71
8-Nonenoic acid, ethyl ester	21.59	948	*c*	0.42	0.11
Butanoic acid	22.84	987	*a*	0.15	0.24
Decanoic acid, ethyl ester	23.25	1000	*a*	2.89	110
Octanoic acid, 3-methylbutyl ester	23.75	1017	*b*	0.04	0.83
2-Furanmethanol	23.89	1022	*a*	0.43	1.38
3-Methyl-butanoic acid	24.12	1029	*a*	0.57	0.40
2-Methyl-butanoic acid	24.15	1031	*b*	0.35	0.18

Table 4 (*continued*)

Compound	Rt (min)	Ri		Conc. (mg/l)	
				0 °C	15 °C
Butanedioic acid, diethyl ester	24.32	1036	*a*	0.05	0.16
Ethyl dec-9-enoate	24.76	1051	*b*	15.5	21.6
α-Terpineol	24.99	1059	*a*	0.04	0.04
3-(Methylthio)-1-propanol	25.58	1079	*a*	0.19	0.45
Naphthalene	25.93	1093	*a*	–	–
3,7-Dimethyl-2,6-octadien-1-ol, acetate, (e)-	26.65	1116	*c*	–	–
Acetic acid, 2-phenylethyl ester	28.33	1173	*a*	5.12	20.9
β-Damascenone/2-buten-1-one, 1-(2,6,6-trimethyl-1,3-Cyclohexadien-1-yl)-, (e)-	28.48	1178	*a*	–	–
Anethole	28.55	1181	*a*	–	–
Dodecanoic acid, ethyl ester	29.13	1200	*a*	0.00	2.73
Hexanoic acid	29.21	1203	*a*	2.46	6.01
Geraniol	29.36	1206	*a*	0.22	0.18
Phenylethyl alcohol	31.09	1274	*a*	18.4	66.9
Heptanoic acid	32.13	1314	*b*	0.10	0.02
2-Methyl-propanoic acid, 2-phenylethyl ester	32.39	1328	*c*	–	–
Phenol	33.50	1366	*b*	–	–
Dihydro-5-pentyl-2(3H)-furanone	34.04	1386	*c*	0.07	0.11
Octanoic acid	34.95	1421	*a*	6.95	37.6
Nonanoic acid	37.59	1521	*b*	0.16	0.16
4-Hydroxy-2-methylacetophenone	38.25	1546	*b*	0.94	3.64
Hexadecanoic acid, ethyl ester	39.67	1600	*a*	–	0.13
Decanoic acid	40.14	1618	*a*	0.53	8.27
Ethyl 9-hexadecanoate	40.30	1624	*b*	–	0.33
Glycerin	41.19	1658	*b*	2.02	3.94
2,3-Dihydro-benzofuran	42.83	1720	*b*	0.36	0.48

Rt – retention time. Ri – ethyl ester retention index. *a* – identification by comparison of retention indices and mass spectral data with those of authentic compounds. *b* – identification by comparison of mass spectral data from Wiley and NIST libraries. *c* – tentative identification.

hol and 4-hydroxy-2-methylacetophenone, whose amounts were considerably higher at 15 °C. Off-flavour compounds, such as hexanal (*green-leaves* odour) and 3-(methylthio)-1-propanol (*hydrogen sulphide* or *sulphur dioxide-like*) were detected in traces in both samples. The sulphur compounds 3-(methylthio)-1-propanol and DMS were found in higher amounts in beers produced at 15 °C.

4. Conclusions

The above results, demonstrate that DBSG is an interesting carrier for cell immobilization, meeting the prerequisites for a cost effective industrial application of cell immobilization in brewing. BSG are a relatively resistant material that is not disrupted in the fermentation environment due to its high fibre content. It is a cheap and easily available raw material of food grade purity, which is fully compatible with beer. The use of psychrotolerant yeast, immobilized on DBSG, significantly increased the rate of wort fermentation and beer productivity, even at extremely low temperatures. The produced green beers had fine quality and, among the tested samples, those produced at very low temperatures (0–5 °C), were characterized as better, with fine flavour and mature character. This may be attributed to the lower higher alcohol, diacetyl and DMS concentrations, as well as the quantitative differences observed in the volatile composition of beers produced at different temperatures, caused either by altered metabolism of the immobilized cells or different speed of chemical transformations. From the results shown in Tables 3 and 4 it can be concluded that the impact of fermentation temperature on the formation of aroma compounds is high, although immobilization itself does not seem to alter the qualitative composition of beer aroma compared to beers produced by free cells, as was also shown in previous works (Kourkoutas et al., 2003). The fine clarity of the products produced by immobilized cells, as shown in previous studies (Bardi et al., 1996; Bekatorou, Koutinas, Psarianos, et al., 2001; Bekatorou et al., 2002; Bekatorou, Soupioni, et al., 2002), in combination with the low vicinal diketone concentrations, may allow the possibility of eliminating the maturation stage, resulting in significant reduction of production and investment costs.

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