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Beer volatile by-product formation at different fermentation temperature using immobilised yeasts

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Abstract

Beers produced by bottom-fermented yeast *Saccharomyces cerevisiae* entrapped in calcium pectate and κ-carrageenan contained lower amounts of diacetyl and higher alcohols at all temperatures studied (from 5 to 20°C). Ester formation was lower at temperatures from 5 to 15°C and acetaldehyde formation at temperatures from 5 to 12°C. The contents of total nitrogen and free amino nitrogen were higher at all temperatures studied due to lower amino acid uptake by entrapped cells. The character of beers produced by yeast adsorbed on DEAE-cellulose at different temperatures was similar to beers produced by free yeast. The concentration of diacetyl in beers fermented by entrapped yeasts decreased as the temperature was increased. In contrast, the diacetyl concentration increased with increasing temperature using free yeast and yeast immobilised on DEAE-cellulose. The concentration of acetaldehyde, higher alcohols and esters increased as the temperature was increased in all beers.

Keywords: *Saccharomyces*; Beer; Flavour; Calcium pectate; κ-Carrageenan; DEAE-cellulose

1. Introduction

Beer flavour is the result of a complex combination of components that give each brew its distinctive personality. Yeast metabolism makes an important contribution to flavour. Higher temperatures increase the rate of yeast metabolism but the quantitative influence of a temperature change will be different for each biochemical reaction, changing the balance of flavour compounds. The production of bottom fermented beer at lower temperatures is in recognition of the fact that fermentation at temperatures above 14°C results in a product with significantly poorer aroma and taste.

Immobilised cell systems increase productivity and improve the economy of bioprocesses, but also influence yeast metabolism and consequently, beer flavour. Differences in specific metabolic activity between free and immobilised yeast have been reported by numerous authors [1–4]. Waste products are formed in a local environment limited by mass transfer. In brewing applications common problems, resulting from metabolic changes or diffusion limitations due to immobilisation are reported to be slow nitrogen utilisation, unbalanced flavour profile, and high diacetyl concentrations. Initial primary fermentations with immobilised yeast cells were reported to produce excessive amounts of diacetyl [5,6] and vicinal diketones that are responsible for undesirable buttery flavour. The conversion of diacetyl to less flavour-active acetoin by immobilised cells is usually slow and therefore considered insufficient. According to Masschelein [7], immobilised cells exhibit lower production of flavour-active compounds due to their low metabolic activity.

The aim of this study was to determine the influence of fermentation temperature and immobilisation on fermentation parameters and beer quality in the first step of wort fermentation using the bottom yeast *Saccharomyces cerevisiae*. A suitable fermentation temperature of yeast immobilised in calcium pectate was sought while maintaining desirable analytical and flavour characteristics of the beer produced.

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2. Materials and methods

2.1. Yeast strains and medium

Saccharomyces cerevisiae W 96, was obtained from a local brewery (the number of the strain refers to Collection of the Research Institute of Brewing and Malting, Prague, Czech Republic) and maintained on the malt-extract agar at 5°C.

Cells for immobilisation were prepared by two-stage batch cultivation. In the first stage, yeasts were grown at 28°C for 24 h under aerobic condition in shake flasks containing 100 ml complete medium. In the second stage the yeast was grown for 18 h, in 500 ml complete medium. The yeast was collected by centrifugation from the medium, washed and re-suspended in sterile distilled water at a concentration of 1×10^{10} cells/ml of water (approx. 55 g cell dry mass per litre of water). This suspension was used for immobilisation.

Yeast concentration was determined as cell dry mass and by cell microscopic counting method.

The complete medium contained (in g/l): glucose 10, $(NH_4)_2SO_4$ 5, yeast extract 3, KH_2PO_4 2, $MgSO_4.7H_2O$ 1, $CaCl_2$ 0.1, NaCl 0.1. The pH value was adjusted to 5.8 before sterilisation. The cultivation medium was wort of original extract 11.5% (w/w) and the content of saccharides was 104 to 105 g/l and the concentration of unfermentable saccharides in the range 19–20 g/l. (Brewery Codecon, Svätý Jur, Slovakia).

2.2. Cell immobilisation

Potassium pectate was prepared by de-esterification of apple pectin (Pektin, Smiřice, Czech Republic) catalysed by KOH (0.1 mol/l). Data characterising some of these materials were published recently [8]. κ-Carrageenan (Genugel type X-0909) was obtained from Copenhagen Pectin (Denmark). Granular diethyl-aminoethyl-cellulose (DEAE) was a commercial anion exchanger of the Sigma Chemical Company (No. D 8382).

Yeast suspension (10^{10} cells per ml) was added to a dispersion of potassium pectate (50 g/l) or κ-carrageenan (40 g/l). Dispersion with pectate was dropped into a 0.1 mol/l $CaCl_2$ solution, κ-carrageenan to a 0.1 mol/l NaCl solution, giving rise to beads of 2 mm diameter. The cell concentration in the beads was 10^9 per 1 ml of gel matrix (approx. 50 g cell dry mass per litre of gel).

Yeast cells were immobilised according to Hehta [9]: 4 g dry DEAE-cellulose was added to 100 ml of 0.5 mol/l HCl, stirred for 30 min at 100 rpm, and the supernatant removed by suction filtration using a Büchner funnel. The DEAE cellulose was then washed with distilled water until the pH of the washing was between 4.0 and 6.0. The washed preparation of DEAE-cellulose was suspended in 100 ml of 0.5 mol/l NaOH and stirred for 30 min at the 100 rpm. The alkali was removed by suction filtration and washed with distilled water until the washings were at neutral pH. The washed DEAE-cellulose was re-suspended in 200 ml of 50 mmol/l sodium phosphate buffer, pH 7, and glutaraldehyde (25%, w/v aqueous solution) added to a final concentration of 1% (w/v). After stirring for 2 h at room temperature, the DEAE-cellulose derivative was recovered by filtration and the excess glutaraldehyde removed by washing with the sodium phosphate buffer (50 mmol/l, pH 7.0). The yeast suspension was added to the activated DEAE-cellulose, re-suspended in 200 ml of the buffer and dispersed by shaking for 2 h at 100 rpm and then kept 12 h at 15°C. The immobilised preparation was washed with sodium phosphate buffer containing 0.5 mol/l NaCl to remove non-covalently bound cells. The final yeast concentration on DEAE-cellulose was approx. 28 g cell dry mass per litre of cellulose (the final volume of cellulose with yeasts was 220 ml).

2.3. Batch fermentation

Batch fermentations were carried out stationary in flasks with an initial yeast concentration in media of approx. 10 g cell dry mass per litre of wort (containing 100 ml of immobilised calcium pectate or κ-carrageenan beads and 400 ml of wort, or 200 ml of immobilised DEAE-cellulose beads and 320 ml of wort), saturated with air at oxygen concentration, of approx. 6 mg/l. The duration of fermentation was 5 days. The results quoted were obtained from duplicate experiments.

2.4. Continuous fermentation

Continuous fermentation was carried out in an up-flow gas-lift bioreactor of volume 482 ml with an internal diameter of 4.6 cm, containing 100 ml of immobilised calcium pectate beads (the initial yeast concentration in medium approx. 10 g cell dry mass per litre of wort). The carrier gas was re-cuperated carbon dioxide formed during wort fermentation and recycled at the flow rate 0.048 l/min. The wort, saturated with air at an oxygen concentration, of approx. 2 mg/l, was supplied from a sterile flask into the reactors using a peristaltic pump. The wort-feeding rate was adjusted to 30.6 ml/h (residence time 12.5 h, concentration of saccharides on the output of reactor was 27 g/l of). The operating temperature was kept at 15°C.

2.5. Analytical methods

Ethanol and other low molecular weight volatile compounds were determined in beer samples by gas chromatography using a flame ionisation detector (FID) and using a column filled with *Porapak QS* for

ethanol. Analysis of volatile compounds after distillation and extraction in Likens–Nickerson apparatus [10] employed a column with a free fatty acid phase (FFAP 8%). Total nitrogen, free amino nitrogen, total polyphenols, bitterness and colour were measured according to the current European Brewery Convention Recommended Methods [11]. Diacetyl concentration was expressed as the total vicinal diketones (VDK), measured after thermal conversion of precursors to VDK as described Acker [12]. The concentration of saccharides was determined, after reaction with sulphuric acid, spectrophotometrically at 488 nm by the phenol method [13]. The yeast cell concentration was expressed as dry mass and determined by drying at 105°C to a constant weight. The cell concentration in gel beads was determined as an increase of biomass dry mass of the beads. Characterisation of beer was determined by a Servo Chem Automatic Beer Analyser (SCABA 5600, Tecator AB, Höganäs, Sweden).

3. Results and discussion

3.1. Fermentation and character of beers

The primary wort fermentation was performed over 5 days, stationary, at temperature from 5 to 20°C using free and immobilised bottom brewery yeast strain *Saccharomyces cerevisiae* W 96. Yeast cells were adsorbed on DEAE-cellulose or entrapped in calcium pectate or κ-carrageenan beads. According to an earlier report on the production of lager-type beer using continuous immobilised systems [14], a gas-lift reactor with yeast entrapped in calcium pectate, working at 15°C was chosen for these experiments. The optimal residence time, with the minimum concentration of saccharides 27 g/l on the output of reactor, was 12.5 h.

Young beers produced at a higher temperature had a fruity aroma, using free cells or cells immobilised on DEAE-cellulose at temperatures higher than 12°C, using entrapped cells at temperatures higher than 17°C. The character of beers produced by yeast adsorbed on DEAE-cellulose at different temperatures was very similar to beers produced by free yeast. The results of beer analyses are summarised in Table 1. Significant differences were obtained in the levels of total nitrogen and free amino nitrogen at all temperatures between beers fermented by yeast immobilised in calcium pectate and κ-carrageenan beads and beers produced by the control free yeast. Differences in fermentation temperature had little effect on nitrogen concentration. The level of nitrogen in beers produced by yeast immobilised on DEAE-cellulose was similar to those in control beers. This can be explained by differences between types of the cell binding. On preformed DEAE-cellulose yeast cells are adsorbed, while in gel matrices cells are entrapped. In the micro-environment of gels, cells exist in different conditions and exhibit modified metabolic activity. Higher levels of free amino nitrogen found in beers produced by entrapped yeast indicate a lower rate of amino acid uptake. Lower amino acid uptake by alginate-immobilised yeast has been frequently reported [5,15,16]. The higher density of immobilised yeast in fermentation medium leads to a lower content of these compounds in beer [17].

Steady-state characteristics of beer produced in the continuous system were similar to batch fermentation characteristics at 15°C using the same carrier for yeast immobilisation (Table 1).

3.2. Diacetyl and pentane-2,3-dione

Diacetyl and pentane-2,3-dione (vicinal diketones) have characteristic aromas and tastes described as 'buttery', 'honey' or 'toffee-like'. They have a very high off-flavour potential, dependent on temperature. The taste threshold concentration for diacetyl in lagers is 0.1–0.14 mg/l. At levels above 1 mg/l it becomes increasingly 'cheese-like' and sharp. Primary wort fermentations with immobilised cells have been reported to produce excessive amounts of diacetyl [5,6]. This fact was not confirmed in our beers. Yeast entrapped in calcium pectate or κ-carrageenan beads produced beers in batch systems with lower diacetyl concentration than the control, and the concentration of diacetyl decreased as the temperature was increased (Fig. 1, Tables 2–5). In contrast, the concentration of diacetyl increased with increasing temperature with free yeast and yeast immobilised on DEAE-cellulose. Bardi [18] reported that the concentration of diacetyl in continuous beer fermenta-

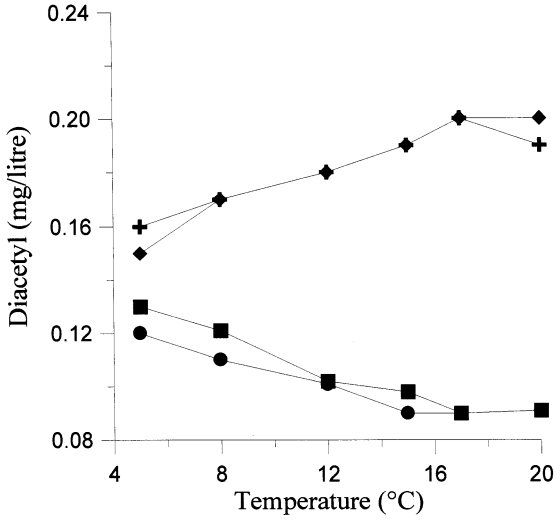

Fig. 1. Diacetyl concentration in beers produced by batch fermentation of 11.5% wort using immobilised yeast cells as a function of temperature. +, Free cells; ◆, DEAE-cellulose; ■, calcium pectate; ●, κ-carrageenan.

Table 1
Characteristics of beers produced in 5-day batch fermentation of wort at different temperatures[a]

T (°C)	Cell carrier	Apparent extract (%)	Real extract (%)	Alcohol (%)	Real attenuation (%)	Colour (°EBC)	pH	Bitterness (BU)	Total nitrogen (mg/100 ml)	Free amino nitrogen (mg/100 ml)	Total polyphenols (mg/l)
5	Free cells	2.71	4.38	4.11	61.9	11.1	4.1	20.1	63.2	16.4	19.8
	DEAE-cellulose	2.70	4.36	4.11	62.0	11.1	4.1	20.0	64.2	17.4	20.4
	Calcium pectate	2.69	4.51	4.12	60.7	13.0	4.1	18.6	91.4	27.6	17.6
	κ-carrageenan	2.67	4.52	4.09	60.6	12.0	4.1	18.4	90.4	26.5	18.1
8	Free cells	2.74	4.41	4.09	61.6	11.3	4.1	19.6	61.3	15.7	19.3
	DEAE-cellulose	2.73	4.37	4.17	61.8	11.2	4.2	19.7	63.4	16.9	19.8
	Calcium pectate	2.70	4.47	4.24	61.1	14.0	4.1	19.1	94.3	26.9	17.4
	κ-carrageenan	2.74	4.49	4.24	60.9	13.0	4.1	18.5	93.2	26.7	18.1
12	Free cells	2.79	4.43	3.94	61.4	12.4	4.2	18.1	58.1	12.1	18.4
	DEAE-cellulose	2.75	4.35	4.16	62.1	11.2	4.2	18.5	59.7	16.1	19.5
	Calcium pectate	2.71	4.50	4.23	60.8	14.0	4.2	19.4	92.6	26.5	18
	κ-carrageenan	2.75	4.50	4.25	60.8	13.0	4.2	19.1	92.5	26.8	18.4
15	Free cells	2.84	4.41	3.86	61.6	12.6	4.3	17.3	54.6	10.4	17.6
	DEAE-cellulose	2.73	4.37	4.15	62.0	11.3	4.3	17.3	55.6	15.4	19.1
	Calcium pectate	2.70	4.53	4.26	60.6	14.0	4.2	19.4	92.5	26.1	18.4
	κ-carrageenan	2.71	4.54	4.24	60.5	14.0	4.2	19.2	92.6	25.9	18.7
17	Free cells	2.87	4.45	3.81	61.3	12.4	4.3	16.9	49.6	9.3	17.4
	DEAE-cellulose	2.69	4.34	4.14	62.2	11.3	4.3	16.7	50.4	13.9	18.5
	Calcium pectate	2.68	4.54	4.23	60.5	14.0	4.2	19.7	91.7	25.4	18.5
	κ-carrageenan	2.64	4.57	4.19	60.2	14.0	4.3	19.4	91.4	25.7	19.1
20	Free cells	2.96	4.47	3.76	61.1	12.6	4.4	15.4	43.2	8.7	17.1
	DEAE-cellulose	2.74	4.37	4.17	62.0	11.2	4.3	15.4	49.8	13.2	17.9
	Calcium pectate	2.6	4.56	4.24	60.3	15.0	4.3	19.6	91.5	25.1	18.9
	κ-carrageenan	2.61	4.56	4.18	60.3	14.0	4.3	19.5	91.6	25.3	19.2
15[b]	Calcium pectate	2.65	3.38	4.67	72.7	14.2	4.1	17.9	82.9	30.1	20.1

[a] Original extract-11.5% (w/w).
[b] Steady-state characteristics of beer produced in immobilised continuous gas-lift bioreactor. Residence time 12.5 h. Analysis measured during 960 h.

Table 2
Volatile products (mg/l) formed in batch fermentation of 11.5% wort using free cells (control) at different temperatures

Temperature (°C)	5	8	12	15	17	20
Diacetyl	0.16	0.17	0.18	0.19	0.20	0.19
Acetaldehyde	3.1	3.3	3.5	3.4	3.6	3.8
Acetic acid	4.1	3.6	3.4	3.1	2.9	2.7
Propionic acid	0.12	0.15	0.14	0.13	0.11	0.5
Butyric acid	0.30	0.31	0.28	0.27	0.25	0.21
i-Butyric acid	3.4	3.6	3.1	2.9	2.6	2.1
Valeric acid	3.1	3.5	3.6	3.7	3.6	3.7
i-Valeric acid	0.21	0.26	0.27	0.29	0.30	0.34
Hexanoic acid	0	0.17	0.21	0.20	0.18	0.16
Octanoic acid	0.31	0.31	0.29	0.30	0.28	0.33
Decanoic acid	0.21	0.24	0.26	0.25	0.24	0.22
Dodecanoric acid	0.09	0.11	0.13	0.12	0.13	0.11
n-Propanol	6.4	6.9	6.8	6.1	5.7	5.4
2-Methylpropanol	31.2	46.4	49.8	51.7	53.8	58.4
i-Butanol	9.6	10.4	11.8	19.6	19.7	20.6
2- and 3-Methylbutanol	80.4	81.7	89.6	93.4	97.5	102.3
2-Phenylethanol	11.4	12.3	14.5	14.6	14.7	14.9
Total alcohols	139	157	172	185	191	201
Ethyl formate	1.42	1.89	2.07	2.14	2.12	2.34
Ethyl acetate	14.7	16.7	16.4	16.8	16.9	17.4
Propyl acetate	1.23	1.30	1.37	1.44	1.57	1.54
2-Methylpropyl acetate	0.4	0.2	0.21	0.20	0.21	0.19
3-Methylbutyl acetate	1.91	1.96	2.06	2.11	2.24	2.34
Ethyl hexanoate	1.17	1.32	1.34	1.39	1.4	1.47
Hexyl acetate	5.1	5.3	5.7	5.9	6.4	6.7
Ethyl lactate	1.9	2.2	2.6	2.9	3.1	3.4
2-Methylpropyl hexanoate	0.31	0.36	0.34	0.37	0.35	0.34
Ethyl octanoate	0.54	0.57	0.58	0.59	0.64	0.67
3-Methylbutyl hexanoate	0.21	0.25	0.21	0.20	0.16	0.19
Ethyl decanoate	2.4	2.6	1.21	0.18	0.13	0.14
Ethyl phenylacetate	0.11	0.14	0.11	0.17	0.18	0.16
Ethyl dodecanoate	0.19	0.21	0.32	0.47	0.44	0.45
Ethyl tetradecanoate	0.05	0.06	0.09	0.13	0.17	0.24
Total esters	31.6	35.0	34.6	34.9	36.1	37.5
Alcohols:eters	4.3:1	4.4:1	4.9:1	5.2:1	5.3:1	5.4:1

tions with yeast immobilised on cellulosic material decreased as the temperature was increased. Diacetyl concentrations reported were in the range of most commercial beers. In the batch system the values of diacetyl remained constant as the temperature was decreased. In continuous system the beer produced had a diacetyl concentration lower than 0.1 mg/l (Table 4).

3.3. Acetaldehyde

Acetaldehyde, the most important aldehyde of beer, is formed as a metabolic branch point in the pathway leading from carbohydrate to ethanol. Its level varies during fermentation and ageing and in beers usually lies in the range 2–20 mg/l. At concentrations of 20–25 mg/l acetaldehyde causes 'green vegetation' or 'vegetable' flavour. In young beers the acetaldehyde concentrations increased as the temperature was increased (Fig. 2 and Tables 2–5). In beers produced by the free yeast and yeast immobilised on DEAE-cellulose, the differences were only very slight: for control 3.1 mg/l at 5°C, 3.8 mg/l at 20°C and for DEAE-cellulose 3.2 mg/l at 5°C, 3.9 mg/l at 20°C. Using entrapped yeast, the differences were more evident: for pectate 2.6 mg/l at 5°C, 4.4 mg/l at 20°C and for κ-carrageenan 2.5 mg/l at 5°C, 4.5 mg/l at 20°C.

3.4. Volatile and fatty acids

Volatile acids are usually present in beer at total concentrations of 20–150 mg/l. Butyric and *iso*-butyric acids in concentration about 6 mg/l may cause a 'butyric' or 'rancid' flavour, valeric and *iso*-valeric acids 'old hop' and 'cheesy' flavour. 6- to 12-carbon fatty

acids the characteristics are 'cheesy', 'goaty' or 'sweaty' flavour. The threshold concentrations of these acids, according to Stempfl [19], are about 5 mg/l for caproic acid (hexanoic) and 10 mg/l for caprylic (octanoic) and capric acid (decanoic). Lauric acid (dodecanoic) in concentration higher than 6 mg/l may causes 'soapy' flavours. The threshold concentration of all acids is higher than the concentration in the experimental beers. The concentrations of these acids were higher in beers produced by entrapped yeast, and most increased with increasing temperature of fermentation (Tables 2–5).

3.5. Higher alcohols

Higher alcohols constitute an important part of the by-products formed during wort fermentation, but like the major product of fermentation, ethanol, have little impact on the flavour of the final beer. Fusel alcohols, such as butyl, amyl and *iso*-amyl alcohol, contribute to the general alcohol warming sensation in the mouth [20]. Their formation is dependent upon the fermentation temperature. An increase in temperature resulted in increased concentrations of higher alcohols, except n-propanol produced by natural fermentation at temperatures higher than 12°C (Table 2) and by yeast adsorbed on DEAE-cellulose at temperatures higher than 15°C (Table 3). In beers produced with entrapped yeast (Tables 4 and 5) the concentrations of n-propanol were higher, compared to control beers, at all temperatures. n-Propanol and 2-methylpropanol may cause 'rough' flavours and harshness of beer [19], but their threshold concentrations are much more higher than their concentration in the experimental beers (800 and 200 mg/l, respectively).

Table 3
Volatile by-products (mg/l) formed in batch fermentation of 11.5% wort using cells immobilised on DEAE-cellulose at different temperature

Temperature (°C)	5	8	12	15	17	20
Diacetyl	0.15	0.17	0.18	0.19	0.20	0.20
Acetaldehyde	3.2	3.4	3.5	3.5	3.6	3.9
Acetic acid	4.2	3.8	3.6	3.5	3.3	3.1
Propionic acid	0.13	0.15	0.14	0.16	0.17	0.18
Butyric acid	0.34	0.32	0.31	0.31	0.29	0.27
i-Butyric acid	4.1	3.9	3.8	3.6	3.4	3.2
Valeric acid	2.9	3.1	3.2	3.2	3.3	3.4
i-Valeric acid	0.24	0.28	0.30	0.30	0.32	0.34
Hexanoic acid	0	0	0	0.09	0.14	0.18
Octanoic acid	0.32	0.34	0.34	0.35	0.36	0.37
Decanoic acid	0.23	0.26	0.23	0.21	0.19	0.17
Dodecanoric acid	0.08	0.13	0.14	0.15	0.15	0.19
n-Propanol	5.9	6.7	6.8	7.1	5.8	4.6
2-Methylpropanol	29.7	38.6	47.8	50.6	54.6	57.9
i-Butanol	9.4	10.5	11.7	18.9	19.7	21.3
2- and 3-Methylbutanol	78.9	80.8	87.9	91.4	96.5	101.3
2-Phenylethanol	9.9	11.2	13.5	14.5	14.2	15.1
Total alcohols	133	147	167	182	190	200
Ethyl formate	1.54	1.87	2.06	2.11	2.20	2.29
Ethyl acetate	14.2	15.8	16.1	16.5	16.6	17.1
Propyl acetate	1.21	1.29	1.34	1.42	1.57	1.61
2-Methylpropyl acetate	0	0.2	0.24	0.19	0.14	0.08
3-Methylbutyl acetate	1.87	1.95	2.01	2.17	2.23	2.31
Ethyl hexanoate	1.09	1.31	1.30	1.34	1.45	1.49
Hexyl acetate	4.9	5.1	5.6	5.9	6.4	6.8
Ethyl lactate	1.7	2.1	2.4	2.9	3.0	3.7
2-Methylpropyl hexanoate	0.28	0.27	0.29	0.31	0.36	0.39
Ethyl octanoate	0.51	0.58	0.59	0.61	0.67	0.71
3-Methylbutyl hexanoate	0.23	0.26	0.28	0.21	0.18	0.15
Ethyl decanoate	2.3	2.7	1.4	0.15	0.12	0
Ethyl phenylacetate	0.14	0.15	0.09	0	0	0
Ethyl dodecanoate	0.17	0.19	0.31	0.45	0.47	0.49
Ethyl tetradecanoate	0	0.09	0.11	0.17	0.19	0.20
Total esters	30.1	33.8	34.1	34.4	35.6	37.3
Alcohols:esters	4.4:1	4.3:1	4.9:1	5.3:1	5.8:1	5.3:1

Table 4
Volatile by-products (mg/l) formed in batch fermentation (CoF-continuous at 15°C) of 11.5% worth using cells entrapped in calcium pectate at different temperatures

Temperature (°C)	5	8	12	15	17	20	CoF
Diacetyl	0.13	0.121	0.102	0.098	0.09	0.091	0.09
Acetaldehyde	2.6	2.9	3.2	3.7	4.1	4.4	2.61
Acetic acid	4.5	4.6	4.5	4.3	3.9	3.6	4.21
Propionic acid	0.11	0.15	0.15	0.16	0.15	0.14	0.11
Butyric acid	0.28	0.31	0.34	0.36	0.39	0.46	0.19
i-Butyric acid	3.9	4.1	4.7	4.8	5.6	5.9	4.4
Valeric acid	3.2	3.1	3.4	3.4	3.5	3.7	3.2
i-Valeric acid	0.14	0.15	0.13	0.12	0.11	0.10	0.22
Hexanoic acid	0.09	0.11	0.12	0.12	0.14	0.15	0.19
Octanoic acid	0.38	0.41	0.49	0.50	0.53	0.51	0.39
Decanoic acid	0.21	0.21	0.24	0.24	0.25	0.24	0.08
Dodecanoric acid	0	0.12	0.28	0.28	0.33	0.34	0.13
n-Propanol	7.6	7.9	8.1	8.7	9.2	9.6	6.4
2-Methylpropanol	38.6	40.7	41..3	45.6	49.8	51.7	36.9
i-Butanol	10.2	11.7	13.9	14.7	16.5	18.4	13.4
2- and 3-Methylbutanol	50.4	53.2	60.2	64.7	71.2	76.5	56.8
2-Phenylethanol	9.8	10.3	12.2	13.6	14.8	15.7	10.1
Total alcohols	116	123	135	147	161	171	123.1
Ethyl formate	1.83	1.90	1.91	1.96	1.94	1.97	1.49
Ethyl acetate	12.3	12.4	12.3	13.6	18.9	19.6	11.6
Propyl acetate	1.21	1.26	1.21	1.11	1.06	0.97	1.19
2-Methylpropyl acetate	0	0.06	0.09	0.10	0.14	0.18	0.25
3-Methylbutyl acetate	1.8	2.3	2.9	2.8	3.4	4.1	3.19
Ethyl hexanoate	1.9	2.1	2.3	2.6	2.9	3.6	1.89
Hexyl acetate	3.8	4.1	4.6	4.3	5.4	5.9	4.49
Ethyl lactate	0	0.6	1.02	1.08	1.3	2.4	1.09
2-Methylpropyl hexanoate	0	0	0	0	0.12	0.21	0.11
Ethyl octanoate	0.11	0.24	0.38	0.32	0.44	0.47	0.21
3-Methylbutyl hexanoate	0.14	0.19	0.26	0.19	0.09	0.08	0.14
Ethyl decanoate	1.8	1.9	2.6	2.4	2.1	1.98	1.9
Ethyl phenylacetate	0	0	0	0.03	0	0	0.15
Ethyl dodecanoate	0.11	0.13	0.19	0.11	0.14	0.19	0.17
Ethyl tetradecanoate	0.14	0.12	0.12	0.13	0.21	0.34	0.12
Total esters	25.1	27.3	29.8	30.7	38.1	41.9	27.99
Alcohols:esters	4.6:1	4.5:1	4.5:1	4.7:1	4.2:1	4.0:1	4.39:1

Amyl alcohols (2- and 3-methylbutanol) cause 'fruity' flavours in threshold concentrations of 50 mg/l for 2-methylbutanol and 1 mg/l for 3-methylbutanol. They can be not separated by gas chromatography using a free fatty acid phase (FFAP), but in bottom-fermented beers 2-methylbutanol and 3-methylbutanol are usually in the ratio of 1:5. In the experimental beers the concentrations of 3-methylbutanol were higher than the taste threshold (Tables 2–5). The concentrations of amyl alcohols were significantly lower in beers prepared using entrapped yeast at higher temperatures.

The concentrations of 2-phenylethanol were lower in all beers produced by immobilised yeast at temperatures below 15°C, but were slightly higher at higher temperatures (except κ-carrageenan). 2-Phenylethanol causes 'sweet' or 'rose' flavours in beer. The threshold concentration of 2-phenylethanol in water is 1 mg/l [19].

The total concentration of higher alcohols increased with increasing temperature in all beers. In beers produced with yeast immobilised in calcium pectate or κ-carrageenan beads the total concentration of higher alcohols were reduced at every temperature compare to controls and beers produced by yeast immobilised on DEAE-cellulose (Fig. 3). Their concentrations were in the range of 140–160 mg/l in beers produced by free yeast at temperatures below 10°C and in beers produced with entrapped yeast at temperatures above 15°C (Tables 2–5). This fact was expected, because of lower amino acid uptake of entrapped yeast, as the biosynthesis of higher alcohols is generally considered to be related to amino acid metabolism and linked to their absorption from the wort.

In continuous fermentation of wort by yeast entrapped in calcium pectate, the content of higher alcohols were lower than in batch fermentation at the same temperature using the same carrier for cell immobilisation (Table 4).

3.6. Esters

Esters in beer contribute several 'fruitlike' and 'floral' aromatics. Ethyl acetate represents approximately one third of all esters in beers. The threshold concentration of ethyl acetate in beer is 30 mg/l [19], but for lager-types beers the recommended concentration is lower than 5 mg/l. Very intensive 'fruity' and 'banana' aromas are caused by *iso*-amyl acetate (3-methylbutyl acetate) in concentrations higher than 2 mg/l. Ethyl hexanoate has a low threshold concentration (0.005 mg/l in water), ethyl octanoate (0.5 mg/l) and ethyl decanoate (1.5 mg/l), giving 'apple' aromas to beer. As shown in Tables 2–5 and Fig. 4, in young beers the concentration of total esters increased as the temperature of fermentation was increased. In beers produced by control free yeast and yeast immobilised on DEAE-cellulose the differences in total esters concentrations were only very slight: for control 31.6 mg/l at 5°C, 37.5 mg/l at 20°C and for DEAE-cellulose 30.1 mg/l at 5°C, 37.3 mg/l at 20°C. Using entrapped yeast, the differences were more evident: for pectate 25.1 mg/l at 5°C, 41.9 mg/l at 20°C and for κ-carrageenan 24.3 mg/l at 5°C, 40.4 mg/l at 20°C.

In the case of continuous fermentation of wort, the content of total esters (similar to the content of total alcohols) were lower than in batch fermentation at the same temperature using the same calcium-pectate-entrapped yeast (Table 4).

Table 5
Volatile by-products (mg/l) formed in batch fermentation of 11.5% wort using cells entrapped in κ-carrageenan at different temperatures

Temperature (°C)	5	8	12	15	17	20
Diacetyl	0.12	0.21	0.101	0.09	0.09	0.091
Acetaldehyde	2.5	2.8	3.3	3.8	4.2	4.5
Acetic acid	4.4	4.5	4.6	4.3	3.8	3.3
Propionic acid	0.09	0.13	0.15	0.17	0.14	0.13
Butyric acid	0.26	0.32	0.35	0.37	0.41	0.47
i-Butyric acid	3.7	4.0	4.5	4.9	5.4	5.8
Valeric acid	3.1	3.4	3.5	3.7	3.8	3.8
i-Valeric acid	0.17	0.14	0.12	0.09	0.06	0
Hexanoic acid	0	0	0	0.08	0.11	0.14
Octanoic acid	0.29	0.34	0.36	0.41	0.47	0.54
Decanoic acid	0.19	0.21	0.21	0.24	0.25	0.26
Dodecanoric acid	0	0	0.18	0.24	0.29	0.34
n-Propanol	7.4	7.7	8.2	8.4	8.7	9.1
2-Methylpropanol	36.5	38.9	41.4	44.3	45.7	48.9
i-Butanol	10.1	10.9	12.4	13.8	14.5	17.5
2- and 3-Methylbutanol	49.8	51.4	58.7	64.2	69.8	72.4
2-Phenylethanol	9.1	9.9	10.3	11.4	12.5	12.8
Total alcohols	112	118	131	142	151	160
Ethyl formate	1.81	1.89	1.92	1.97	1.99	2.03
Ethyl acetate	11.7	12.4	12.1	13.4	15.8	18.9
Propyl acetate	1.11	1.24	1.13	0.98	0.67	0.58
2-Methylpropyl acetate	0	0	0	0.08	0.14	0.19
3-Methylbutyl acetate	1.6	2.1	2.4	2.7	3.1	3.4
Ethyl hexanoate	1.7	2.2	2.6	2.9	3.4	3.8
Hexyl acetate	3.5	4.2	4.7	4.9	5.7	6.1
Ethyl lactate	0	0	0.54	1.12	1.34	1.48
2-Methylpropyl hexanoate	0	0	0	0	0	0.11
Ethyl octanoate	0.9	0.21	0.34	0.31	0.47	0.56
3-Methylbutyl hexanoate	0.13	0.21	0.25	0.14	0.11	0
Ethyl decanoate	1.7	1.8	1.9	1.8	2.1	2.5
Ethyl phenylacetate	0	0	0	0.12	0.24	0.34
Ethyl dodecanoate	0.09	0.14	0.21	0.10	0.15	0.19
Ethyl tetradecanoate	0.11	0.14	0.16	0.17	0.19	0.21
Total esters	24.3	26.5	28.2	30.7	35.4	40.4
Alcohols:esters	4.6:1	4.5:1	4.6:1	4.6:1	4.3:1	3.9:1

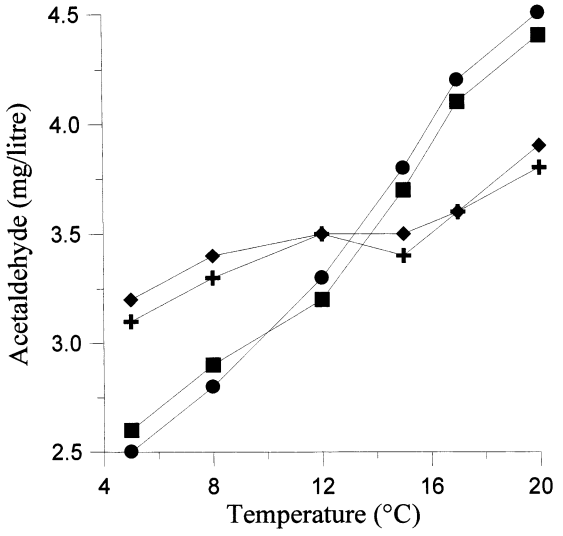

Fig. 2. Acetaldehyde concentration in beers produced by batch fermentation of 11.5% wort using immobilised yeast cells as a function of temperature. +, Free cells; ◆, DEAE-cellulose; ■, calcium pectate; ●, κ-carrageenan.

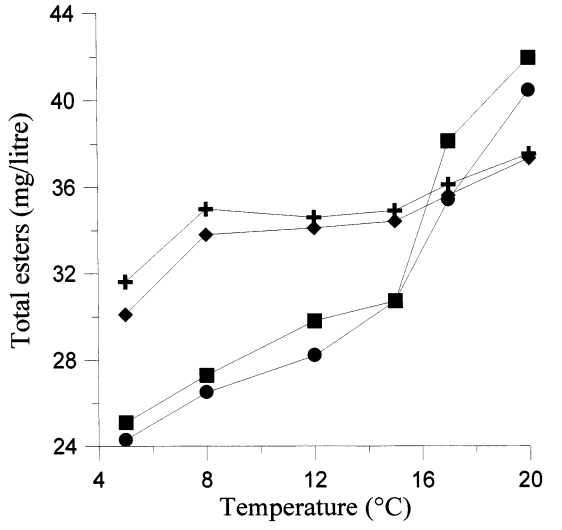

Fig. 4. Total esters concentration in beers produced by batch fermentation of 11.5% wort using immobilised yeast cells as a function of temperature. +, Free cells; ◆, DEAE-cellulose; ■, calcium pectate; ●, κ-carrageenan.

3.7. Higher alcohols-to-esters ratio

The overall flavour of a beer depends on the relative contents of these compounds. The optimum higher alcohols-to-esters ratio for lagers is 4.1 to 4.7:1 according to Poledníková [15]. In experimental beers, these were strongly dependent on temperature. Beers produced by yeast immobilised in calcium pectate (Table 4) and in κ-carrageenan beads (Table 5) had suitable higher alcohols-to-esters ratios at temperatures from 5 to 17°C, beers produced by yeast immobilised on DEAE-cellulose and control yeast only at temperatures of 5 and 8°C.

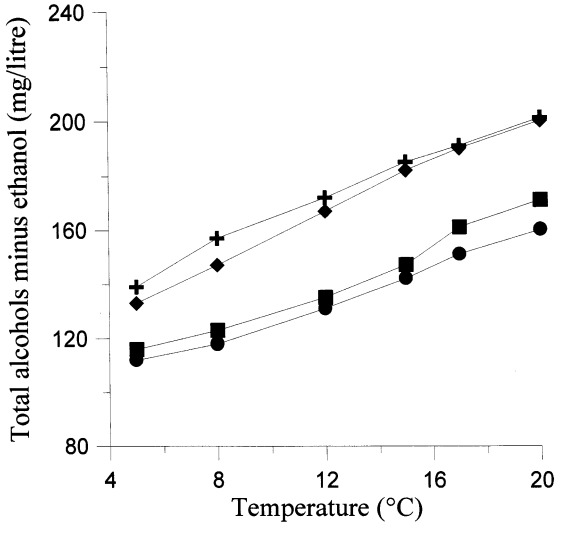

Fig. 3. Total alcohols concentration in beers produced by batch fermentation of 11.5% wort using immobilised yeast cells as a function of temperature. +, Free cells; ◆, DEAE-cellulose; ■, calcium pectate; ●, κ-carrageenan.

According to these data, all three carriers tested for immobilisation were suitable for beer fermentation. The behaviour of yeast immobilised by adsorption on DEAE-cellulose was very similar to that of free yeast at all temperatures, while entrapped yeast showed metabolic differences. The aroma and flavour of beers produced by yeast entrapped in calcium pectate or κ-carrageenan at temperatures around 15°C were similar to beers produced at lower temperatures using free yeast. Seven of ten tasters considered beer produced using calcium-pectate-entrapped yeast cells at 15°C in our continuous system to be comparable to a beer produced by classical fermentation technology. This fact has high practical significance, because fermentation at higher temperatures is much cheaper and the process using immobilised cells in continuous fermentation can be carried out with significantly reduced residence times [14].

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