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The impact of process parameters on flavour profile of alcohol-free beer from a single-stage continuous gas-lift reactor with immobilized yeast

Radek Lehnert[1], Michal Kuřec[1], Tomáš Brányik[1] & José A. Teixeira[2]

[1]ICT, Department of Fermentation Chemistry and Bioengineering, Technická 5, CZ-166 28 Prague, Czech Republic e-mail: tomas.branyik@vscht.cz
[2]Universidade do Minho, Centro de Engenharia Biológica, Braga, Portugal

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SUMMARY

In order to study the formation and conversion of the most important flavour compounds, the real wort used in alcohol-free beer fermentation was mimicked by a complex model medium containing glucose, yeast extract and aldehydes (hexanal, 2-methyl propanal, 3-methyl butanal, furfural). Fermentation experiments were carried out in a continuously operating gas-lift reactor with brewing yeast immobilized on spent grains. During continuous experiment, parameters such as oxygen supply, residence time (Rt) and temperature (T) were changed to find the optimal conditions for alcohol-free beer production. The formation of ethanol, higher alcohols (HA), esters (ES), reduction of aldehydes, and consumption of glucose were observed. The results suggest that the process parameters, particularly the oxygen supply, represent a powerful tool in controlling the degree of fermentation and flavour formation carried out by immobilized biocatalyst. Under optimal conditions in the continuous immobilized cell reactor it was possible obtain a fermented model medium with a composition approaching commercial alcohol-free beers.

INTRODUCTION

In EU countries a beer is considered alcohol-free when its alcohol concentration does not exceed 0.5 % volumetric (3.945 g/l). Although still representing a minor product of brewing industry, the increasing market share of alcohol-free beer reflects the global trend for healthier lifestyle. There are two main strategies of its production. One is based on the removal of alcohol from regular beer (dialysis, reverse osmosis, vacuum distillation or evaporation). This approach requires special equipment for alcohol removal, which increases both investment and running costs. The other strategy comprises methods of suppressed or controlled alcohol formation. The production of alcohol-free beer using immobilized yeast cell systems rank among methods of controlled fermentation using short contact between immobilized yeasts and wort. The continuous alcohol-free beer fermentation can outperform the rival technologies in various aspects (productivity, investment and operating costs).

However, it is essential that the continuous system produces a competitive final product.

Alcohol-free beers are usually characterized by worty off-flavours and lack of pleasant fruity (estery) aroma found in regular beers. Such defects may stem from a fermentation procedure that fails to reduce the chemical compounds responsible for the worty off-flavour and to produce fusel alcohols and esters. Several alcohols, other than ethanol are formed in beer during fermentation, among which n-propanol, iso-butanol and isoamyl alcohols (2-methyl and 3-methyl butanol) contribute most significantly to beer flavour. Control of higher alcohol formation in continuous systems can be well balanced by the choice of an appropriate yeast strain [8, 14], wort composition, fermentation conditions, immobilization method and reactor design [11, 20]. The synthesis of aroma-active esters by yeast is of a great importance because they represent the largest group of flavour active compounds in beer. Fundamentally, two factors are important for the rate of ester formation: the availability of the two substrates (acetyl/acyl-CoA and fusel alcohols), and the activity of enzymes involved in the formation of esters. Consequently, all parameters that affect enzyme activity or substrate concentrations will influence ester production [18].

Several wort carbonyls are proposed to contribute to the unpleasant worty taste of alcohol-free beers [5, 12]. However, yeast metabolism is known to bring about the reduction of these substances to less flavour active ones. Vicinal diketones (diacetyl and 2,3-pentanedione) are a specific group of carbonyls because of their low taste threshold [2].

Fermentation experiments were carried out in a continuously operating gas-lift reactor with brewing yeast immobilized on spent grains, a brewing by-product. In order to study the formation and conversion of the most important flavour compounds, the real wort used in alcohol-free beer fermentation was simulated by a defined complex medium containing glucose, yeast extract and aldehydes (2-methyl propanal, 3-methyl butanal, hexanal and furfural). During the optimization study, parameters such as aeration, residence time (Rt) and temperature (T) were changed to elucidate their influence on the continuous production of alcohol-free beer with balanced flavour.

METHODOLOGY

Microorganisms and medium

A brewing yeast strain (*Saccharomyces cerevisiae subsp. carlsbergensis*) supplied by the company Unicer (Bebidas de Portugal, S.A., S. Mamede de Infesta) was used throughout the experiments. Due to high volumetric medium consumption, the yeast were cultivated in a complex model medium (CMM) mimicking an average wort composition used for alcohol-free beer production. The composition of CMM was (in g/l): 5, KH_2PO_4; 2, $(NH_4)_2SO_4$; 0.4, $MgSO_4.7H_2O$; 2, yeast extract (Merck, Darmstadt, Germany); 20, glucose. Aldehydes were added in the following concentrations (in μg/l): 100, 2-methyl propanal; 200, 3-methyl butanal; 100, hexanal; 100, furfural (Fluka Chemie GmbH, Steinheim, Switzerland). Barrels with 20 l of CMM were sterilized at 121 °C, 100 kPa for 30 min.

The immobilized cell reactor (ICR)

The gas-lift reactor (GLR) used in this work is of the concentric draught tube type with an enlarged top section for degassing and a total working volume of 2.9 l. The dimensions of the reactor are: total height-76 cm; down comer's lengh-44 cm and inside diameter-7 cm; draught tube length-41 cm, diameter-3.2 cm and thickness-0.4

cm; cylindrical part's length-8 cm and diameter-14 cm. The angle between the conical sector and the main body was 51°. Gas was injected through a perforated plate (diameter 1 cm) with seven holes (diameter 0.5 mm) and placed 2.5 cm below the annulus of the riser. The outflow of the reactor was placed behind a sedimentation barrier, thus minimizing carrier losses. The whole ICR was placed into a thermostated cold room (figure 1). The desired gas flow (air or CO_2+air mixture) was adjusted with a mass flow controller (Aalborg GFC17, Aalborg Instruments, Orangeburg, New York, USA). Dry spent grains were cleaned by acidic hydrolysis (3 % vol. HCl) followed by a delignification in 2 % w/v NaOH. Prior to use, the carrier was washed with water until pH 7 and dried [3].

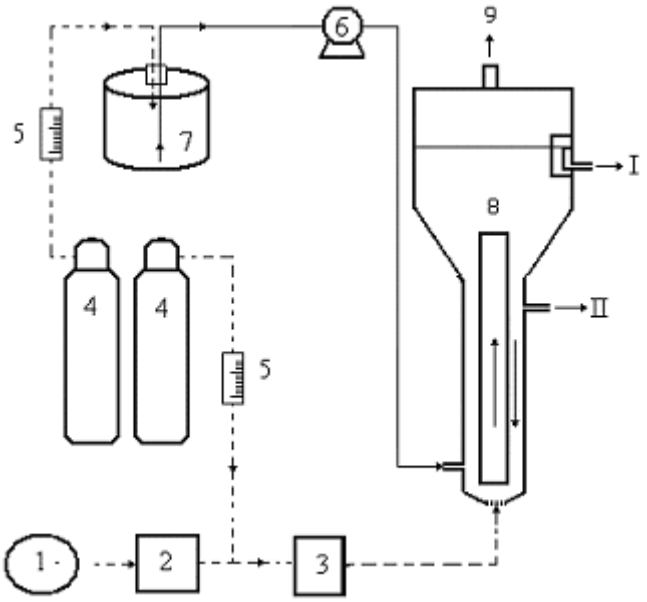

Figure 1: Schematic diagram of the continuous immobilized cell reactor system (ICR) kept in a thermostated cold room: 1 air supply, 2 mass flow controller, 3 gas sterilization filter, 4 CO_2 gas cylinder, 5 rotameter, 6 peristaltic pump, 7 barrel with complex model medium, 8 gas-lift reactor (GLR), 9 gas outlet; I medium outflow, II sampling port.

Starting and operating of ICR

The Plexiglas GLR was sterilized using sodium hypochlorite solution (2 % active chlorine) at least 4 days prior to fermentation. After draining the reactor the sterile air supply into GLR was started at a total flow rate of 0.25 l/min and the GLR was washed with 30 l of sterile water. Prior to inoculation, the reactor was filled with sterilized slurry consisting of spent grains (40 g dry state) in distilled water (1.5 l). Subsequently, the GLR was charged with CMM and then inoculated with 500 ml of yeast cell suspension grown on a rotary shaker at 20 °C for 24 hours. After 24 h of batch growth, the start-up period of the ICR initiated. The CMM was fed at a total residence time of 7 h and the temperature in inside GLR was maintained at 15 °C. Within 10 days a fully developed yeast biofilm was formed around the spent grain particles and afterwards the process parameters (residence time, temperature, aeration) could be changed without causing harm to immobilized yeast biofilm. In order to prevent contamination and oxidation, CMM was kept during the whole experiment in a refrigeration unit at 6-8 °C while CO_2 was sparged into the wort barrel (50 ml/min).

The continuous system was considered to be in steady state after a period of 5 residence times (Rt).

Determination of k_La

The volumetric mass transfer coefficient k_La characterizing the 3 phase system GLR used in this work was determined by the dynamic gassing-out method [4]. The 3 phase system in GLR was consisting of complex model medium (CMM), gas (N_2 or air) and solid support (40 g dry weight/reactor). First, oxygen was desorbed from the liquid phase inside GLR by feeding with nitrogen (250 ml/min). When zero oxygen concentration was reached, the nitrogen flow was stopped and air was fed into the liquid (250 ml/min). The change of oxygen concentration was measured by an oxygen probe (XB4-K/S, Gryf s.r.o., Havlíčkův Brod, Czech republic). The obtained k_La values were used to calculate the oxygen transfer rates [7].

Analytical methods

Ethanol and glucose were analysed by HPLC (Pump LCP 4000, Column oven LCO 101, ECOM Ltd.) using an Polymer IEX Ca form column (250 × 8 mm, Watrex International Inc., San Francisco, USA), and a RIDK 102 refraction index detector (Laboratorní přístroje Praha, Prague, Czech Republic). Elution was performed with Nanopure-filtered water at 85 °C and a flow rate was 0.7 ml/min. The flavour and aroma compounds (higher alcohols and esters) were measured according to the current European Brewery Convention recommended methods [1] while vicinal diketones were determined according to Pivovarsko-sladarska analytika [2]. The aldehydes were determined by solid-phase microextraction using on-fiber derivatization with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBOA). The carbonyl compounds selectively reacted with PFBOA, and the oximes formed were desorbed into a gas chromatograph injection port and quantified by mass spectrometry [19].

RESULTS AND DISCUSSION

Perhaps the most important factor affecting flavour formation in continuous fermentation systems is aeration. Although ethanol production is an anaerobic process, some oxygen is essential for yeast growth, unsaturated fatty acid and sterol synthesis [9]. However, in order to supply the optimum oxygen into bioreactors, the knowledge of volumetric oxygen mass transfer coefficients (k_La) under real fermentation conditions is indispensable. By determining the k_La it was possible to adjust the proper oxygen transfer rate (OTR) and thus to avoid the often observed under- or over-aeration in continuous beer fermentation systems resulting in excessive or poor flavour formation.

The volumetric mass transfer coefficient was determined for a three-phase internal-loop airlift reactor with an enlarged degassing zone. It was shown that the volumetric mass transfer coefficient diminishes with the increase of solids loading due to an increase in bubble coalescence [7]. Reduction of k_La by 65 % was obtained with the introduction of 14 g/l of solids comparing to the two-phase system in GLR without carrier (data not shown). The k_La determined for the maximum solid loading (0.977 h^{-1}) was applied for the estimation of the oxygen transfer rate (OTR) during real fermentation experiments at different mixtures of air with CO_2.

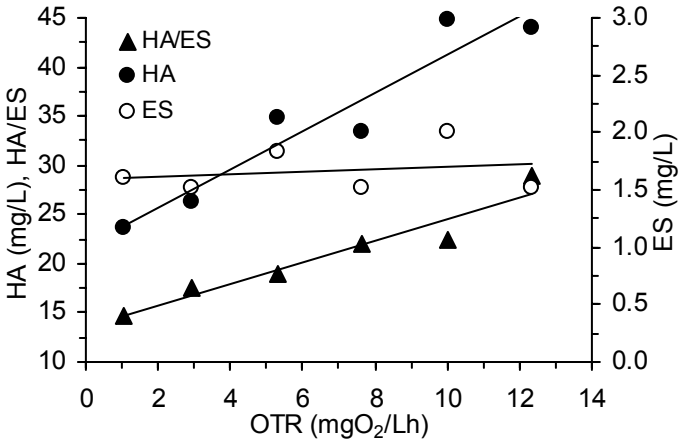

Figure 2: Influence of oxygen transfer rate (OTR) on the concentration of two groups of flavour active compounds (HA-total higher alcohols, ES-total esters) and their mutual ratio (HA/ES) at a constant fermentation temperature (8 °C) and residence time (6 h) in ICR. The ethanol content in the outflow from GLR was approximately 1 % vol.

The experiments studying the influence of OTR on the conversion of flavour active compounds were carried out under conditions (8 °C, residence time 6 h) leading to ca. 1 % vol. ethanol content in fermented CMM (figure 2). The concentration of total higher alcohols and esters at optimum OTR (1 mg O_2/lh) in fermented CMM obtained after dilution to legally admitted level of ethanol in alcohol free beer (0.5 % vol.) was 11.8 and 0.83 mg/l, respectively. These concentrations were in the range of total higher alcohol (9.3-16.9 mg/l) and ester (0.2-1.8 mg/l) concentrations in three commercially available alcohol-free beers produced by traditional batch fermentation (table 1).

The total amount of oxygen delivered into the GLR influenced the concentration of most important flavour active compounds in the outflow from continuous fermentation system. For instance, by increasing the OTR, the total higher alcohols to total esters (HA/ES) ratio increased in the range from approximately 14 to 30 (figure 2). Since the HA/ES ratio in Pilsner type beers is usually between 4 and 6, approaching the same values in alcohol-free beers would be considered as an improvement of the sensorial quality. Therefore, the best ratio of higher alcohols to esters achieved in the continuous system was the one at the lowest oxygen supply (figure 2). The same quality criteria (HA/ES) found in three commercial alcohol-free beers produced by traditional short batch fermentation method was 9.3, 13.3 and 55 rendering the minimum HA/ES ratio obtained in our experiments rather satisfactory.

The interventions based on the stimulation of growth intensity, e.g. dissolved oxygen concentration and temperature, are expected to enhance the higher alcohol formation [16]. Therefore, the HA/ES ratio was influenced mainly by the increasing concentration of HA at higher aeration rate (figure 2).

At the conditions of alcohol-free beer production the total ester concentration both in the products from ICR and commercial samples was rather low (table 1). Simultaneously, the total ester formation in ICR seemed to be independent on OTR (figure 2).

Parameter	CONT	COM1	COM2	COM3	Threshold[a]
Ethanol (v/v %)	0.5	≤ 0.5	≤ 0.5	≤ 0.5	1.77
n-Propanol (mg/l)	1.6	2.0	2.7	2.1	800
Isobutanol (mg/l)	1.6	1.8	1.0	3.85	200
Amyl alcohols (mg/l)	8.6	7.65	5.6	10.95	70
Total higher alcohols (mg/l)	11.8	11.45	9.3	16.9	-
Ethyl acetate (mg/l)	0.58	0.78	0.13	1.44	30
Amyl acetates (mg/l)	0.02	0.06	0.02	0.35	1.2
Ethyl caproate (mg/l)	0.01	0.01	0.01	0.01	0.23
Ethyl caprylate (mg/l)	0.22	0.01	0.01	0.02	0.9
Total esters (mg/l)	0.83	0.86	0.17	1.82	-
HA/ES[c]	14.2	13.7	54.7	9.3	-
Acetaldehyde (mg/l)	8.5	6.9	7.4	0.7	10
Hexanal (µg/l)	0.4[b]	0.2	0.1	0.4	300
2-methyl propanal (µg/l)	0.9[b]	0.3	1.6	4.3	1000
3-methyl butanal (µg/l)	3.5[b]	1.6	7.4	6.8	600
Furfural (µg/l)	0.6[b]	2.7	3.6	5.9	150 000
Total aldehydes (µg/l)	5.4[b]	4.8	12.7	17.4	-

Table 1: Chemical composition of fermented complex model medium (diluted to 0.5 % vol. ethanol content) produced in the continuous immobilized cell reactor system (ICR) and of three commercial alcohol-free beers (AFBs) produced by traditional industrial batch fermentation. CONT: fermented CMM (OTR = 1 mg O_2/lh, temperature 8 °C and 6 h residence time); COM1-3: commercial AFBs available in the Czech republic; [a] Flavour thresholds [10]; [b] Concentration of aldehydes in the undiluted inflow CMM (µg/l): 100, 2-methyl propanal; 200, 3-methyl butanal; 100, hexanal; 100, furfural; [c] total higher alcohols/total esters ratio.

Some authors are regarding the ester formation as a metabolic pathway using superfluous substrates (acyl-CoA and alcohols) at non-growing conditions [18]. According to them, higher wort aeration can affect esters synthesis on one side negatively through reduced availability of acetyl-CoA (used for growth and lipid synthesis) and inhibition of acyltransferases, and on the other side positively by the increased availability of higher alcohols. Thus it can be assumed, that the final ester formation in ICR resulted from the interplay of both enhancing and inhibiting factors. Moreover, the final ester concentration depends, besides on cell physiology, immobilization method and yeast strain, also on wort composition [6]. Therefore, the prediction of ester formation for real alcohol-free beer fermentation using wort from the results obtained in ICR fed by CMM is rather difficult.

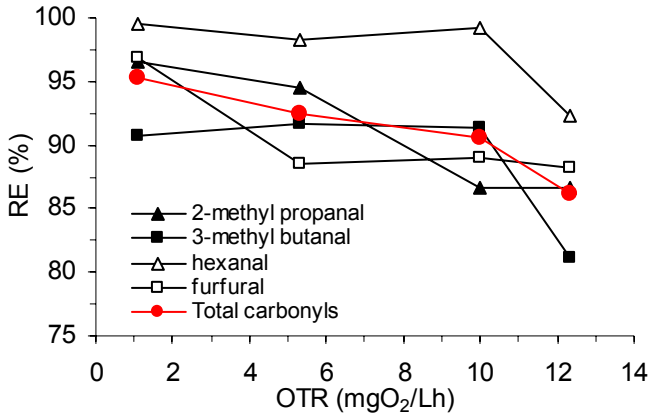

Figure 3: Reduction efficiency (RE) of individual and total aldehydes vs. oxygen transfer rare (OTR) at a constant fermentation temperature (8 °C) and residence time (6 h) in ICR.

A significant wort aldehyde reduction was observed in ICR. The total aldehyde reduction varied in the range from 86 to 95 % with higher reduction efficiency at lower aerations (figure 3). This is in accordance with former observations concluding that fermentative metabolism is more efficient with regard to aldehyde reduction. Another earlier observation, namely that the most rapidly reduced are the linear saturated aldehydes, was also proved [5]. In general, the aldehyde reduction potential of the immobilized and free biomass inside ICR seems to be sufficient to reduce the aldehydes of real wort used for alcohol free beer production. This statement is based on the fact that the total aldehyde content of a 8 °P wort (brewed at the ICT microbrewery, Prague, Czech Republic) was by ca. 50 % lower than that of the CMM applied in this work (data not shown). The aldehyde reduction efficiency of cells in ICR was better or equal to that from the continuous system with silicon carbide and DEAE-cellulose as immobilization materials [5]. However, it can be assumed that a significant part of aldehydes are not reduced only through fermentation but are removed chemically by binding to amino acids or proteins of CMM [13].

Conversely to afore mentioned aldehydes, other carbonyl compounds with sensorial activity such as vicinal diketones (VDKs = diacetyl and 2,3-pentanedione) and acetaldehyde are mostly formed during the active growth phase of yeast. However, there is only little biomass growth and glucose consumption under conditions of 1.0 % vol. ethanol formation (8 °C, OTR = 1 mg O_2/lh, Rt = 6 h). Consequently the fermented CMM diluted to 0.5 % vol. ethanol contained 0.05 mg/l total VDKs and 8.5 mg/l acetaldehyde. At the given level, the total VDKs content does not represent any serious flavour issue since the taste threshold limit of VDKs (0.25 mg/l) is much higher [2]. Although the acetaldehyde content in CMM (0.5 % vol. ethanol) approaches the taste threshold limit of 10 mg/l (table 1) it does not exceed it. Higher OTRs, however, stimulate the acetaldehyde synthesis leading to a concentration of 23 mg/l at 12 mg O_2/lh. Whether the higher acetaldehyde formation is a particularity of the used yeast strain or an effect of the CMM composition will have to be verified by further experiments.

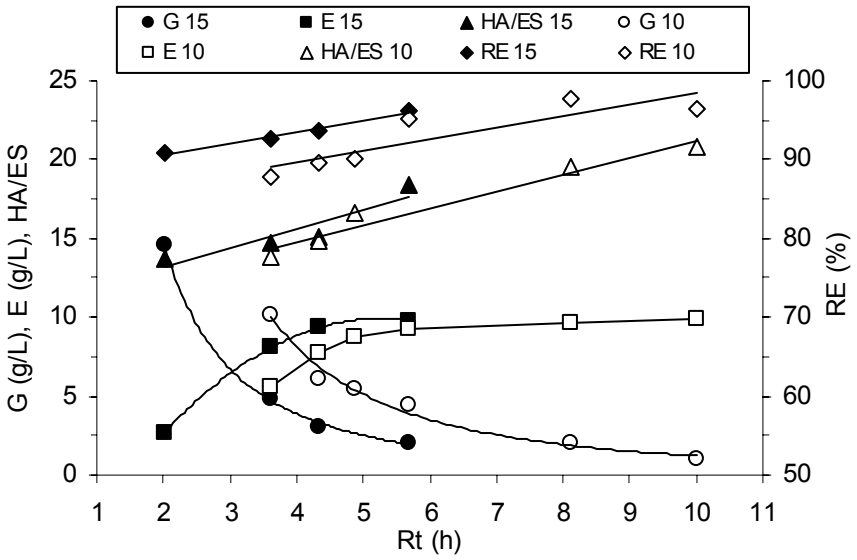

Figure 4: Selected system parameters in the effluent (E-ethanol, G-glucose, HA/ES-total higher alcohols to total esters ratio, RE-total aldehyde reduction efficiency) vs. Rt-residence time in ICR at 10 and 15 °C and constant oxygen transfer rate (OTR) of 1 mg O_2/lh.

The increasing residence time (Rt) of the complex model medium (CMM) resulted in a gradual increase of the degree of fermentation, noticeable from the increasing glucose consumption and ethanol formation (figure 4). Besides, the influence of temperature (10 °C and 15 °C) and residence time (Rt) in ICR on the formation of flavour active volatiles and reduction of aldehydes was also studied (figure 4).
Simultaneously with the progress in the degree of fermentation, the HA/ES ratio increased too. By changing the fermentation temperature and Rt, the HA/ES ratio varied in the range from 14 to 21 (figure 4). Not surprisingly, since the final concentration of higher alcohols is determined also by the extent of carbohydrate metabolism [15]. In other words, the increasing HA/ES ratio can be ascribed mainly to more intensive higher alcohol formation as a result of the progress in fermentation. Hence, the ratio of volatile fermentation by-products (HA/ES) is more favourable at lower degree of fermentation leading to 0.5 % vol. ethanol (figure 4).
The total aldehyde reduction efficiency (RE) in fermented complex model medium (CMM) containing 0.5 % vol. ethanol (3.945 g/l) was approximately 91 % and 87 % at 15 °C and 10 °C, respectively (figure 4). Although the RE increases with the prolonging contact (residence time) between cells and CMM, the RE values at 0.5 % vol. ethanol in fermented CMM can be considered satisfactory taking into account that aldehydes were added into CMM in excess.

CONCLUSIONS

Generally it can be concluded, that the process parameters (oxygen transfer rate, residence time, temperature) are instruments which allow us to some extent control the flavour formation in continuous immobilized cell reactor system (ICR). The following conclusions can be stated as regards the operation of ICR and flavour composition of continuously fermented model medium mimicking the production of alcohol-free beer:

(i) The volumetric productivity of the bioreactor was found to increase with increasing temperature of fermentation.
(ii) The total fusel alcohols over total esters ratio (HA/ES) increased both with prolonged residence time and higher oxygen supply. The adjustment of oxygen supply, based on k_La determination in 3 phase GLR, was particularly suitable for fine tuning the HA/AS ratio. The lowest HA/ES ratio found in continuously fermented model medium (14) was comparable with those found in three commercial alcohol-free beers (9.3, 13.3 and 55).
(iii) The wort aldehyde reduction capacity of the ICR is assumed to be sufficient. It is based on the fact that the reduction of total aldehydes, added in excess into model medium, was very efficient (85-95 %).
(iv) The concentration of flavour active compounds such as vicinal diketones and acetaldehyde was in the complex model medium fermented under optimized conditions below the taste threshold.

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