Preliminary optimization study of alcohol-free beer production in a continuous immobilized cell reactor system

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Introduction

In EU countries a beer is considered alcohol-free when its alcohol concentration does not exceed 0.5 % volumetric. 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 alcohol formation. The production of alcohol-free beer using immobilised yeast cell systems rank among methods of controlled fermentation using short but intensive contact between immobilized yeasts and wort. The approaches taking advantage of the suppression of alcohol formation or its early interruption are economically more attractive.

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 flavour and to produce fusel alcohols and esters. In order to study the formation and conversion of the most important off-flavours, the real wort used in alcohol-free beer fermentation was simulated by a defined synthetic medium containing glucose, yeast extract and aldehydes (hexanal, pentanal, 3-methybutanal, furfural).

Several carbonyl compounds present in wort have high flavor potency (3-methyl butanal, 2-methyl butanal, hexanal, heptanal etc.). They contribute largely to the worty off-flavors detected particularly in low-alcohol beer produced by limited fermentation. Beer aldehydes arise mainly during wort production (mashing, boiling) and partially are formed during fermentation from yeast oxoacid pools both via anabolic process from carbon source and catabolic pathway from exogenous amino acids. The reduction of wort aldehydes is crucial namely in alcohol free beer production[1,2] .

In fermentations of alcohol-free beer it is also important to control the level of VDKs (diacetyl and 2,3-pentanedione) because their relatively low taste threshold (0.25 mg/l)[3]. Diacetyl and 2,3-pentanedione are by-products of the pathways leading to the formation of the amino acids valine and isoleucine, respectively. The α-acetohydroxy acids are partially excreted into the fermenting wort where they undergo spontaneous oxidative decarboxylation, giving rise to vicinal diketones. Diacetyl is then taken up by the yeast and reduced to acetoin and then 2,3-butanediol. The flavor thresholds of the latter compounds are relatively high[4].

Several alcohols, other than ethanol are formed in beer during fermentation, among which n-propanol, iso-butanol and isoamyl alcohols (2-methyl and 3-methly 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[5,6], wort composition, fermentation conditions, immobilization method and reactor design[7,8].

The synthesis of aroma-active esters by yeast is of 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 AAT synthesis and activity or substrate concentrations will influence ester production[9].

Fermentation experiments were carried out in a gaslift reactor with brewing yeast immobilized on spent grains. During continuous experiment, parameters such as dilution rate (D), temperature (T) and aeration were changed to find the optimal conditions for alcohol-free beer production. The formation ethanol, VDKs, reduction of aldehydes, and consumption of glucose were observed during the long-term fermentation experiment.

Experimental

Microorganisms and Medium

The brewing yeast strain (Saccharomyces cerevisiae) supplied by the company UNICER (Bebidas de Portugal, S.A., S. Mamede de Infesta) was used. The yeast were cultivated in a complex model medium (CMM) which composition was (in g/ L): 5, KH_2PO_4; 2, $(NH4)_2SO_4$; 0,04, $MgSO_4.7H_2O$; 2, yeast extract (Merck, Darmstadt, Germany); 20, glucose. Aldehydes were added in the following concentrations (in µg/ L): 30, 3-methyl butanal; 1, pentanal; 1, hexanal; 7, furfural (Fluka Chemie GmbH, Steinheim, Switzerland). Barrels with 20 litres of CMM were sterilized by autoclaving at 121 ºC, 100 kPa for 30 min. Antifoam A (Fluka Chemie, Steinheim, Switzerland) was added to CMM prior to sterilization (0.01mL/L).

The immobilized cell reactor system (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 51º. Gas was injected through a perforated plate (diameter-1 cm) with seven holes (diameter-0.5 mm, each) and placed 2.5 cm bellow the annulus of the riser. The outflow of the reactor was placed behind a sedimentation barrier, thus minimizing carrier losses. The reactor was placed into a refrigerator maintaining the desired temperature inside the reactor (Fig.1). The desired gas flow (CO_2, air or both mixed) was adjusted with a mass flow controller (Aalborg GFC17, Aalborg Instruments, Orangeburg, New York, USA).

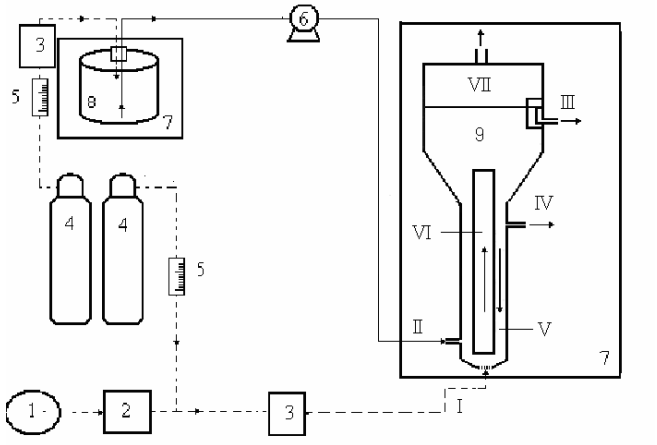

Fig.1: Schematic diagram of the continuous immobilized cell reactor system (ICR) and its set up: 1 air supply, 2 mass flow controller, 3 gas sterilization filter, 4 CO_2 gas cylinder, 5 rotameter, 6 peristaltic pump, 7 refrigeration unit, 8 barrel with complex model medium, 9 gas-lift reactor (GLR); I gas inflow, II medium inflow, III outflow of fermented medium, IV sampling port, V downcomer, VI riser, VII degassing zone

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 reactors the sterile gas supply into GLR was started at a total flow rate of 0.25 l min^{-1} 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 synthetic medium started being fed into GLR at a total residence of 9 h, which was after 120 h of operation decreased to 7.5 h. In order to prevent contamination and oxidation, wort was kept during the whole experiment in a refrigeration unit at 6 - 8 °C while CO_2 was sparged into the wort barrel. During fermentation the total gas flow rate (GF) in the reactor (mixture of air and CO_2) was kept at 0.25 l min^{-1}, with different proportions of air flow (AF) in the mixture. The continuous system was considered to be in steady state conditions after a period of 5 residence times (Rt).

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 (250x8 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 (Analytica-EBC, 2000)[10].

Vicinal diketones were determined according to Pivovarsko-sladarska analytika III.

Analysis of the immobilized cells from the continuous experiment: the biocatalyst (carrier + immobilized cells) was taken from the reactor through the sampling port and washed with 2 × 100 mL of distilled water, then 50 mL of synthetic medium without glucose and yeast extract were added and agitated with a magnetic stirrer (2 cm bar, 200 rpm) for 20 min. After the agitation the biocatalyst was allowed to sediment for 4 min and the released biomass from the supernatant was either used for viability staining measurement or underwent a fixation process (cells were centrifugated consequently resuspended in 70% ethanol and kept at 4°C for 24 hours) prior to further analyses of glycogen. The flow cytometric measurements were taken using a Partec Pas III (Partec GmbH, Münster, Germany) analyzer equipped with an argon ion laser (15 mW laser power with excitation wavelength 488 nm).

The relative content of glycogen in cells was determined using Acriflavine, which is a fluorescent dye able to covalently bind to polysaccharide glycogen after permeabilisation of the cell membrane by ethanol (fixation). An aliquot of 0.5 mL (1.10^6 cells/ml) of the sample was removed and 10 µL of Acriflavine (Sigma Aldrich, Germany) solution (1 mg/mL in PBS, stored at 4ºC) ware added. Incubation occurred at room temperature in darkness for 30 min. The sample was then analyzed by flow cytometry in FL1 channel (530 nm).

The viability was determined by flow cytometry using fluorescent probe Propidium Iodide (PI). Cells (200-400 cells/s) were stained with 10 µl PI (1mg/ml in PBS) for 1 min. The fluorescence signal intensity was acquired in FL3 channel (630 nm).

Results and discussion

Glucose consumption, formation of ethanol and flavour components, and aldehyde reduction were monitored during the continuous fermentations of complex model medium (CMM). The continuous immobilized cell reactor system (ICR) showed complete glucose consumption until residence times (Rt) 6 and 8 hours at temperatures 15 and 10 °C, respectively (Fig. 2).

During fermentations at 10°C and 15°C it was found that the increasing Rt in ICR resulted in decreasing ratio of fusel alcohols over esters (A/E) (Fig. 2). This tendency was caused by lower fusel alcohol and higher ester formation at long Rt (Fig. 3). If the ester formation is regarded as a metabolic pathway using superfluous substrates at non-growing conditions (acyl-CoA and alcohols) the increased ester formation at high Rt and complete glucose consumption is expected. However, there is only a slight increase of ester formation at longer contact of CMM with cells. On the contrary, the higher alcohol in the fermented medium decreased more significantly resulting in lower A/E ratio at high Rt. It can only be speculated weather it was caused by fusel alcohol re-assimilation or stripping from the reactor by driving gas. The sudden drop of fusel alcohol and ester formation at short Rt can be ascribed to growth conditions at incomplete glucose consumption (Fig. 3). At these conditions the substrates of ester synthesis are utilized for growth and/or the alcohol acyltransferase, responsible for ester synthesis, is inhibited. Although, during the experiment the appropriate A/E ratio for pilsner type of a lager beer has not been achieved, it is essential to say that the ratio of aroma compounds in alcohol-free beers is usually different from that in regular beers.

Significant wort aldehyde reduction was observed in ICR. The total aldehyde reduction varied in the range from 40 to 70 % with higher reduction efficiency at longer Rt (Fig. 4). It is expected that reduction of aldehydes decreases significantly in the absence of glucose[11]. Indeed, reduction of aldehydes was significantly lower at longer Rt when glucose was consumed. Of course, the residence time also determines the degree of aldehyde reduction. This can explain the sudden decrease at short Rt (Fig. 4).

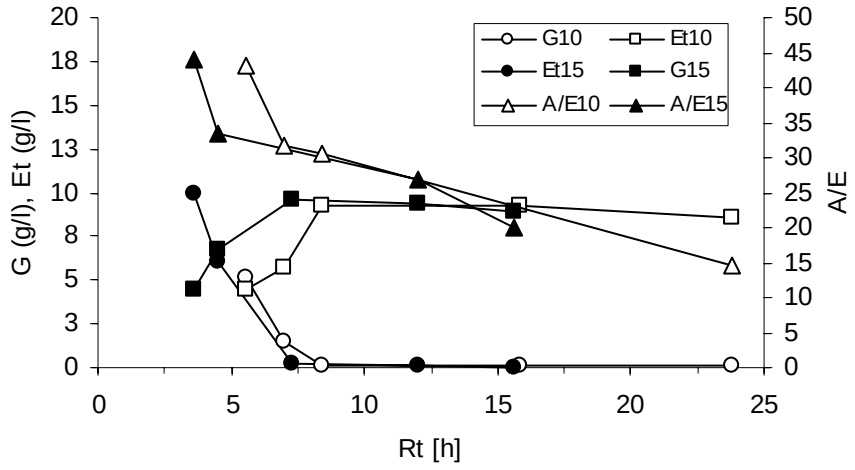

Fig. 2 Selected system parameters in the effluent (Et-ethanol, G-glucose, A/E-fusel alcohols to ester ratio) vs. Rt-residence time in ICR at 10 and 15°C.

Productivity of VDKs increased with shorter Rt (increasing dilution rate) (Fig. 4).Under the conditions of steady state the productivity of VDKs is equal to the growth rate of yeast. The higher is the growth rate the higher is the production of VDKs because α-acetohydroxy acids (precursors of VDKs) are linked to anabolic pathways of amino acids[12].

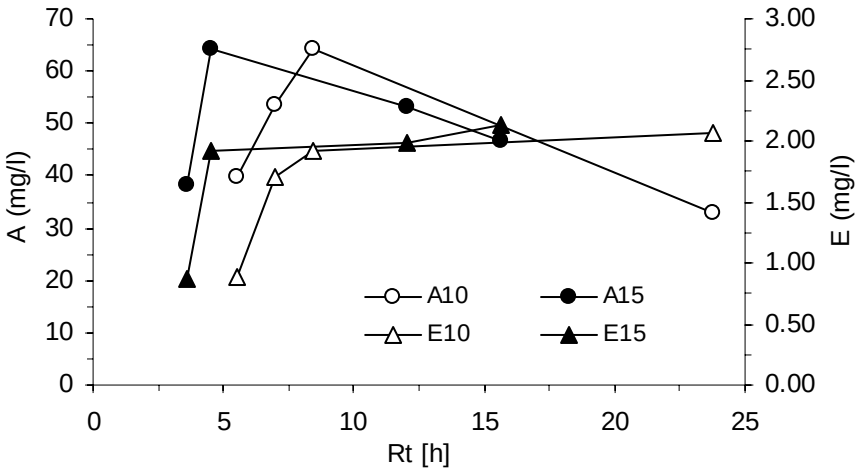

Fig. 3 Formation of higher alcohols and esters (E-esters, A-higher (fusel) alcohols) vs. Rt-residence time in ICR at 10 and 15°C.

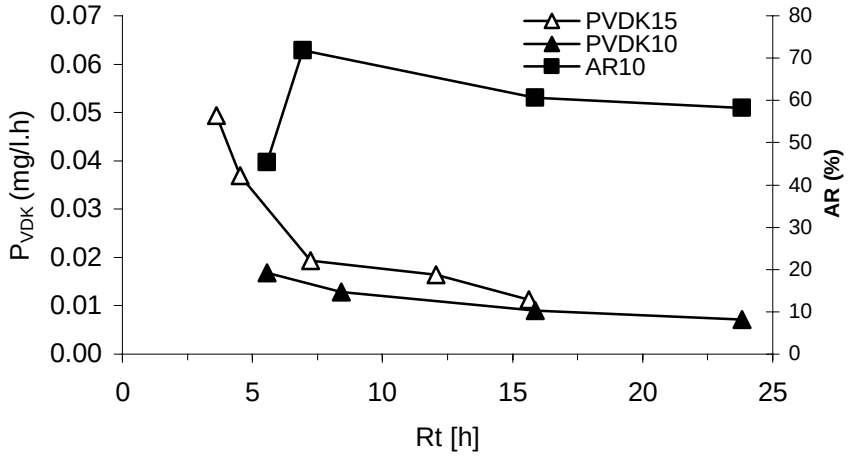

Fig. 4 Productivity of vicinal diketones (P_{VDK}) and total aldehyde reduction (AR) vs. Rt-residence time in ICR at 10 and 15°C.

The ethanol concentrations in the fermented complex medium, which correspond to the legally admitted level in alcohol free-beer (0.5% vol. or 3.945 g/L), were at different temperatures produced at different medium flow rates (D) in ICR (Fig. 5). Under these conditions the glucose consumption was incomplete (Fig. 2) while the yield of glucose conversion to ethanol was almost constant and independent on temperature (Fig. 5). VDK formation also increased with temperature; however, its values in the model medium never exceeded the taste threshold (0.25 mg/l)[3]. Two hypotheses can be formulated concerning the positive influence of temperature on VDK formation: (i) the conversion of α-acetohydroxy acids to VDKs is a temperature dependent chemical decarboxylation reaction and therefore the final VDK concentration increased with temperature and (ii) since the VDK formation is linked with biomass growth, the higher requirements for maintenance energy at low temperatures decreased the biomass yield ($Y_{X/S}$) and thus the total amount of excreted VDK precursors. A decreased α-acetohydroxy acids formation during alcohol-free beer production at low temperatures was observed by other authors as well [13].

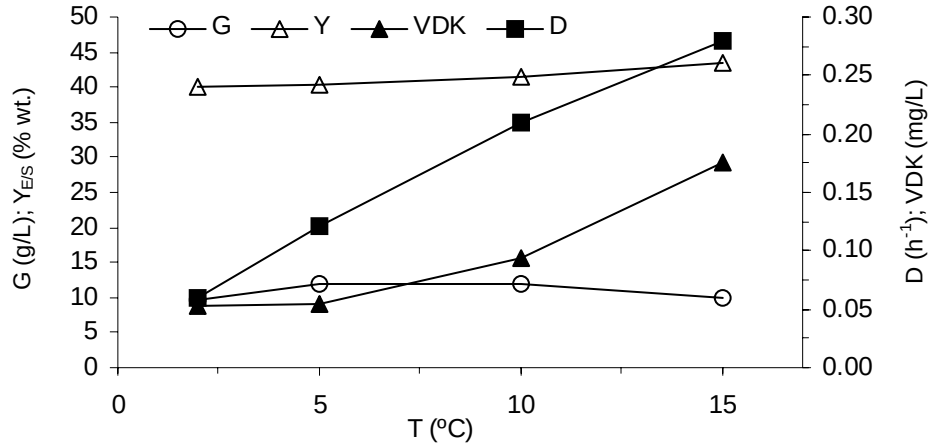

Fig. 5 Selected system parameters in the effluent (G-glucose, $Y_{E/S}$- ethanol yield, VDK-vicinal diketones, D-dilution rate) at constant ethanol concentration in the outflow from GLR (0.5 % vol.) at different temperatures.

During long-term fermentation experiment the immobilized cells viability was decreasing. In order to avoid the undesirable effect of high dead cell number on product quality, a part of the "old" biocatalyst was regularly replaced by new carrier. This biocatalyst renewal strategy is based on the ability of brewing yeast to colonize the spent grain particles[14].The biocatalyst replacement resulted in regular decrease of immobilized cell numbers and subsequent immobilization of new carrier. Hence, the immobilized dead cell numbers decreased prolonging thus the operational stability of ICR (Fig. 6). Apart from bioreactor long-term stability an interesting relationship between viability and intracellular glycogen content of immobilized cells was observed. Seemingly, the glycogen accumulation in immobilized cells precedes the increase of dead cell numbers as if the decrease of cell vitality would induce the accumulation of intracellular reserve polysaccharides. This phenomenon could be in the future exploited as an indicator of immobilized cell aging signalizing the necessity of biocatalyst replacement.

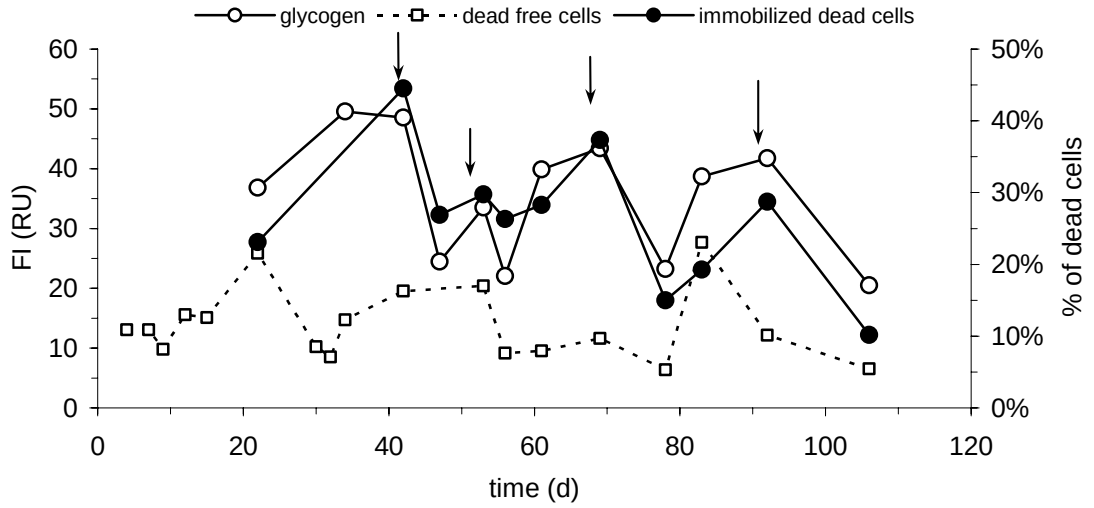

Fig. 6. Intracellular glycogen content, expressed as fluorescence intensity (FI) in relative units (RU), of immobilized cells and viability of free and immobilized cells during long-term continuous fermentation experiment. Arrows indicate replacements of "old" biocatalyst by clean carrier.

Conclusion:

Generally it can be concluded, that process parameters (dilution rate, temperature) represent a powerful tool in controlling the degree of fermentation and flavour formation carried out by immobilized biocatalyst. The productivity of the bioreactor was found to increase with increasing temperature of fermentation. The fusel alcohols over esters decreased with prolonged residence time and the reduction of total aldehydes varied in the range 40 – 70 %. A relationship between the cell glycogen content and cell viability was found and this parameter will be studied for possible use in scheduling the regular replacement of carrier with old immobilized cells. These results allow pre-selecting the conditions suitable to perform real alcohol-free beer fermentation experiment. However, further experiment will have to be carried out in order to optimize aeration and biocatalyst replacement.

Acknowledgement

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