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Flavor formation and cell physiology during the production of alcohol-free beer with immobilized *Saccharomyces cerevisiae*

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Production of alcohol-free beer by limited fermentation is optimally performed in a packed-bed reactor operating in downflow. This ensures a highly controllable system with optimal reactor design. In the present study, we report on changes in the physiology of immobilized yeast cells in the reactor. During production, a simultaneous increase in the activity of alcohol acetyl transferase, and formation of the esters ethyl acetate and isoamyl acetate were observed. In addition, the amount of unsaturated fatty acids decreased significantly. Since these phenomena coincided with a significant decrease in growth rate, we conclude that the anaerobic conditions, and the absence of substantial levels of unsaturated fatty acids in wort limit cell growth during production and stimulate formation of acetate esters. Low temperatures (2°C) appeared to suppress production of α-acetolactate whereas at 12°C, substantial levels were obtained. An optimal and constant flavor profile of the alcohol-free beer can be achieved by introduction of regular aerobic periods to stimulate yeast growth. Temperature can be used to control the rate of growth as well as the rate of flavor formation.

Keywords: Brewer's yeast; DEAE-cellulose; immobilization; physiology; alcohol-free beer; flavor; process control

Introduction

Over the last 5–10 years, several methods have been developed for production of low-alcohol or alcohol-free beer.[1] Both removal of alcohol as well as limited fermentation are successfully applied. In the case of limited fermentation, production is most efficient when using an immobilized system due to the advantages of immobilized systems to suspended batch systems, viz. high controllability, flexibility, and high biomass concentration which allows short contact times.[2–4] Such a system was developed and is being applied successfully for several years already.[5,6] During the production of alcohol-free beer, wort aldehydes are reduced by the activity of the alcohol dehydrogenases of the yeast,[7] and optimally a beer flavor is formed without substantial levels of ethanol or off-flavors such as diacetyl.[8]

Fermenting cells of *Saccharomyces cerevisiae* generally produce high levels of estery flavors. Final levels in the end-product are dependent on environmental conditions as well as strain characteristics. Generally, factors that lead to more vigorous fermentations such as high sugar concentrations, increased temperature and low levels of oxygen will increase ester formation.[9] The acetate esters, such as ethyl acetate and isoamyl acetate are the most pronounced group and are synthesized by alcoholysis of acetyl-coenzyme A (acetyl-CoA). Since acetyl-CoA is an intermediate in lipid biosynthesis, ester production is closely linked to the metabolism of lipids. As a result, production is low during the

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growth phase when lipids are accumulated and enhances significantly upon the increase of intracellular concentrations of acetyl-CoA during stationary phase.[9] Recently, Malcorps *et al.*[10] suggested that ester synthesis is related to the induction of alcohol acetyl transferase (AAT) rather than to increased availability of acetyl-CoA. This was corroborated by Fujii *et al.*[11], who showed that expression of the AAT gene is suppressed by oxygen and unsaturated fatty acids.

In fermentations performed by *S. cerevisiae*, α-acetolactate, an intermediate in amino acid metabolism, is synthesized from pyruvate by acetohydroxy acid synthase (AHAS).[12,13] Overproduction will lead to leakage from the cell into the medium where it decarboxylizes into diacetyl, an important off-flavor in beer. Both higher temperatures and increased oxygen concentrations were reported to increase the amount of α-acetolactate by influencing the rate and extent of yeast growth.[14] In addition, a clear correlation exists between the maximum concentration of vicinal diketones and the minimum concentration of free amino nitrogen.[15] In the present article, we report on the production of alcohol-free beer by a Brewer's yeast strain of *S. cerevisiae*. In the reactor, flavor formation and yeast physiology are changed due to the anaerobic conditions and immobilization. Manipulating the flavor of the outflow from the reactor by controlling temperature and oxygen is discussed.

Materials and methods

Chemicals

Bis[2-hydroxyethyl]-imino-tris[hydroxymethyl]-methane (Bis-Tris), 2-[*N*-morpholino]-ethanesulfonic acid (MES), acetyl CoA, and dithiothreitol (DTT) were supplied by Sigma Chemical Company (St Louis, MO). Thiamin pyrophosphate (TPP) and coenzymes (except acetyl CoA) were purchased from Boehringer (Mannheim, Germany).

Stain and growth conditions

A bottom-fermenting Brewer's yeast strain of *Saccharomyces cerevisiae* var. *uvarum* strain W34 was provided by the Institute of Weihenstephan (Technical University of Munich, Germany). *S. cerevisiae* W34 was grown in the wort (12°Plato). Aerobic batch growth was performed in a temperature-controlled rotary shaker operated at 150 rpm using shake flasks. Anaerobic growth was performed in the same wort; ergosterol and unsaturated fatty acids were supplied as mentioned by Visser *et al.*[16] Growth was followed by measuring the optical density (OD) increase at 600 nm.

Limited fermentation

Production of alcohol-free beer was performed by a limited fermentation using a packed-bed reactor, operated under downflow conditions.[5,6,8] The reactor volume of 1.5 m^3 is loaded with 1 m^3 of carrier. The flow rate and temperature are set approximately 0.5 m^3 h^{-1} and 4°C. Dependent on yeast growth and product concentration, temperature and flow rate are adjusted to values of 0–2°C and 2 m^3 h^{-1}. Generally, the reactor is operated for 5–7 months.

In the reactor, the yeast cells are attached to the surface of the carrier. This carrier is a granular material consisting of polystyrene coated with DEAE-cellulose, and is provided by Cultor® Ltd., Finnsugar Bioproducts (Helsinki, Finland). The material is non-porous and not-compressible and particle sizes range from 0.3 to 0.8 mm. The carrier is inert and can be sterilized at 80°C with NaOH (2% w/v). The characteristics enable its use in a packed-bed reactor.

Cell sampling and preparation of cell extracts

For determination of enzyme activities and cell composition, carrier material was regularly taken from the reactor. Cells were detached by washing the carrier in a physiological salt solution and sonifying it mildly in a waterbath-sonicator. During the entire procedure, samples were kept on ice. In case of aerobic or anaerobic batch growth, cells were harvested at the end of the logarithmic growth phase. Cells were washed with 0.1 M BisTris buffer pH 7.0, 1 mM DTT, and stored in the same buffer at −80°C.

Cells were broken by vortexing with an equal volume of glass beads (diameter varying from 0.1 to 1 mm). Samples were vortexed (maximum speed) five times for 45-s periods, alternating with 45-s cooling periods (on ice). Cell debris was analyzed microscopically, and generally more than 90% of the cells were broken. Cell debris was removed by centrifugation (45,000 *g* for 20 min twice), and the cell extract (CE) was stored in different aliquots at −80°C. The activities of the enzymes did not decrease significantly during the storage period.

Enzyme assays

During the *in vitro* enzyme assays, all activities were linearly proportional with the amount of protein and with time. Reaction conditions for enzyme assays were as follows.

Acetohydroxy acid synthase (AHAS). 100 mM KP_i, pH 8.0, 5 mM $MgCl_2$, 1 mM TPP, 100 mM pyruvate, and 0.2 mM FAD. The reaction was stopped by addition of 3.5 μl 3 M H_2SO_4 per 200 μl reaction mixture. The α-acetolactate formed in the reaction is readily decarboxylated to acetoin or diacetyl upon heating in acid.[17] Both were determined by gas chromatographic analysis of the static headspace (GC-HS).

Alcohol acetyl transferase (AAT). The assay to determine the activity of alcohol acetyl transferase (AAT) was adopted from Malcorps and Dufour;[18] 50 mM KP_i, pH 7.5, 15 mM isoamyl alcohol, 0.8 mM acetyl-CoA. The reaction was stopped by lowering the pH to approximately 3 by addition of 2 μl 3 M H_2SO_4 per 200 μl reaction mixture. The amount of isoamyl acetate was determined by GC-HS. One unit is defined as the activity which produces 1 μmol isoamyl acetate min^{-1}.

Rate assays

The rates of formation of esters and α-acetolactate were determined per unit biomass. Carrier material was taken from the reactor and was incubated anaerobically in wort of

12°P at two different temperatures, 2°C and 12°C. At regular time intervals, samples were taken, filtered over a 0.45-μm filter, and subsequently frozen. No influence of storage procedure on the flavor composition of the samples was observed. Esters and α-acetolactate were analyzed by gas chromatography as described in the following.

Analytical methods

The concentration of the flavor compounds was determined by gas chromatographic analysis of the static headspace (GC-HS). The gas chromatograph (HRGC 5300 Mega series, Carlo Erba Instruments, Milan, Italy) was supplied with a cold trap. Samples were incubated for 30 min at 60°C in a temperature incubator (HS800, Fison Instruments, Interscience, Breda, The Netherlands) and were rotated for periods of 30 s at 1,500 rpm with 30-s time intervals. During this incubation, α-acetolactate is readily converted to diacetyl.[19] Headspace (2.5 ml) was taken and injected in the cold trap (−110°C). After purging the cold-trap, the sample was injected on the column by heating to 240°C (MFA 815, Fison Instruments). Carrier gas was helium (0.3 kPa), the column was DB-Wax, 30 m × 0.542 mm, film 0.1 μm (J & W, Interscience) and a temperature program was used starting at 30°C and heating the oven at 2.5°C min^{-1} to 110°C. Detection occurred with an FID-detector (EL 980, Fison Instruments). The detection limit of this system for diacetyl is estimated at 0.2 μM.

Protein concentrations were determined according to the method of Lowry *et al*.[20] Dry weights (DW) were measured by filtration over predried and preweighed nitro-cellulose filters (0.45 μm). Filters were dried at 80°C.

Analysis of fatty acids and sterols

Cells were harvested, washed in 0.1 M MES buffer pH 6.0, and spun down. Lipids were separated by three subsequent extractions with methanol/chloroform (ratio 1:2). The chloroform fractions were pooled and dried with a stream of N_2 gas. The fatty acid composition in the lipid fraction was determined by gas chromatography of the methyl esters following the methylization of the lipid fraction with anhydrous, acid methanol. Experimental details are according to Verheul *et al*.[21] Sterol composition was determined by direct injection of the lipid fraction onto a capillary, apolar GC column SGE BP-1, 12 m × 0.53 mm, film 0.1 μm (SGE, Milton Keynes, U.K.). Sterols were separated with a temperature gradient of 220–300°C using helium as a carrier gas (0.21 kPa).

Results and discussion

Fermentation and growth

Production of alcohol-free beer is performed at low temperature and under anaerobic conditions.[6] These conditions significantly influence cell composition and metabolism; therefore, the fatty acid composition of immobilized cells, harvested during alcohol-free beer production, was determined. The composition changed significantly. Both the average length and the amount of unsaturated fatty acids decreased substantially (*Figure 1*). In addition, a small increase was observed in the total amount of fatty acids (data not shown).

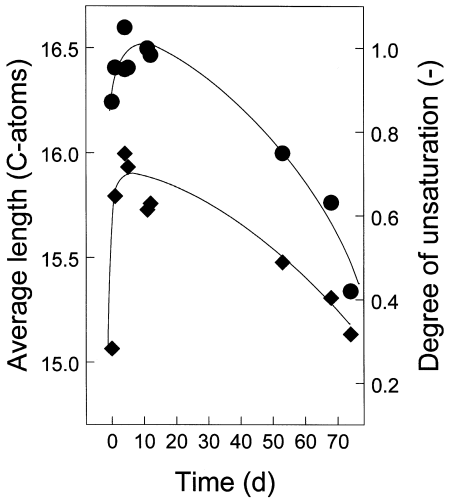

Figure 1 Average chain length (●) and degree of unsaturation (♦) of fatty acids from *S. cerevisiae*. Cells were harvested during production of alcohol-free beer, detached from the carrier and fatty acids were determined according to *Materials and methods*

In addition to fatty acid analysis, the sterol composition was determined during production. A significant increase was observed in the relative concentration of squalene after a prolonged production period whereas concentrations of ergosterol and lanosterol decreased (*Table 1*). *S. cerevisiae* needs molecular oxygen to synthesize unsaturated fatty acids and sterols. In the synthesis of sterols, squalene is the last intermediate prior to the reactions which involve oxygen.[22] With suspended batch-grown cells, it has been shown that squalene accumulates during anaerobic conditions.[23] Apparently, this is also the case with immobilized *S. cerevisiae*.

In theory, the decrease in unsaturated fatty acids, as seen with cells growing in the anaerobic bioreactor, would result in a decrease in the fluidity of the membrane whereas the simultaneous decrease in chain length has an opposite effect. The changes in degree of unsaturation and chain length apparently enable the cells to grow and maintain membrane function for several division cycles. When growth was determined in the bioreactor, cells indeed appeared to be able to grow for a period of approximately 20 days.[24] Although

Table 1 Relative concentrations of squalene, ergosterol, and lanosterol in *S. cerevisiae* during the production of alcohol-free beer

Time (day)	Squalene (%)	Ergosterol (%)	Lanosterol (%)
0	77	14	8
5	57	33	10
53	86	11	3
68	89	9	2
74	88	11	2

cells were continuously supplied with substrates and nutrients present in the wort, growth decreased after this period since the wort only contained very limited amounts of unsaturated fatty acids (Van Dieren, personal communication).

The decrease in growth and the changes in membrane composition coincided with an increase in the formation of acetyl esters (*Figure 2*). Both production of ethyl acetate and the production of isoamyl acetate were low during the first 20 days, but increased significantly following the decrease in growth rate. At a higher fermentation temperature, an additional increase was observed. Production rates at 12°C were approximately two- to fourfold higher compared to rates at 2°C (*Figure 2*).

Ideally, flavor formation is already high from the start of the production period; therefore, we determined the activity of the ester-producing enzymes, i.e., the AAT. It appeared that upon the decrease in the amount of unsaturated fatty acids, an increase in the activity of AAT was observed (*Figure 3*). In addition, we determined AAT activity in batch-grown cells (12°C). Under aerobic conditions, activity was approximately 0.005 ± 0.001 U g^{-1} protein, but was increased significantly under anaerobic conditions to 0.053 ± 0.007 U g^{-1} protein. Activity appeared to be comparable to the activity observed during prolonged alcohol-free beer production. The increase in AAT under anaerobic conditions coincides well with the low amount of unsaturated fatty acids since enzyme synthesis is induced at low concentrations of these fatty acids.[11,18] Therefore, we may conclude that during alcohol-free beer production, ester formation increases as a result of a decrease in unsaturated fatty acids.

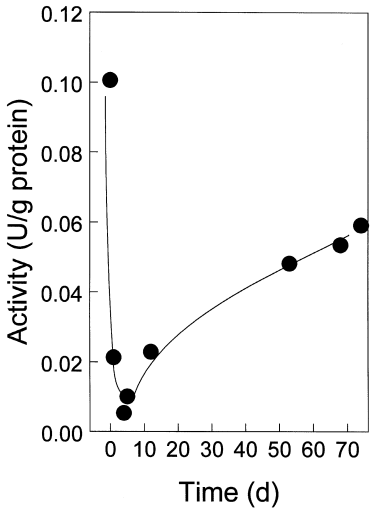

Figure 3 Alcohol acetyl transferase (AAT) in cell extract from *S. cerevisiae*. Cells were harvested during alcohol-free beer production and AAT was measured according to *Materials and methods*

Acetohydroxy acid synthesis

The flavor of beer is strongly affected by the amount of α-acetolactate since during storage this is converted to the strong off-flavor diacetyl; therefore, the *in vitro* activity of AHAS was determined. It appeared that enzyme activity was significant (38 ± 5 U g^{-1} protein), but comparable to values determined with batch grown cells (29 ± 3 and 25 ± 4 U g^{-1} protein for aerobically and anaerobically grown cells, respectively).

In addition, production of α-acetolactate was studied *in vivo* by fermentation of the wort with cells harvested from the reactor (*Figure 4*). Surprisingly, a clear influence of incubation temperature was observed. At 12°C, high levels of α-acetolactate were reached within a short time period; however, in the fermentations at 2°C, no increase in α-acetolactate was observed and concentrations remained below the detection limit of the FID detector (0.2 μM). This confirmed results that were obtained during production of alcohol-free beer where at the low production temperatures no α-acetolactate was observed (data not shown).

Since α-acetolactate is entirely produced by anabolic processes, the influence of temperature on synthesis can be explained by changes in metabolism. Apparently, the yeast cell is better able to balance the production of this compound to the demand for the production of isoleucine, leucine, and valine at low temperatures compared to higher temperatures.[25]

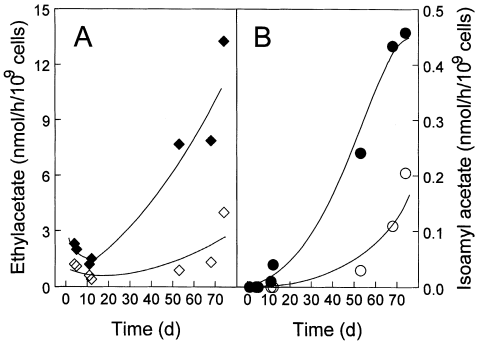

Figure 2 Rate of ester formation by immobilized *S. cerevisiae*. Cells were harvested during production of alcohol-free beer and subsequently incubated in the wort at 12°C (closed) at 2°C (open symbols) under anaerobic conditions. Formation of ethylacetate (♦,◇) [A]. Formation of isoamyl acetate (●,○) [B]

Process control

During the production of alcohol-free beer, yeast metabolism is continuously affected by environmental conditions and wort composition. This enables the brewer to interfere and optimize the flavor profile of the final product. The anaerobic conditions inhibit growth and stimulate ester production whereas oxygen stimulates growth but may cause oxidative off-flavors. By increasing temperature, yeast metabolism and ester

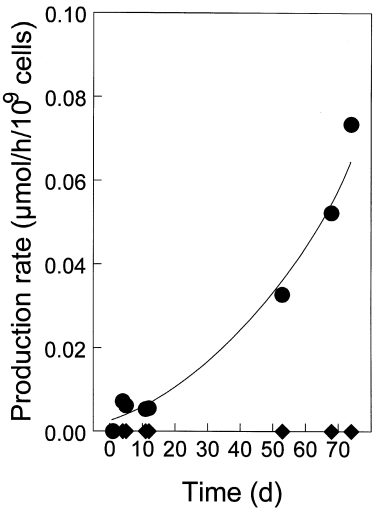

Figure 4 Production rate of α-acetolactate by *S. cerevisiae*. Cells were harvested during production of alcohol-free beer, and subsequently incubated in the wort of 12°P at 12°C (●) or 2°C (♦) under anaerobic conditions

production will increase; however, to suppress acetohydroxy acid formation, temperature must remain sufficiently low (preferably between 0 and 4°C).

By introduction of regular aerobic intervals, an optimum can be reached between the supply of oxygen for yeast growth and the prevention of oxidation of the alcohol-free beer. Intermittently, yeast metabolism is stimulated and cells are able to multiply. By varying the time of interval between two successive periods, cell physiology can be manipulated such that ester formation is constant during the entire production period. The continuous need for oxygen by the yeast will accomplish the complete removal of oxygen from the wort during alcohol-free beer production. In addition, the flow rate and wort composition are used to control flavor concentration. Early in production, biomass concentration is low, and thus flow rate is reduced. Depending on the increase in biomass, and subsequently the degree of fermentation (specific gravity) and production of flavors, the flow rate will be increased. For successful, reproducible fermentations leading to consistent beer quality, it is imperative that production is carefully monitored and controlled. The parameters mentioned above enable a constant and optimal flavor profile of the alcohol-free beer.

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