Journal of Industrial Microbiology, (1995) 14, 495–501

Continuous production of non-alcohol beer by immobilized yeast at low temperature

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(Received 14 July 1994; accepted 30 September 1994)

Key words: Saccharomyces cerevisiae; Non-alcohol beer; Wort; Immobilization; DEAE-cellulose carrier; Low temperature

SUMMARY

A system for production of non-alcohol beer is described. A limited fermentation is carried out with immobilized cells of *Saccharomyces cerevisiae* in a packed bed reactor. In the reactor, combined stress factors such as low temperature (2–4 °C) and anaerobic conditions limit cell metabolism. Of the available sugars only a small amount of glucose is metabolized, resulting in low concentrations of ethanol (<0.08%). The absence of oxygen affects the redox balance of the yeast cell, and thus stimulates formation of esters and higher alcohols. Products are formed by reduction of wort aldehydes, as well as reduction of intracellular metabolites. Despite the stress conditions, biomass increases during prolonged production periods. In batch experiments, *S. cerevisiae* strain W34 grows at low temperatures and a minimum growth temperature of –2 °C was found, indicating that a further reduction of temperature during production will not inhibit growth. The characteristics of the system allow its use in very different applications. Potential applications of the immobilized system are discussed.

INTRODUCTION

Production of low, or even non-alcohol, beers is possible by two basically different methods. The first method is based on the production of a high-alcohol beer. The alcohol is subsequently removed by such techniques as reverse osmosis, dialysis, or evaporation [2].

In contrast, other methods have been developed, based on limited fermentation, resulting in a reduced alcohol content [9,21,22]. During limited fermentation, high temperatures (15–20 °C) are combined with short fermentation times (0.5–8 h). However, low temperatures are used as well (0–5 °C), often in combination with longer fermentation times (up to 24 h). In most cases, a high yeast cell concentration is used ($>10^8$ cells ml^{-1}); thus a thick yeast slurry is mixed with high-gravity wort. A disadvantage of the limited fermentation is that the yeast slurry, which is used for inoculation, may contain a relatively high ethanol concentration (6.5% v/v) [9]. Especially during the short contact times, it is extremely important to have strict control of fermentation in order to prevent an overshoot in alcohol production. In addition, an inhomogeneous mixture of wort and cells may give problems such as reduced flavor development and cell death.

To overcome these problems, an immobilized-cell system was developed [7,16]. Generally, the volume of yeast biomass can increase from about 2% in batch systems up to 15% in immobilized systems, thereby allowing a tenfold reduction in fermentation time [17]. In the new system, three aspects are important: (i) the Cultor® carrier, a DEAE-cellulose based granular material, is used in a packed bed reactor, which is operated under down-flow. The yeast cells are bound to the rough surface in a monolayer, and cells are not subject to starvation by substrate limitation. By packing the carrier particles in a bed reactor, and operating under down-flow, a flexible and easily controlled system is obtained. (ii) A low temperature is used (2–4 °C). Yeast growth, which might clog the reactor after long production periods, is suppressed and metabolism is limited; however substrate conversion and product formation are still sufficient. Because of the low temperature, viability remains high over long periods. (iii) Anaerobic conditions are maintained which also suppress yeast growth, and prevent oxidation of wort lipids to carbonyl off-flavors. The combined stress factors thus suppress yeast growth and sugar metabolism and also decrease the risk of contaminants developing in the reactor [12].

To make future applications successful, more insight is needed into the physiological state of the yeast cells under the combined stress conditions. Several articles have appeared which focus on either low temperature stress, i.e. the cold-shock response [10,11], or on anaerobic conditions [8]. However, physiological changes caused by combining these factors are not described. In this article, we describe the characteristics of the system and their influence on metabolism of the yeast cells. This information should enable us to improve existing applications, as well as to develop new biotechnological processes.

This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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MATERIALS AND METHODS

Strain

A bottom-fermenting brewers' yeast strain of *Saccharomyces cerevisiae* (W34) was provided by the Bavaria Brewery, Lieshout, The Netherlands.

Media

S. cerevisiae W34 was grown in stationary cultures in a MYGP medium containing malt extract (3 g L^{-1}), yeast extract (3 g L^{-1}), glucose (10 g L^{-1}) and mycological peptone (5 g L^{-1}). When streak plates were used, the medium was solidified with 1.5% agar.

Measurement of growth

Growth in stationary cultures was followed by plating and counting the number of colonies (CFU). Growth in the bioreactor (see below) was followed by sonification of carrier particles, and counting the yeast cells with a Thoma counting chamber (Rofa Mavi, Beverwijk, NL). Growth rates were calculated by fitting the curves with a modified Gompertz equation [26].

Carrier-particles

The carrier (FDA approved) was provided by Cultor® Ltd, Finnsugar Bioproducts, Helsinki, Finland. It is a granular material consisting of polystyrene coated with DEAE-cellulose. The carrier is non-porous and not-compressible and has a particle size ranging from 0.3–0.8 mm [15]. The particle is inert and resistant to brewery chemicals. These characteristics enable sterilization at 80 °C with hot caustic as well as regeneration after several production periods.

Operation procedure

Fermentation is carried out in a packed-bed reactor operated under down-flow conditions (Fig. 1). The volume of the reactor is 1.5 m^3 and it is usually loaded with 1 m^3 of carrier. In a hydration tank, carrier particles are first hydrated with water at 65 °C, sterilized with 2% (w/v) NaOH at 80 °C, and neutralized with dilute acid and sterile, carbonated water. When the particles have settled in the bioreactor, a yeast suspension in wort is added. After 24 h, unbound yeast cells are removed from the reactor and production can start. Wort of 12 °P is used. At first, flow rate and temperature were set at 0.5 m^3 h^{-1} and 4 °C, respectively. Dependent on the overall performance of the reactor, temperature and flow rate were adjusted to values of 2 °C and 2 m^3 h^{-1}.

Whenever necessary, production may be stopped for a few weeks. During this period, wort is circulated over the reactor at low temperature. After a short activation step (rise in temperature, addition of fresh wort and oxygen), production can be resumed.

After 5–7 months, the entire reactor, including carrier particles, is cleaned and sterilized with 2% NaOH (80 °C), and loading and production start again.

GC dynamic headspace analysis

Volatile compounds were analyzed by dynamic headspace sampling, and GC analysis [6]. Samples (15 ml) were diluted with 50 ml distilled and Nanopure-filtered water. Volatiles were purged by a N_2-flow (40 °C, 25 ml min^{-1}, 30 min); 0.1 g Tenax-TA (Alltech, Zwijndrecht, The Netherlands) was conditioned by purging it with N_2 (250 °C, 4 h, 2.5 ml min^{-1}) and was used to trap the volatiles.

The gas chromatograph (GC 5300 MEGA, Carlo Erba Instruments, Milan, Italy) was equipped with a thermodesorption and cold trap unit (−120 °C with liquid nitrogen, Chrompack, Middelburg, NL), a Supelcowax 10 capillary column (0.25 μm Carbowax 20 M, 60 m × 0.25 mm, Supelco, Bellefonte, PA, USA), helium carrier gas (0.6 ml min^{-1}, 160 kPa), and a flame ionization detector (temperature 275 °C). The Tenax tube was heated at 180 °C for 10 min, volatiles were

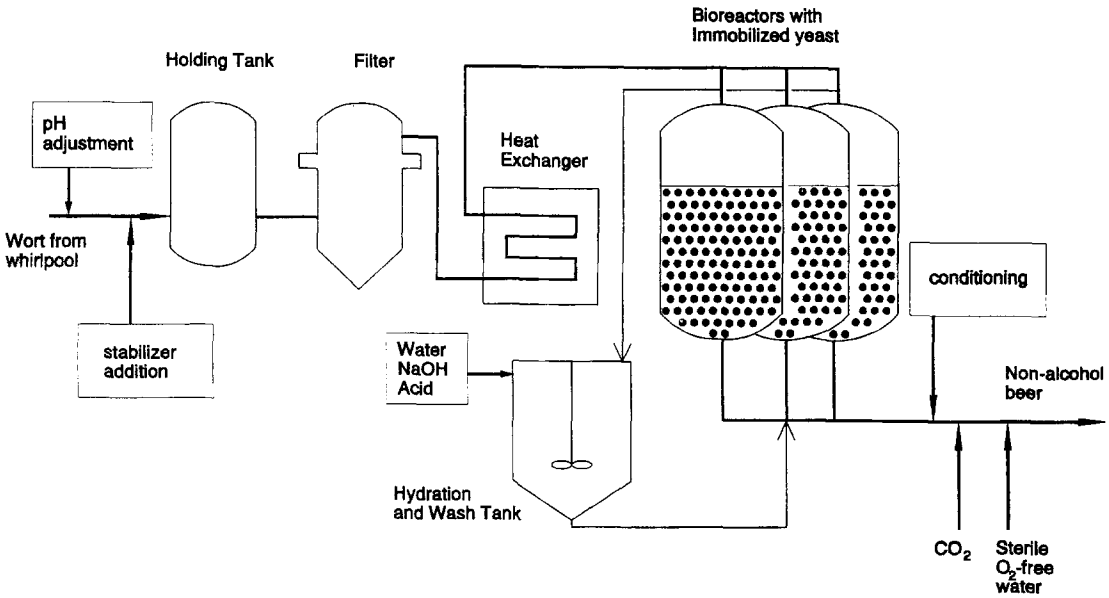

Fig. 1. Flow diagram of the production of non-alcohol beer using an immobilized yeast reactor.

collected in the cold trap. Subsequent heating of the cold trap (5 min, 240 °C) injected the compounds on the column. The starting temperature was 40 °C. After 4 min, this was increased to 92 °C at 2 °C min^{-1}, and subsequently to 272 °C at 6 °C min^{-1}.

Components were identified by GC-MS as described above with a VG MM7070F mass spectrometer in the 70 eV ionization mode (VG, Altrincham, UK).

Sugar analysis

Samples were prepared by filtration over a 0.45-μm pore size filter and dilution to the appropriate concentration with Nanopure-filtered water.

Sugar composition of wort samples was determined by high-performance anion-exchange chromatography (HPAEC) with pulsed-amperometric detection. Chromatography was performed using a Dionex Bio-LC system equipped with a Carbopac PA 100 column (4 × 250 mm) (Dionex, Sunnyvale, CA, USA). Separation was done with a combined gradient of three eluents: 0.1 M NaOH, 1 M sodium acetate in 0.1 M NaOH, and distilled water. All eluents were prepared with distilled water that had been filtered through a Nanopure-system.

RESULTS

Reactor performance

For about one month, the performance of the reactor was followed with respect to development of biomass and product formation (Fig. 2). Production of ethanol and *iso*-butanol clearly accompanied the increase in biomass, which was most significant during the first 40 days (Fig. 2). This increase was also seen with other higher alcohols and esters (data not shown). By adjusting the flow rate and temperature, a constant concentration of ethanol in the efflux, less than 0.1% (v/v), was obtained.

Growth and sugar conversion

Samples of carrier taken from the reactor showed high numbers of immobilized yeast cells (Fig. 3). The growth rate in the bioreactor was calculated from data in Fig. 2 and was approximately 0.012 h^{-1}. Growth was also followed in batch cultures at various temperatures (Fig. 4). *S. cerevisiae* W34 grew at very low temperatures, and growth rates at 2 °C and 4 °C were 0.007 h^{-1} and 0.022 h^{-1}, respectively. Thus, the growth rate of *S. cerevisiae* W34 in the reactor agrees well with the rates determined in batch culture. Temperature and the square root of the growth rate are linearly related to each other [18]. The minimal growth temperature, calculated by linear regression over a temperature range of 2–20 °C, is −2 °C.

Sugar analysis was performed on the influx and efflux of the reactor (Table 1). It appears that sucrose (12.9 ± 0.7 mM) was partially hydrolyzed (approximately 64%), which would result in an increase of both the glucose and fructose concentration by approximately 8.2 mM each. However, the concentration of glucose increased only slightly, while that of fructose increased by approximately 8.2 mM. From these results, it can be calculated that about 7 mM of glucose was taken up by the yeast cells, which is 19–21% of the total amount (Table 1). The concentrations of other fermentable sugars, viz. maltose and maltotriose, did not decrease significantly.

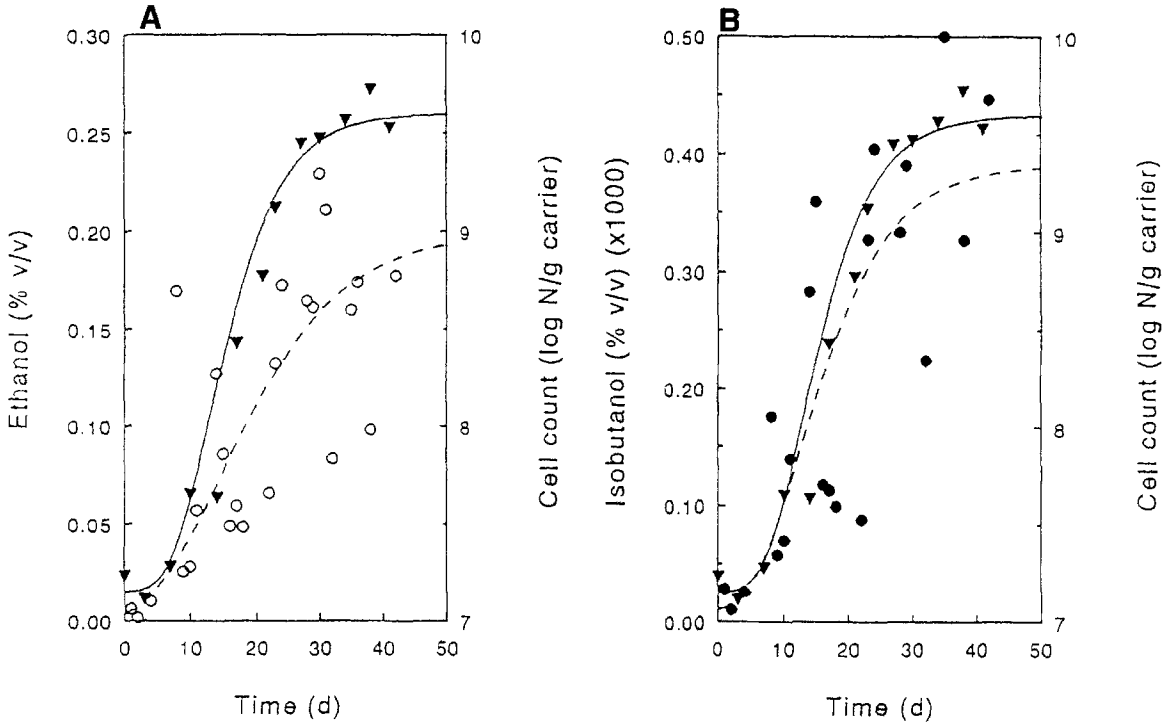

Fig. 2. Increase in product concentration during prolonged operation of the immobilized reactor. Samples were analyzed by purge and trap gas chromatography; (A) ethanol (–○–), (B) *iso*-butanol (—●—). Biomass (–▼–) is expressed as cell count per gram dry weight of carrier.

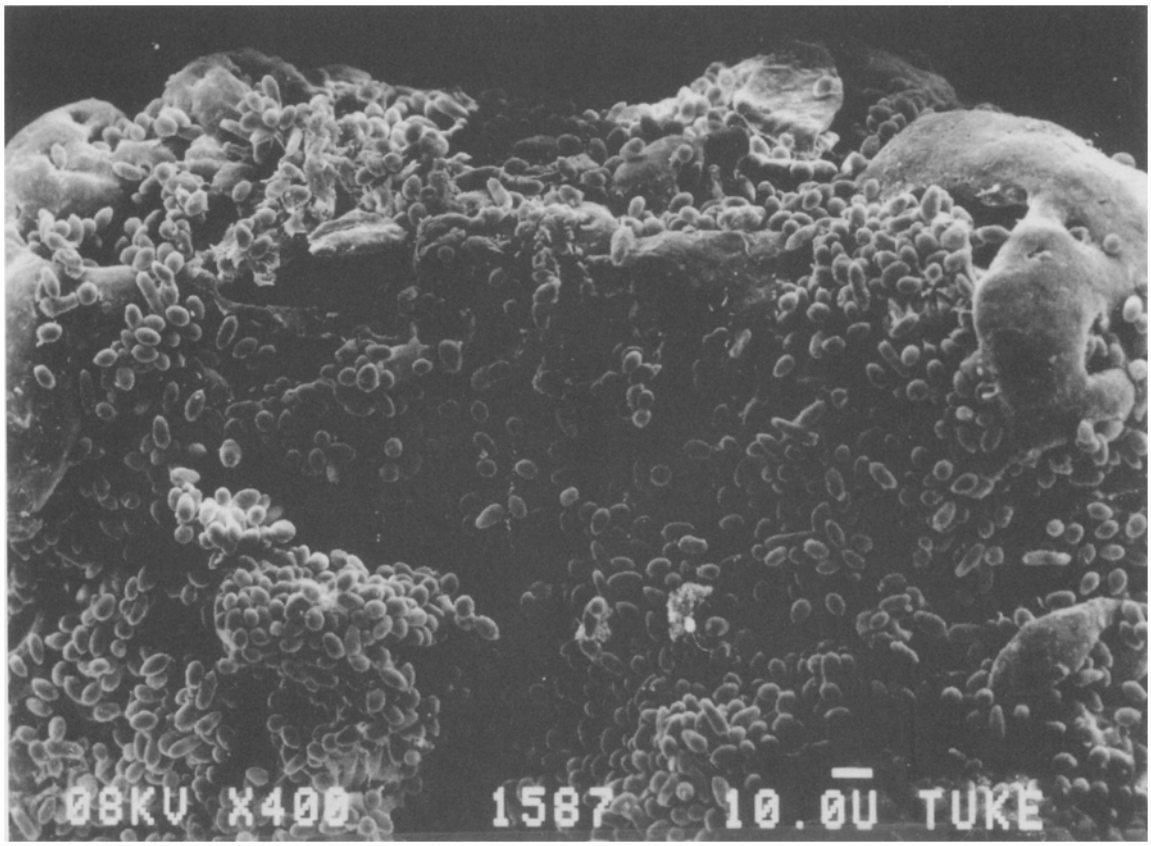

Fig. 3. Scanning-electron micrograph of yeast cells immobilized to a carrier particle. Bar represents 10 μm.

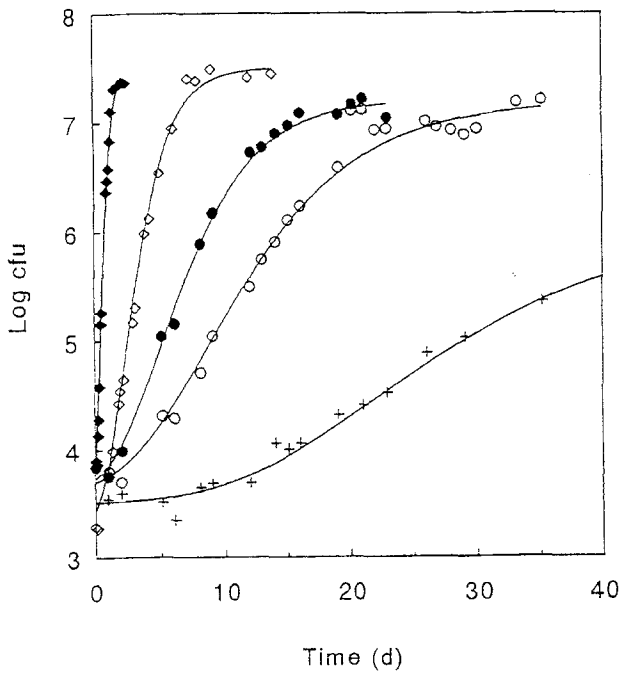

Fig. 4. Growth of *S. cerevisiae* at different temperatures as determined by plate-counting. Growth was in MYGP in stationary cultures at 2 °C (+), 4 °C (○), 6 °C (●), 10 °C (◇) and 25 °C (◆).

TABLE 1

Concentrations of fermentable sugars in the wort (influx) and non-alcohol beer (efflux), temperature 3 °C, flow rate 1 $m^3 h^{-1}$

	Sugar concentration		Change in sugar concentration (mM)
	influx (mM)	efflux (mM)	
Glucose	32.3 ± 1.1	33.8 ± 1.2	1.5
Fructose	5.6 ± 0.2	13.9 ± 0.5	8.3
Sucrose	12.9 ± 0.7	4.7 ± 0.3	−8.2
Maltose	141 ± 2.8	139 ± 2.8	−2
Maltotriose	18.3 ± 0.5	18.2 ± 0.5	−0.1

Flavor development

The composition of the wort (influx) and non-alcohol beer (efflux) was analyzed by dynamic headspace analysis (Table 2). Linear and branched aldehydes were reduced during the limited fermentation. Branched ketones however, were not converted. Several higher alcohols, esters and organic acids were produced, of which some are closely related to each other; e.g. 2- and 3-methyl-butanol, 2- and 3-methylbutanoic acid and 2- and 3-methylbutyl acetate. With mass spectrometry, both 2- and 3-methylbutanoic acid were detected, but these compounds cannot be separated by gas chromatography. Therefore the sum of these compounds is given in Table 2.

TABLE 2

Concentrations of volatile components in the influx and efflux of the reactor (temperature 3 °C, flow rate 1 $m^3 h^{-1}$). Sample composition was determined by dynamic headspace analysis

	Concentration in		Reduction (%)
	influx ($\mu g L^{-1}$)	efflux ($\mu g L^{-1}$)	
CARBONYLS			
2-methyl propanal	2.8	–[a]	100
2-methyl butanal	7.9	3.1	61
3-methyl butanal	27	14	48
4-methyl-2-pentanon	3.5	3.5	0
3-methyl-2-pentanon	1.3	1.3	0
hexanal	1.0	0.6	40
heptanal	1.0	0.6	40
octanal	1.3	1.3	0
nonanal	4.5	3.6	20
decanal	9.3	8.6	8
ALCOHOLS			
ethanol	–	>100[b]	
2-methyl-1-propanol	0.9	3	
1-butanol	–	0.3	
2-methyl butanol	–	3	
3-methyl butanol	–	13	
1-nonanol	–	0.9	
ESTERS			
ethyl acetate	–	6.9	
2-methylbutyl acetate	–	0.7	
3-methylbutyl acetate	0.4	7.7	
ethylbutyrate	–	0.7	
ethylhexanoate	–	17	
ethylheptanoate	–	0.3	
ethylcaprylate	–	1.4	
ACIDS			
methyl butanoic acid[c]	–	6.3	
SULFUR COMPOUNDS			
dimethyl disulfide	1.0	0.3	70
dimethyl trisulfide	1.5	0.3	80
methyl thioacetate	–	0.3	
dimethyl sulfoxide	4.3	0.5	88

[a]Not detectable.
[b]Off scale.
[c]Consists of 2-methyl butanoic and 3-methyl butanoic acids.

The concentration of various sulfur compounds, such as dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and dimethyl sulfoxide (DMSO) decreased during passage through the reactor. Simultaneously, a small increase was seen in methyl thioacetate.

DISCUSSION

Growth at low temperature

Growth in the reactor (Fig. 2) and in batch cultures (Fig. 4) show the ability of the brewers' yeast strain to grow at low temperature. Cells metabolize glucose (Table 1) and reduce numerous wort components to alcohols and esters (Table 2). Growth, even at 0 °C, limits the lifetime of the reactor, due to slowly increasing cell mass, ultimately resulting in blockage of the down-flow.

Growth at extremely low temperature, as compared to that at optimum temperature, requires changes in cell physiology. Cell composition changes; the phospholipid composition of membranes shows a higher ratio of unsaturated to saturated and short-chain to long-chain fatty acids, and higher protein and RNA concentrations have been reported [19,25]. Comparison of mesophilic and psychrophilic bacteria revealed that the former were able to metabolize only endogenous substrates at temperatures below their minimal growth temperature, while the latter were also able to metabolize exogenous substrates. This might suggest that inhibition of transport processes is responsible for absence of growth. Besides transport processes, low temperature affects protein synthesis, and as a result, the so-called cold-shock response is induced [10,11]. In the case of large temperature shifts, the cold shock is more pronounced and cells need more time to adjust [23], as indicated by the significant increase in lag time (Fig. 4). We also examined the influence of a temperature shift on transport of glucose. It appears that in cells subjected to a cold-shock (a shift from 25 °C to 4 °C), glucose transport decreased from 91 to 15 μmol min^{-1} (g protein)$^{-1}$. Cells grown at 4 °C took up glucose at this low temperature at a rate comparable to that observed in cells grown at 25 °C (data not shown). Thus, the lag time that occurs after transfer of cells from high to low temperature is not caused by total inhibition of sugar transport but is most likely due to inhibition of transcription or translation [10,11].

Saccharomyces cerevisiae preferentially takes up glucose compared to other sugars, as can be concluded from data in Table 1. The organism hydrolyzes sucrose using invertase in a mixture of different sugars [1]. Due to glucose repression of the maltose and maltotriose transport systems, these sugars are not metabolized [5,14]. Apparently, also at low temperature, regulation of sugar metabolism is centered around glucose which is metabolized preferentially. Of the total amount of glucose, approximately 20% is used. From 7 mM of glucose, no more than 0.08% (v/v) of ethanol can be produced. This is in agreement with results obtained from HPLC analysis of the efflux of the reactor (data not shown).

Flavor development

Synthesis or degradation of flavor components often involves oxidation and reduction steps, e.g. reduction of 3-methyl butanal to 3-methyl butanol. Reduction of several aldehydes occurs in the reactor (Table 2). However, the total amount of higher alcohols, esters and organic acids that are produced is larger than the decrease in the concentrations of related aldehydes. During fermentation of beers in the normal or high alcohol range, several conditions, such as the absence of oxygen, the use of high-gravity wort, low concentrations of unsaturated fatty acids and high concentrations of amino acids stimulate formation of higher alcohols and esters [4]. This is explained by a rapid transamination of amino acids which gen-

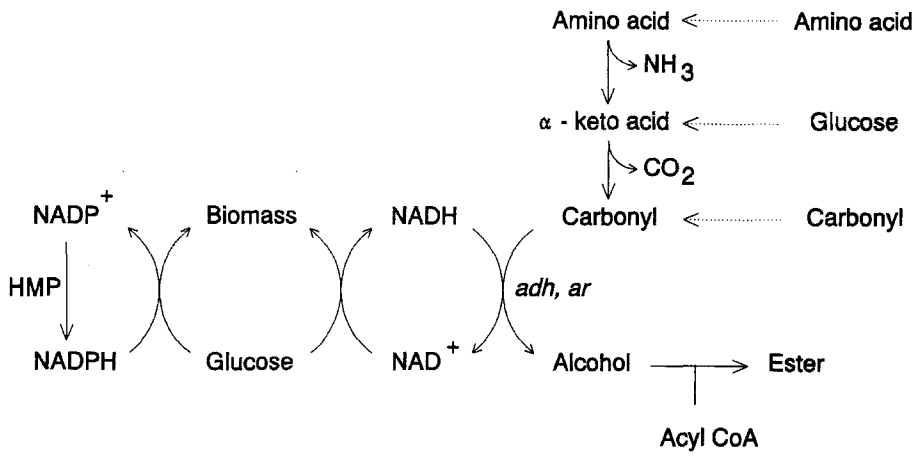

Fig. 5. Coupling between growth on glucose and reduction of carbonyl components under anaerobic conditions. Abbreviations: HMP: hexose monophosphate pathway; *adh*: alcohol dehydrogenase; *ar*: aldehyde reductase. Dashed arrow indicates uptake.

erates a large oxoacid pool. Via decarboxylation and subsequent reduction by alcohol dehydrogenase, higher alcohols are formed. *Iso*-butanol is produced from valine, while 2-methyl and 3-methyl butanol are derived from isoleucine and leucine respectively [4].

The involvement of reduction and oxidation steps might implicate the importance of the redox-balance in flavor development. A high ratio of reduction equivalents NAD(P)H over $NAD(P)^+$ is necessary for overall production of reduced flavor compounds [24]. During aerobic growth of *S. cerevisiae* on glucose, excess reducing requivalents (NADH) are oxidized via the respiratory chain. Under anaerobic conditions, the $NADH/NAD^+$ ratio is maintained by glycerol formation, in which case one NADH is reoxidized at the expense of one ATP [8,24]. However, acetoin has been shown to allow growth of fermentative yeasts after a sudden shift from aerobic to anaerobic conditions [20]. Thus, reoxidation might also occur by simultaneous reduction of other organic compounds, e.g. the reduction of carbonyl compounds to higher alcohols (Fig. 5). The occurrence of higher alcohols, esters and organic acids, all derived from the same carbon backbone structure, points to reduction of the corresponding aldehyde [4]. Although formation of higher alcohols is strain dependent, difficulties in maintaining a proper redox-balance under oxygen-limiting conditions might be the cause of increased higher alcohol and ester formation.

Potential applications

For several years, the system described in this paper has been used for the secondary fermentation of green beer [15], for primary fermentation [13], and now for the production of non-alcohol beer. The different production processes can be further optimized, e.g. by selecting yeast strains. Other possible applications are in the production of secondary metabolites, and production of heterologous proteins with genetically engineered strains.

Recently a new application was developed. Back and Pittner [3] used the carrier for immobilization of lactic acid bacteria and continuous production of biologically acidified wort. The immobilization of lactic acid bacteria shows the potential use of the system in other biotechnological fields. Immobilization does not affect the viability of the cells, which makes the reactor a powerful tool in biotechnological production systems.

ACKNOWLEDGEMENTS

The assistance of J.L. Cozijnsen with gas chromatographic analysis is greatly appreciated. We thank Dr M.A. Posthumus (Department of Organic Chemistry, Wageningen Agricultural University) for performing the GC-MS analyses.

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