Appl Microbiol Biotechnol (2009) 84:453–464
DOI 10.1007/s00253-009-1930-y

Physiological characterization of brewer's yeast in high-gravity beer fermentations with glucose or maltose syrups as adjuncts

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Received: 2 October 2008 / Revised: 23 February 2009 / Accepted: 24 February 2009 / Published online: 3 April 2009

Abstract High-gravity brewing, which can decrease production costs by increasing brewery yields, has become an attractive alternative to traditional brewing methods. However, as higher sugar concentration is required, the yeast is exposed to various stresses during fermentation. We evaluated the influence of high-gravity brewing on the fermentation performance of the brewer's yeast under model brewing conditions. The lager brewer's strain Weihenstephan 34/70 strain was characterized at three different gravities by adding either glucose or maltose syrups to the basic wort. We observed that increased gravity resulted in a lower specific growth rate, a longer lag phase before initiation of ethanol production, incomplete sugar utilization, and an increase in the concentrations of ethyl acetate and isoamyl acetate in the final beer. Increasing the gravity by adding maltose syrup as opposed to glucose syrup resulted in more balanced fermentation performance in terms of higher cell numbers, respectively, higher wort fermentability and a more favorable flavor profile of the final beer. Our study underlines the effects of the various stress factors on brewer's yeast metabolism and the influence of the type of sugar syrups on the fermentation performance and the flavor profile of the final beer.

Keyword High-gravity brewing · Brewer's yeast · Sugar syrups · Adjuncts · Stress

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Introduction

When higher productivity and substantial economic savings are the key aspects in process development, high-gravity brewing is an attractive approach. It requires the use of wort with higher sugar concentration, and because of the higher ethanol concentrations produced, at the end, the beer is diluted to the desired ethanol content. In brewing, the unit of gravity used is degrees Plato. Brewing wort at 12°Plato (equivalent to 12 g extract per 100 g liquid) ferments to produce beer of 5% (*v*/*v*) ethanol, whereas brewing wort of 18°P produces beer with 7.5% (*v*/*v*) ethanol content. Such beer is diluted further to achieve a final ethanol content of 5% (*v*/*v*; Blieck et al. 2007). The use of high-gravity brewing technology has the advantages of increasing brewery capacity by 20–30% without additional expenditures for facilities, reducing the cost of energy and labor, and improving the beer smoothness, flavor, and haze stability (Stewart 2007a, b). The relatively high ethanol concentrations formed during fermentation promotes increased precipitation of the complex polyphenol–protein material; thus, high-gravity-produced beer has better colloidal stability than standard-gravity fermented beer (Boulton and Quain 2006). High-gravity brewing also offers a flexibility of the beer type produced as products

with different sugar content (light beer) and alcohol levels (low/high alcohol beer) can be produced (Stewart 2007a, b).

The negative effects of high-gravity brewing on the brewer's yeast performance are increased osmotic pressure due to high sugar concentrations at the beginning of the fermentations and elevated ethanol concentrations toward the end of the fermentations. Both of those factors have been implicated as limiting for reduced yeast viability and vitality (Pratt et al. 2003). As a result, reduced fermentation rates and incomplete fermentations in wort with more than 18°P may be observed. Such fermentations result in high residual sugars, mainly maltotriose and maltose, lower ethanol yield, and modified flavor profiles, and the extent of the osmotic pressure depends on the concentrations of solutes surrounding the medium.

The use of certain adjunct types to supplement the wort is another cost-saving approach used in the high-gravity brewing industry. However, unlike wort, adjuncts contain only carbohydrates and not other nutrients; consequently, the addition of adjunct to the wort at the beginning of beer fermentation results in modified nutrient balance compared to normal-gravity fermentations. Thus, increased initial gravity will result in beer fermentations with reduced cell number and lower specific growth rate (Casey et al. 1985). The use of sugar syrups as adjuncts reduces also the available amino acid content, and the lower specific growth rates lead to lower amino acid uptake rates. Such fermentations are also associated with a modified (abnormal) pattern of sugar uptake and altered levels of production for some of the flavor compounds (Dragone et al. 2007; Boulton and Quain 2006). The modified flavor and aroma compound profile in the final beer produced from the high-gravity brewing process is especially pronounced with regards to elevated levels of ethyl acetate and isoamyl acetate, compared to beer produced from lower gravities, but with the same alcohol content.

The objective of the present study was to evaluate the influence of high-gravity fermentation conditions, in terms of modified carbon to nitrogen ratio on the brewer's yeast physiology and fermentation characteristics. Furthermore, the addition of glucose versus maltose syrup adjuncts in high-gravity beer fermentations and their influence on brewer's yeast physiology and the flavor compound formation in the final beer was investigated.

Materials and methods

Wort

All-malt wort with a gravity of 14°P and pH=5.2 (purchased from Alectia A/S, Denmark) was used for all fermentations. The wort contained 90% carbohydrates of which the fermentable carbohydrates consisted of 4.4% fructose, 12.5% glucose, 66.5% maltose, and 16.7% maltotriose (*w*/*v*). The wort also contained non-fermentable carbon sources such as dextrins and β-glucan. $ZnSO_4.7H_2O$ was added to a concentration of 0.1 ppm Zn. To adjust the wort to higher gravities 21°Plato and 24° Plato, respectively, highly fermentable syrups Clearsweet® 95% Refined Liquid Dextrose Corn Syrup (95.5% glucose, 2,5% maltose, 1% maltotriose, 1% higher saccharides, present in % dry basis (*w*/*w*)) and Satin Sweet® 65% High Maltose Corn Syrup (70% maltose, 18% maltotriose, 2% glucose, 9% higher saccharides, present in % dry basis) were used as adjuncts. Both syrups were kindly provided by Cargill Nordic A/S. The resulting sugar composition of the different fermentation media used in this study, as measured by HPLC analysis, is summarized in Table 1. Prior to inoculation, the wort was oxygenated with air until it reached 100% saturation.

Wort density

Wort density was measured using an A. Paar density meter DMA 4500 apparatus (Anton Paar GmbH, Germany), and the gravity was expressed in degree Plato. One degree Plato corresponds to 1 g of extract per 100 g of liquid solution, where extract include both fermentable sugars and non-

Table 1 Sugar composition and free amino nitrogen (FAN) content of the wort at different gravities used in the present study

Wort °P	Glucose (g l^{-1})	Fructose (g l^{-1})	Maltose (g l^{-1})	Maltotriose (g l^{-1})	Total sugars (g l^{-1})	FAN (g l^{-1})
14	12.5	4.4	67.1	16.9	100.9	240
21Gl	98.8	5.2	78.6	18.7	201	210
21M	14.5	4.7	135	35.7	190	220
24Gl	144	5.7	68.2	16.5	235	192
24M	16	5.0	157	42.4	220	201

The numbers 14, 21, and 24, respectively, represent the corresponding gravity in the fermentation media

Gl supplementation with glucose syrup, *M* supplementation with maltose syrup

fermentable carbon sources such as dextrins and β-glucan (Blieck et al. 2007).

Strain

The flocculent bottom fermenting industrial beer yeast strain Weihenstephan 34/70 (Hefebank Weihenstephan, Freising, Germany) was used in this study. The strain was maintained as a frozen stock culture in 40% (*v*/*v*) glycerol.

Fermentation conditions

For the precultures, the yeast from the stock culture was propagated on YPD plates at 30°C for 4 days. A single yeast colony was transferred to 20 ml of 14°P wort in a sterile 50 ml Falcon tube and incubated at 25°C in a rotary shaker at 150 rpm. After 48 h, the preculture was transferred to a shake flask with 375 ml of fresh wort and incubated for 72 h.

All fermentations were performed in 2.2 l bioreactor (Biostat B5; Braun Biotech International, Melsungen, Germany) with a working volume of 1.5 l. Dissolved oxygen was monitored with an autoclavable polarographic oxygen electrode. The fermentors were integrated with Braun Biotech Multi-Fermenter Control System for data acquisition. Silicone-based antifoam agent FD20P in concentration of 0.1 ml/l (Basildon Chemicals, England) with a food grade quality was used in the fermentations. The reactors were inoculated with a volume of preculture, corresponding to 1×10^7 cells/ml. During the cultivation, the temperature was maintained at 14°C, and the stirring was set to 90 rpm. Prior to sampling, the stirring was increased to 300 rpm for 2 min. The higher stirring allowed better mixing and homogenization of the media and ensured representative sampling. The pH was recorded on-line, but not controlled. After the fermentation was completed, the whole fermentation broth was transferred to a sterile vessel and stored for 14 days at 0°C, for further maturation.

Methylene blue staining

Viability tests were done using the methylene blue staining method according to EBC Analytica method (1992), and the viable yeast cells were counted in a Bűrker-Tűrk cell counting chamber.

Sampling

Samples for analysis of sugars, alcohols, and amino acids were collected every 24 h throughout the fermentation. For measuring the wort density and free amino nitrogen content, samples were collected from the first and the final day of the primary fermentation. For the all of the above analyses, 2–10 ml of fermentation samples was withdrawn from the fermentor, immediately filtered through a Cameo 0.20-μm pore size acetate/glass filters (Sartorius AG, Germany), and stored at −20°C prior to analysis. Samples for flavor compound analyses were collected after 14 days of maturation, filtered, and stored at −20°C prior to analysis. For determination of the intracellular trehalose content, cell mass samples were collected from the exponential and from the stationary phases of the fermentations. Cells from 5 ml of fermentation broth was harvested by centrifugation, washed three times with cold 0.9% (*w*/*v*) NaCl, and dissolved in 0.2 M sodium citrate buffer, pH 4.8. The samples were frozen in liquid nitrogen and stored at −20°C. Standard deviations between the duplicate fermentation samples were determined to be lower than 5% of the average value for the analysis of sugars, alcohols, amino acids, wort density, and flavor compounds determination and lower than 10% of the average value for the analysis of free amino nitrogen content.

HPLC analysis

Carbohydrates and alcohols

A Dionex Summit HPLC system (Synnyvale, CA, USA) was used for analysis of sugars and metabolites from the extracellular medium. All metabolites were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore, Milford, MA, USA) after separation using Aminex HPX-87H column (Biorad, Hercules, CA, USA) at a temperature of 60°C using 5 mM H_2SO_4 as eluent. To allow the separation of the sugars with different degree of polymerization, two Aminex columns were mounted in serial with isocratic elution at 0.40 ml/min. To account for the metabolite concentration, both non-diluted and 15 times diluted in eluent samples were analyzed. External standards of maltotriose (DP3), maltose (DP2), glucose (DP1), fructose (DP1), glycerol, and ethanol were used for external quantification at six different levels.

Amino acids

The 20 essential individual amino acids were quantified on Dionex Summit HPLC system after derivatization in alkaline buffer with *o*-phthalaldehyde (primary amino acids) and 9-flourenylmethyl chloroformate (secondary amino acids; Herbert et al. 2001).

The amino acid derivatives were separated on a 150× 4.60 mm, 3 μm, Gemini C_{18} column (Phenomenex, Torrance, CA, USA). Detection was performed using a RF200 fluorescent detector, using 288 nm as excitation wavelength and 305 nm as emission wavelength. Separation was performed using a linear binary gradient 1.0 ml/min of

A (20 mM triethylamine adjusted to pH 7.5 using acetic acid) and B (45% acetonitrile, 45% methanol, and 10% water). The gradient started with 12% B for 12 min and was then linearly increased to 40% B at 38 min and further increased to 61% for 9 min, after which increased to 100% in 1 min where it was maintained for 2 min before reverting to the starting conditions in 4 min.

Free amino nitrogen analysis

The levels of free amino nitrogen (FAN) of the unfermented worts and from the last day of the beer fermentation were determined using the ninhydrin method at 570 nm (EBC 1998). Glycine was used as a standard.

Total protein

For determination of the total protein content, 50 µl of the supernatant was treated with 3 ml Bradfort reagent (Sigma), and the samples were measured on spectrophotometer at 595 nm. Bovine serum albumin was used as a standard at three levels.

Trehalose determination

Prior to extraction, the samples were thawed by rapid boiling for 5 min, and the cells were disintegrated using glass beads with size (0.25–0.5 mm) in a Savant Fast Prep FP120 (Savant Industries, NY, USA; Schulze et al. 1995). After centrifuging, the supernatant was treated with trehalase (0.148 U/ml; Sigma) in 0.2 M sodium citrate buffer pH 5.7, overnight at 57°C with 150 rpm shaking. Liberated glucose was measured in duplicate samples using an enzymatic kit (Unimate 5 Gluc HK, Roche, Switzerland) on an automatic analysis robot (Cobas Mira, Roche, Switzerland), and the samples were normalized with the total protein content. Calibration with glucose as a standard at three different levels was used.

Headspace analysis of esters and higher alcohols

GC-FID analysis of the esters and higher alcohols of the final beer samples was performed using a Perkin-Elmer Autosystem XL gas chromatograph equipped with automatic HS40 XL headspace autosampler. Samples of 5.0 ml were transferred to 20 ml autosampler vials and capped with butyl-PFTE seals. Samples were thermostated for 30 min at 60°C, pressurized with 3.5 bar helium, and transferred to the GC through a 0.25 mm ID deactivated fused silica held at 90°C (transfer line). Injection to the analytical column DB-5 (60 m, 0.25 mm, ID 1.0 µm film; J&W Scientific) was done in split mode at a ratio of 1:10 for 1.2 min. The initial oven temperature was set at 35°C for 2 min, followed by an increase with 6°C/min until the temperature reached 200°C. The flame ionization temperature was 250°C, and helium was used as the carrier gas at a constant flow of 35 cm/s. Perkin-Elmer Turbochrom Navigator software was used for instrument control. *N*-Butanol was used as an internal standard and calibrated against all six studied compounds at six levels.

Calculations of specific growth rate, yield coefficients, and wort fermentability

The specific growth rate was determined as the slope from the linear function of the natural logarithmic function (ln) of the cell number (cells/ml) and the fermentation time (h) during the exponential growth phase. The yield coefficients were determined as the slope from the linear regression on the corresponding pairs of substrate (total saccharides) and product concentration (glycerol and ethanol).

The percentage fermentability of the wort is the proportion of the wort dissolved solids (extract), which is fermented during the course of the beer fermentation. The percentage fermentability is calculated using the following formula (Briggs et al. 2004):

$$\text{Fermentability}(\%) = [(\text{original gravity} - \text{final gravity})/(\text{original gravity})] \times 100$$

Results

The physiological characterization of the industrial lager beer yeast strain Weihenstephan 34/70 was performed in basic wort at 14°P and in high-gravity wort at 21°P and 24°P, achieved with the addition of glucose or maltose-rich syrups to the basic wort (for sugar compositions in the different media, see Table 1).

Effect of the gravity increase and type of sugar syrups on the brewer's yeast growth

The main products of beer fermentations are ethanol, carbon dioxide, glycerol, and yeast biomass. In all of the five studied wort conditions, but especially pronounced for the fermentations at higher gravities, the fermentation profile can be separated into three phases (Fig. 1). During the first lag phase, little to no cell growth occurred. Then, the cells entered the exponential growth phase, where cell growth, sugar consumption, and product formation take place. After deceleration, the brewer's yeast cells entered the stationary phase, and the cell number remained

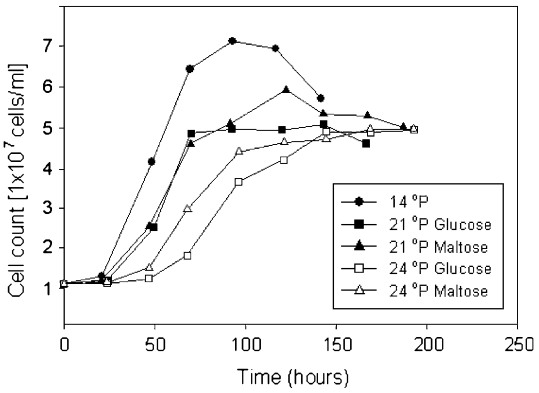

Fig. 1 Time course of the cell number from the exponential growth phase of the studied fermentations. The number 14, 21, and 24 correspond to the gravity of the wort. Gl and M represents glucose and maltose syrup supplementation, respectively

constant. With the advance of the stationary phase, the cell numbers decreased. Especially, pronounced at the higher gravities, in the stationary phase although there was no active cell growth, the sugar utilization and ethanol and glycerol formation continued at a lower rate than during the exponential growth phase (Fig. 2). In all cases, the pH profile of the fermentations declined from 5.3 to 5.1 at the start of the fermentation to 4.2–3.9 (data not shown). With increase in the gravity, the sugar syrups slightly acidified the wort; thus, the starting pH for high-gravity fermentation was slightly lower, but the final pH was not lower than 3.9.

With increase in the gravity from 14°P to 24°P, both for the glucose and for the maltose syrup-supplemented fermentations, both the final cell numbers and the specific growth rate decreased (Table 2). Among the five studied conditions, the highest specific growth rates and the highest cell numbers in the exponential growth phase were observed for the 14°Plato fermentations 0.067 (h^{-1}) and 7.1×10^7 cells/ml, respectively. When maltose syrup was used as a supplement to the wort of 21°P and 24°P, higher specific growth rate 0.064 (h^{-1}) and 0.049 (h^{-1}), respectively, and higher maximum cell numbers 4.9×10^7 and 4.6×10^7 cells/ml were observed, compared to the glucose syrup-supplemented wort of the same gravity 4.8×10^7 cells/ml and specific growth rate of 0.05 (h^{-1}) for the 21° P fermentations and 4.1×10^7 cells/ml and specific growth rate of 0.044 (h^{-1}) for the 24°P fermentations. With increase in the gravities, also longer time was necessary for the cells to enter the exponential growth phase and for the initiation of the ethanol production. The yeast cells from 14°P and 21°P fermentations entered the exponential growth phase at around 20 and 25 h of fermentation, respectively. For the 24°P fermentations, the cells entered exponential growth phase first after 46 h of fermentation.

Comparison of sugar utilization and alcohol production with increase in the gravity

The sugar uptake in brewer's yeast fermentations is an ordered, complex, and highly regulated process. Glucose, fructose, maltose, and maltotriose were the four main fermentable sugars present in the wort (Table 1). Glucose and fructose were consumed first, followed by maltose. After maltose was consumed to certain extent, then maltotriose started to be utilized. With increase in the gravity, the order of sugar uptake remained the same, but the rate of sugar utilization decreased (Fig. 2). While at 14°P the fermentations ended with almost complete utilization for all sugars and very small amount of residual sugars left, at the end of the 21°P and 24°P fermentations, higher amount of residual sugar remained (Table 2). While for the 21°P fermentations, more complete fermentations in terms of sugar utilization were observed for the maltose syrup-supplemented fermentations, for the 24°P fermentations, both glucose and maltose syrup-supplemented fermentations had similar amounts of residual maltose and maltotriose left at the end of the fermentation.

The highest wort fermentability, 80% was achieved for the 21°P fermentations with maltose syrups supplementation, followed by the 14°P and 21°P fermentations with glucose syrups supplementation, both with similar wort fermentability, 76% and 75%, respectively. As expected, 67% wort fermentability was observed for both of the 24°P fermentations with glucose and maltose syrups supplementation. This low fermentability was explained by the high carbohydrate concentrations and low free amino nitrogen concentrations prevailing at these conditions.

The highest ethanol yield based on consumed sugars was observed for the 21°P fermentations, followed by the 24°P fermentations. For each of the corresponding gravities, slightly higher ethanol yield was observed for the maltose syrup-supplemented fermentations 0.49 (g/g) and 0.47 (g/g) for the 21°P and 24°P, respectively, and 0.48 (g/g) and 0.46 (g/g) for the 21°P and 24°P glucose syrup-supplemented fermentations, respectively. Lowest ethanol yield 0.45 (g/g) was observed for the 14°P fermentations. The glycerol yield was found to be higher in the glucose syrup-supplemented fermentations 0.026 (g/g) and 0.027 (g/g) for 21°P and 24°P, respectively, and lower 0.018 (g/g) and 0.022 (g/g) for the 21°P and 24°P maltose syrup-supplemented fermentations, respectively.

Trehalose accumulation with increase in gravity

To assess the intracellular trehalose accumulation, samples from the early exponential phase and from the stationary phase for each of the fermentations were collected. With increased gravity, the amount of intracellular trehalose increased as well. In the stationary phase, both for the

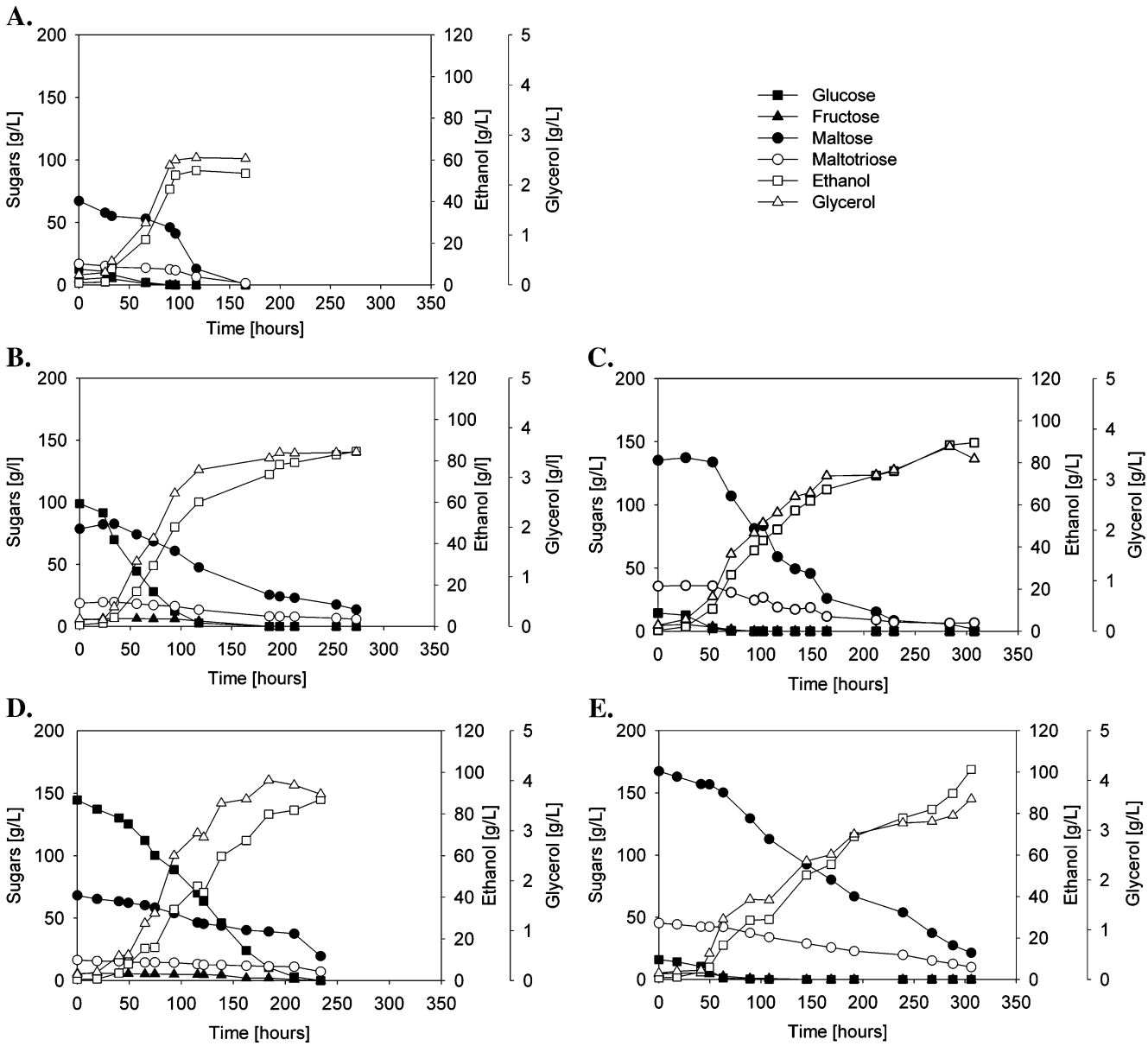

Fig. 2 Fermentation profiles of the studied fermentations, including sugars, ethanol, and glycerol concentrations: **a** 14°Plato, **b** 21°P with glucose syrup supplementation, **c** 21°P with maltose syrup supplementation, **d** 24°P with glucose syrup supplementation, **e** 24°P with maltose syrup supplementation. All present values are based on the average of duplicate experiments

21°P and 24°P fermentations, higher intracellular trehalose concentration (Table 2) was observed for the fermentations with maltose supplementation, 1.98 and 2.69 (g (glucose)/mg (proteins)), respectively, compared to the glucose syrup-supplemented fermentations at 21°P and 24°P, 1.82 and 1.94 (g (glucose)/mg (proteins)), respectively.

Amino acid consumption

As with the sugar metabolism, the amino acid metabolism in brewer's yeast is also an ordered process. Previously, the amino acids in beer fermentation have been divided into four groups A, B, C, and D (Pierce 1987) depending on the order of their uptake. Comparison across the 14°P, 21°P, and 24°P fermentations showed that the order of amino acid uptake remained the same at all gravities (Fig. 3). The only observed difference was that with increased gravity, related to the decreased specific growth rate, the time for complete amino acids utilization for each of the groups increased. Comparison of amino acid uptake across the different gravities showed that all amino acids from group A were consumed in the first 60 to 70 fermentation hours for the

Table 2 Growth and wort characteristics of the studied fermentations

Wort °P	14°P	21°P glucose	21°P maltose	24°P glucose	24°P maltose
Final °Plato wort	3.49	5.17	4.29	7.96	7.93
Fermentability (%)	76	75	80	67	67
Final FAN (mg l^{-1})	49	55	52	75	68
Consumed FAN (mg l^{-1})	190	155	148	117	133
% Assimilated FAN	79.6	73.8	76.4	61	66.2
Specific growth rate (h^{-1})	0.067	0.05	0.064	0.044	0.049
Lag phase to ethanol production (h)	21	25	25	40	41
Ethanol yield (g/g)	0.45	0.48	0.49	0.46	0.47
Glycerol yield (g/g)	0.021	0.026	0.018	0.027	0.022
Intracellular trehalose (g glucose/mg protein) exponential phase	0.9	1.15	0.97	1.46	1.32
Intracellular trehalose (g glucose/mg protein) stationary phase	1.45	1.82	1.98	1.94	2.69

Values are the average of two independent batch cultivations performed in duplicate (n=2). Standard deviations were determined to be lower than 5% of the average value. Yields are calculated as gram of products (ethanol, glycerol) produced per gram of total sugars consumed from the exponential phase. The numbers 14, 21, and 24, respectively, represent the corresponding gravity of the wort. Gl and M represents glucose and maltose suryp supplementation, respectively

14°P and 21°P fermentations and 90 h for the 24°P fermentations. The amino acids from group B were consumed first by the 90–95 h for the 14°P and 21°P fermentations and by the 140-h point for the 24°P fermentations. For the amino acids from group C, complete uptake was observed after 110–120 h for the 14°P and 21°P fermentations, while for the 24°P, complete uptake for the amino acids from group C was observed after 140 h. While proline uptake was not observed for the 14°P fermentations, some proline uptake at a minor rate was observed for the 21°P and 24°P fermentations (data not shown). The pattern of the amino acid uptake was very similar between the glucose and maltose syrup-supplemented fermentations at the corresponding gravities.

Effect of the increased gravity on the free amino nitrogen content

One of the biggest limitations in high-gravity beer fermentation is that the use of sugar syrups as adjuncts to increase the gravity dilutes the wort's nitrogen content. Therefore, with the increase in gravity, the levels of free amino nitrogen decrease (Table 2). In the present study, the initial levels of free amino nitrogen at the beginning of the beer fermentation and the final levels at the end of the beer fermentation were measured. In general, the values for percentage assimilated FAN (% FAN) correlated with the values for percentage fermentability (% fermentability), the specific growth rate, and the maximum cell numbers observed. The levels of FAN were highest for the 14°P fermentations, and those fermentations also resulted in the highest amount of consumed FAN (the difference of FAN from the initial and final stage of beer fermentation). Through the course of fermentation, around 190 mg l^{-1} of FAN was consumed for the 14°P fermentations, resulting in 80% assimilated FAN. The fermentations at 21°P resulted in consumed FAN in the range of 150–155 mg l^{-1}. Assimilated FAN of 76% was observed for the 21°P maltose syrup-supplemented fermentations, while the % assimilated FAN was 74% for the 21°P glucose syrup-supplemented fermentations. The 24°P fermentations contained the lowest FAN available at the beginning of the fermentation (190–200 mg l^{-1}) and resulted in the highest amount of FAN left at the end of the fermentation (68–72 mg l^{-1}), thus resulting in consumed FAN in the range of 120–130 mg l^{-1}. Again, higher percentage of assimilated nitrogen was observed for the 24°P fermentations supplemented with maltose syrup—66%—and lower for the 24°P fermentations with glucose syrup supplementation—61%.

Effect of the increased gravity on the flavor profile of the final beer

To assess the flavor profile of the final beer and the influence of increasing gravity on it, the concentration of the esters/ethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate, higher alcohols propanol, isobutanol, and 3-methyl-butanol and acetaldehyde from the final beer were determined (Table 3). As high-gravity beer fermentations result in final beer with very high alcohol content, in order to compare the concentrations of the flavor compounds at different gravities, the present values of the flavor compounds from the 21°P and 24°P fermentations were

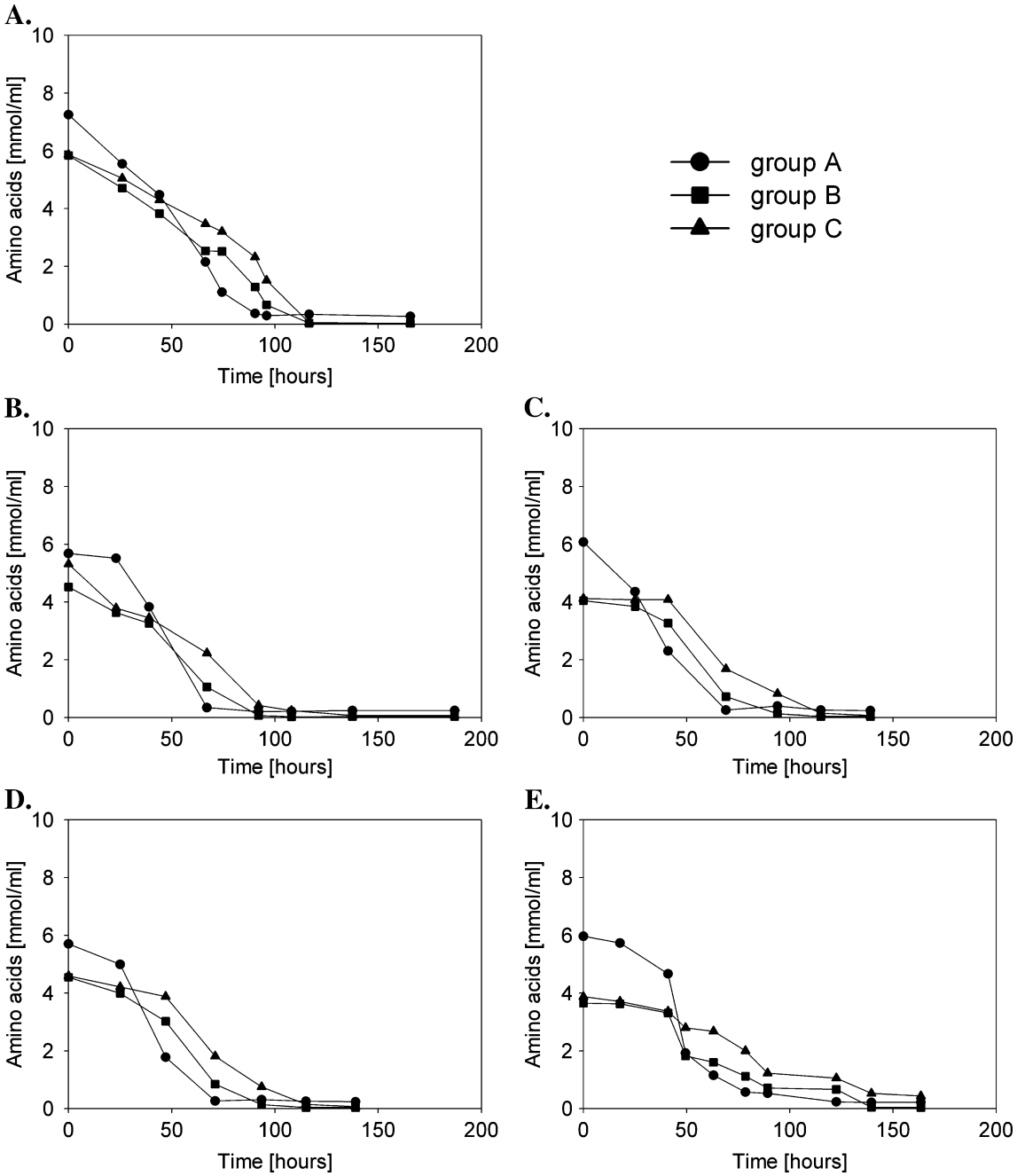

Fig. 3 Amino acid uptake in the studied fermentations. *Group A* includes aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, lysine, and arginine. *Group B* includes valine, methionine, leucine, isoleucine, and histidine. *Group C* includes glycine, phenylalanine, tyrosine, tryptophan, and alanine; **a** 14°Plato, **b** 21°Plato with glucose supplementation, **c** 21°Plato with maltose supplementation, **d** 24°Plato with glucose supplementation, **e** 24°Plato with maltose supplementation

normalized to the same ethanol content as of the 14°P fermentations. With increase in gravity, the biggest difference in the concentrations of the flavor compounds was observed for the ethyl acetate, isoamyl acetate, and acetaldehyde. In general, among the esters, with increase in the gravity, the concentrations of ethyl acetate and isoamyl acetate increased from 14.5 and 1.1 mg l^{-1}, respectively, for the 14°P fermentations to 35–39 and 1.6–2.4 mg l^{-1}, respectively, for the 21°P and 24°P fermentations. The highest concentrations for both esters at each of the corresponding gravities were observed in the glucose syrup-supplemented fermentations. The concentrations of ethyl hexanoate and ethyl octanoate remained similar. With increase in gravity, the acetaldehyde concentrations in the final beer also increased two- to fourfold. The increase in the acetaldehyde concentrations was also especially pro-

nounced for the glucose syrup-supplemented fermentations at the corresponding gravities. With increase in gravity, the concentrations of propanol and isobutanol decreased, while the concentrations of 3-methyl-butanol was slightly higher for the 21°P fermentations and slightly lower for the 24°P fermentations, compared to its concentration at 14°P (Table 3).

Discussion

Effect of the increased gravity on the brewer's yeast sugar metabolism

We observed that by increasing the gravity of the wort, there was a lower specific growth rate, a longer lag phase before initiation of ethanol production, and incomplete sugar utilization during fermentation. The three main fermentable sugars in brewing fermentation are glucose, maltose, and maltotriose (Dietvorst et al. 2007). Both maltose and maltotriose transport have shown complex kinetics involving high- and low-affinity transporters, and both of those systems are sensitive to glucose-induced inactivation.

Previous research indicated that the genes required for maltose utilisation are repressed by glucose, fructose, and sucrose and high levels of maltose and maltotriose remained in the beer when 30% *w*/*v* glucose concentrations were used as a high-gravity wort adjunct (Stewart 1999). During conditions of limited nitrogen in the wort, which is common in high-gravity fermentations, glucose and maltose themselves induce catabolite inactivation of maltose transporters by proteolysis (Rautio and Londesborough 2003).

In the studied fermentations, we observed higher amounts of residual sugars with increase in gravity both for the glucose and maltose syrup-supplemented fermentations compared to the normal-gravity fermentations (Table 2). This effect was especially pronounced for the high-gravity glucose syrup-supplemented fermentations. The glucose repression in *Saccharomyces cerevisiae* is a complex regulatory system that effects the expression of many genes, including those involved in sugar uptake and fermentation (Klein et al. 1998). Thus, the observed high residual maltose concentrations at the end of high-gravity fermentations at 21°P and 24°P are associated with glucose repression (Stewart et al. 1988) and glucose-triggered catabolite inactivation of existing maltose transporter proteins. High ethanol concentration toward the end of the beer fermentation may also play a role in this inactivation, with the known function of ethanol to inhibit the endocytosis and subsequent proteolysis of maltose transporters (Briggs et al. 2004).

In addition, wort fermentability was an important parameter for successful beer fermentation. Besides the obvious economic disadvantages for breweries, in terms of unused substrate and lower final ethanol concentration, incomplete sugar utilization could result in a beer with a higher risk for contamination by spoilage microorganisms. Especially pronounced at higher gravities, the fermentability dropped from 75% to 80% for the 21°P fermentations to 67% for the 24°P fermentations (Table 2). With increase in the gravity from 21°P to 24°P, the effect of the addition of glucose versus maltose syrups diminishes, and for both conditions, the fermentations resulted in similar fermentability. Thus, the reduced fermentability at 24°P is possibly a combination of increased osmotic pressure because of the higher sugar content, reduced FAN content, and increased ethanol toxicity due to the higher ethanol content.

Trehalose accumulation

Trehalose is a non-reducing disaccharide that has been considered to play the role of storage carbohydrate (Lillie and Pringle 1980), and its accumulation is associated with a variety of stress factors such as osmotic

Table 3 Concentration of the flavor and aroma components in the final beer

	Ethyl acetate (mgl^{-1})	Isoamyl acetate (mgl^{-1})	Ethyl hexanoate (mgl^{-1})	Ethyl octanoate (mgl^{-1})	Acetaldehyde (mgl^{-1})	Propanol (mgl^{-1})	Isobutanol (mgl^{-1})	3-Methyl-butanol (mgl^{-1})
Threshold value	21–30	1.4	0.21	0.9	10	600	100	50–70
14°P	14.5	1.1	0.10	1.70	1.7	24.5	15.4	55.7
21°P glucose	37.4	2.5	0.10	0.87	7.2	17.7	14.3	68.5
21°P maltose	36.0	1.9	0.08	0.70	3.1	16.9	13.8	63.1
24°P glucose	39.4	2.1	0.09	0.62	6.3	14.8	9.85	45.5
24°P maltose	37.8	1.6	0.05	0.92	5.2	16.9	10.8	47.5

The values for the 21°P and 24°P fermentations are reported after data normalization (correction) to the same ethanol content 5% (*v*/*v*) as 14°P fermentations. Values are the average of two independent batch cultivations performed in duplicate (n=2). Standard deviations were determined to be lower than 5% of the average value

stress, temperature shock, high ethanol concentration, low levels of water activity, growth restriction, and nitrogen limitation (Briggs et al. 2004). In our fermentations, the intracellular trehalose content increased with increasing gravity. For both the 21°P and 24°P fermentations, we observed higher intracellular trehalose content for the maltose syrup-supplemented fermentations.

Previous research (Majara et al. 1996; Boulton and Quain 2006) has also revealed that the accumulation of trehalose at the late fermentation stages is proportional to the gravity of the wort. Similar findings (Stewart 1999) in high-gravity beer fermentations suggested that intracellular trehalose accumulates to higher concentrations in the fermentations with the addition of maltose syrup to the basic wort. For each of the corresponding gravities (Beney et al. 2001), the cells grown in high-gravity maltose wort exhibited higher viabilities than the cells grown in high-gravity glucose wort.

In glucose-rich media, trehalose accumulation occurs during the transition between exponential growth and entry into the stationary phase and when other nutrients are limited or during the onset of diauxie. The higher intracellular trehalose concentrations observed in the stationary phase of the fermentations are a response to the increased stress caused by the combination of the high ethanol concentrations and enhanced nitrogen limitation (Briggs et al. 2004).

We observed that the physiological state of the brewer's yeast cells is extremely important, especially considering the fact that in the industrial beer production process the brewer's yeast is reinoculated ("repitched") for several fermentation cycles. One of the disadvantages of high-gravity beer fermentation is that the extreme conditions imposed on the brewer's yeast reduces the number of repitching cycles (Boulton and Quain 2006). Therefore, intracellular trehalose content is an important parameter of the yeast's physiological state.

Reduced free amino nitrogen and amino acids utilization

The degree of FAN utilization during the course of beer fermentation is one of the indicators for a successful fermentation. However, the use of pure sugar syrups to increase the gravity dilutes the available free amino nitrogen concentrations as well as other important nutrients in the wort such as biotin, zinc, calcium, magnesium, and other microelements and growth factors.

In the present study, the increase in the gravity by the addition of glucose or maltose-rich syrups to the media resulted in free amino nitrogen concentrations below the levels necessary for successful fermentation performance. As FAN also partially measures compounds that cannot be utilized by the yeast, typical FAN values from the end of the beer fermentations are in the range of 40–50 mg l^{-1}. Similar values were observed in the present study for the 14°P and 21°P fermentations but higher amounts of FAN in the range of 68–75 mg l^{-1} were left at the end of the 24°P fermentations.

The higher amount of residual FAN left at the end of the fermentations could be correlated with the lower specific growth rate and is possibly an indicator of yeast growth-limiting factors other than nitrogen content in the 24°P fermentations. Maltose supplementation in high-gravity beer fermentation at 20°P using dry yeast of Weihenstephan 34/70 has been reported to result in a higher percentage of assimilable FAN, 76%, compared to the glucose syrup-supplemented fermentations, 63.5% (De Rouck et al. 2007). Our results, using fresh inoculums of Weihenstephan 34/70 in the 21°P fermentations, showed a similar trend though the difference in fermentability was not as pronounced. However, at the 24°P fermentations, there was no significant difference between the percentage of assimilable nitrogen for the glucose and maltose syrup-supplemented fermentations. Thus, the effect of more pronounced nitrogen limitation and increased ethanol concentrations at 24°P fermentations diminishes the effect of the type of the carbon source in the studied very-high-gravity fermentations.

The yeast growth and the fermentation profile are influenced by both limiting and excessive presence of assimilable nitrogen. The usable FAN in the wort is directly associated with the formation of flavors such as the production of esters and higher alcohols and is important for a production of beer with good organoleptic characteristics. There is a risk that diluted free amino nitrogen concentrations result in beer with elevated levels of higher alcohols (Boulton and Quain 2006). On the other hand, high levels of FAN in the finished beer of the 24°P fermentations could also result in higher concentrations of aldehydes such as acetaldehyde, 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal and contribute to the staling flavor of the aged beer (De Rouck et al. 2007).

Amino acid uptake of the brewer's yeast

Free amino acids affect the cell mass accumulation, the yeast vitality, and ethanol production (Lekkas et al. 2007). The amino acids present in wort are divided into four classes based on their order of assimilation from the wort during the course of beer fermentation. The amino acids from the first two groups A and B are required for anabolic processes (for example protein synthesis), and they are taken up by permeases that are not affected by the nitrogen catabolite repression. It is known that during the uptake of amino acids of group C, nitrogen catabolite repression is active (Briggs et al. 2004). Our results also confirm that the amino acids from group C are taken up after the amino

acids from group A have been consumed (Fig. 3). Proline is the only amino acid present in group D, and it is the least preferred amino acid by brewer's yeast. Proline normally is not utilized during beer fermentation since its oxidation requires mitochondrial oxidase (Wang and Brandriss 1987), and as beer fermentation is an anaerobic process, the synthesis of this enzyme is repressed. However, with increase in the gravity from 14°P toward 24°P, we observed some proline uptake (data not shown) for the 21°P and 24°P fermentations. Under our experimental conditions, the fermentations were not strictly anaerobic but rather micro-aerobic; thus, the observed partial proline uptake is an indicator of pronounced limitation of assimilable nitrogen sources present in the wort as the cells attempt consumption of the least preferred nitrogen source. This observation points to the need for further elaboration of the role of proline in brewer's yeast metabolism.

Flavors

The most abundant esters and higher alcohols in beer are ethyl acetate and isoamyl acetate, the amyl alcohols, and isobutanol (Younis and Stewart 1998). In this study, with increase in gravity, the concentration of the higher alcohols—propanol, isobutanol, and 3-methyl-butanol slightly decreased. There were no significant differences in the concentrations of glucose and maltose syrup-supplemented fermentations at the corresponding gravities.

Higher alcohols are the precursors of the most flavor active esters, and their synthesis is linked to protein production. The main root for the ester formation is catalyzed by alcohol acyltransferase, with an alcohol and an activated fatty-acyl CoA molecule as substrates (Briggs et al. 2004).

In our fermentations, with increased gravity, the most significant changes in the flavor compounds from the final beer diluted to the same alcohol content 5% (*v*/*v*) were observed in increased concentrations of ethyl acetate, isoamyl acetate, and acetaldehyde. Ethyl acetate contributes with fruity flavor, and isoamyl acetate contributes with banana flavor. However, when present in excessive concentrations, their flavor influence is most often negatively accepted by the consumer. They are normally found in beer produced from average gravity 10–12°P in concentrations below their threshold values of 30 and 2 mg l^{-1}, respectively (Casey et al. 1985). In this study, the ethyl acetate and isoamyl acetate concentrations of the final beer from the 14°P fermentations were in the concentration range normally found in commercial lager beer. However, by increasing the gravity from 14°P to 24°P, the concentration of ethyl acetate and isoamyl acetate in the final beer increased more than twofold. It has previously been shown that high-gravity brewing (>16°Plato) is associated with disproportionate higher levels of esters, particularly ethyl acetate and isoamyl acetate (Stewart 2007b).

(Saerens et al. 2008) have also found that the acetate ester levels in fermentation with synthetic medium using maltose as the carbon source and the lager beer yeast strain CMBS SS01 increased 50% when the carbon content of the fermentation medium was increased from 8% maltose to 20% maltose. Both for the 21°P and 24°P fermentations in this study, the ethyl acetate and isoamyl acetate concentration of the final beer were slightly lower for the maltose syrup-supplemented fermentations compared to that of the glucose syrup-supplemented fermentations. Previous work (Younis and Stewart 1998) using synthetic media suggested that fermentations of very-high-gravity maltose predominant wort results in lower levels of these volatiles compared to fermentations with glucose and fructose predominant wort, with similar FAN levels.

There are a number of reasons, which have been attributed to the cause of lower levels of esters from the maltose syrup-supplemented fermentations. One of those is maltose inhibition on the transport of the volatile compounds out of the cell by altering the plasma membrane (Younis and Stewart 1998); another possibility is that as a result of the maltose metabolism, lower levels of acetyl-CoA have been produced, thus resulting in lower esters concentrations as a result of lack of substrate (Shindo et al. 1992).

Acetaldehyde formation occurs in the exponential growth phase but later on, during the stationary phase, its production usually declines. Acetaldehyde is an important intermediate of the glycolysis and during formation of ethanol. Thus, as expected, higher acetaldehyde concentrations were observed for the high-gravity fermentations than normal-gravity fermentation. Acetaldehyde's flavor threshold value is in the range of 10–20 ppm, and its concentration normally found in commercial lager beer is either below or in the range of its threshold value. In some cases, acetaldehyde can persist in beer above that value resulting in an unpleasant "grassy" flavor (Boulton and Quain 2006). The use of adjuncts with high glucose concentrations inhibits the fermentation rate and yeast growth, and a disproportionate increase in the formation of acetaldehyde has been observed (Briggs et al. 2004). Even though higher acetaldehyde concentrations were observed in the glucose syrup-supplemented fermentations, for all of the studied fermentations in the present study, the acetaldehyde concentrations remained within the desired concentration range.

During the course of high-gravity beer fermentation, brewer's yeast is exposed to a number of stressful conditions such as high osmotic pressure caused by the high glucose concentrations at the beginning of the fermentations and ethanol stress imposed by the elevated ethanol concentrations toward the end of the fermentation. When sugar syrups are used as adjuncts to achieve higher

gravity, the obtained wort contains lower than the minimum required free amino nitrogen concentration for achieving efficient brewer's yeast fermentation. Thus, brewer's yeast is exposed to an additional stress, caused by the induced nitrogen limitation and possible limitation of other nutrients, resulting in restricted growth. Our study underlines the effect of the various stress factors on the brewer's yeast metabolism and the influence of the type of sugar syrups on the fermentation performance and the flavor profile of the final beer. The combination of the above mentioned factors led to poorer yeast growth, longer fermentation times, and incomplete fermentation with higher amounts of residual sugars at the end of the fermentations. Additionally, the flavor profile of the final beer was adversely affected by the high concentrations of ethyl acetate and isoamyl acetate. In order to minimize the effect of the negative fermentation performance, the choice of the type of sugar syrups, used to increase the gravity, is of important consideration. The use of maltose syrup to increase the gravity, assuming that there is no additional FAN supplementation, resulted in a more balanced fermentation performance in the 21°P fermentations in terms of higher cell numbers, a higher specific growth rate, respectively, higher wort fermentability, and a more favored flavor profile of the final beer in terms of lower ethyl acetate, isoamyl acetate, and acetaldehyde concentrations.

The high amount of residual FAN at the end of the 24°P fermentations also revealed that there are other growth-limiting factors in the beer fermentations when sugar syrups are used to achieve higher gravities. Thus, further investigation is needed to unravel the mechanisms behind the effect of stuck fermentations and the complexity of the metabolic yeast response to both nitrogen and other growth factor limitations and glucose repression.

Acknowledgments M. P. Piddocke acknowledges FOOD research school at the Center for Advanced Food Studies, Denmark and Novozymes A/S for the awarded scholarships. The authors thank Lise Schultz and Preben Andersen from the Brewing and Alcoholic Beverage Department at Novozymes A/S for the assisted help for the amino acid and free amino nitrogen analyses.

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