

Isolation and Characterization of Brewer's Yeast Variants with Improved Fermentation Performance under High-Gravity Conditions[∇]

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To save energy, space, and time, today's breweries make use of high-gravity brewing in which concentrated medium (wort) is fermented, resulting in a product with higher ethanol content. After fermentation, the product is diluted to obtain beer with the desired alcohol content. While economically desirable, the use of wort with an even higher sugar concentration is limited by the inability of brewer's yeast (*Saccharomyces pastorianus*) to efficiently ferment such concentrated medium. Here, we describe a successful strategy to obtain yeast variants with significantly improved fermentation capacity under high-gravity conditions. We isolated better-performing variants of the industrial lager strain CMBS33 by subjecting a pool of UV-induced variants to consecutive rounds of fermentation in very-high-gravity wort (>22° Plato). Two variants (GT336 and GT344) showing faster fermentation rates and/or more-complete attenuation as well as improved viability under high ethanol conditions were identified. The variants displayed the same advantages in a pilot-scale stirred fermenter under high-gravity conditions at 11°C. Microarray analysis identified several genes whose altered expression may be responsible for the superior performance of the variants. The role of some of these candidate genes was confirmed by genetic transformation. Our study shows that proper selection conditions allow the isolation of variants of commercial brewer's yeast with superior fermentation characteristics. Moreover, it is the first study to identify genes that affect fermentation performance under high-gravity conditions. The results are of interest to the beer and bioethanol industries, where the use of more-concentrated medium is economically advantageous.

Today's need to produce quality beers in a short time and in the least-expensive way has led most breweries to switch to high-gravity brewing. Traditionally, brewing worts of 12° Plato (12°P) (i.e., 12 g extract per 100 g liquid) are fermented to produce beers of 5% (vol/vol) ethanol. However, substantial cost savings can be realized by increasing the wort density (called "gravity") from 12°P to 18°P and diluting the 7.5% (vol/vol) ethanol-containing product in order to obtain beer with regular ethanol content (5%). The use of this technology increases the brewery capacity by 50% (for 18°P wort) without the need for any investments, reduces the costs in energy and labor (because liquid volumes are reduced), improves the stability of the beer, and enhances the recovery of ethanol per unit of fermentable sugar ("extract") (15, 44). However, the technology is limited by the stress resistance and fermentation performance of the brewer's lager yeast *Saccharomyces pasto-*

rianus. *S. cerevisiae* (used for ale and wine fermentations) can cope much better with such stress conditions. Wine must typically contains up to 25% (wt/wt) sugar, but a major difference from beer production is that the yeast is only used once in wine fermentation whereas it is used multiple times in beer fermentation. In addition, wine must is very different from wort in its composition, and wine fermentation is carried out at higher temperatures. By contrast, beer fermentation using initial wort gravities above 18°P has a negative impact on yeast performance and often results in sluggish and incomplete fermentations (31). In addition, the viability of the yeast in successive rounds of fermentation drops significantly faster than that seen with traditional brewing, thereby compromising recycling of the yeast for successive rounds of fermentation (6, 7). Recycling of yeast is general practice in beer brewing because of the more reliable quality of the beer in the second and next fermentation rounds and because of the cost savings compared to the use of fresh yeast for every fermentation. In addition, undesirable changes in flavor profile (2) and reduction in head retention (9) have been reported for high-gravity brewing.

The frequent sluggish or stuck fermentations encountered in high-gravity brewing are probably caused by several stress factors that are not present or are at least less pronounced compared to traditional brewing results (19, 21, 29, 30). In particular, the high osmolarity at the beginning of the fermentation

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and the high ethanol content combined with nutrient starvation at the end of the fermentation are thought to be the major culprits. Moreover, the combination of high-gravity brewing with other modern practices, such as the use of tall cylindrical fermenters, results in increased hydrostatic pressure and carbon dioxide levels and decreased oxygen levels. Recently it was shown that fungal adenylate cyclases function as CO₂ sensors (1, 3, 22, 24). Thus, different levels of CO₂ may influence yeast fermentation, as there is a direct link between adenylate cyclase activity and glycolysis (13, 26).

The disadvantages of brewing at higher density could be overcome by using more robust yeast strains that are tolerant of the associated stress conditions. Successful strains should be able to ferment the high-gravity wort faster and to a greater extent than the control strain. In addition, the viability of the yeast crop at the end of the fermentation should remain high in order for the yeast to be suitable for recycling. Other characteristics important in brewing, such as flavor formation and flocculation behavior, should remain the same as with the control strain in regular gravity fermentations. When those requirements are fulfilled the strain could be used successfully to produce beer under high-gravity conditions that retains the original brand character.

In this study, a pool of variants of the industrial lager brewing yeast CMBS33 was generated by means of UV mutagenesis or by spontaneous selection. Selection for better-performing strains was then carried out under conditions closely matching the industrial high-gravity conditions, in which the yeast is exposed to a combination of several stress factors. Two variants with better fermentation characteristics under these stringent conditions, GT336 and GT344, were identified. Genome-wide gene expression analysis with these strains revealed genes that are important for faster fermentation under high-gravity brewing conditions.

MATERIALS AND METHODS

Microbial strains and plasmids. The industrial and laboratory yeast strains used in this study are listed in Table 1. The variants derived from strain CMBS33 were stable over several generations. The strains were restreaked on fresh yeast extract-peptone-dextrose (YEPD) plates at least 10 times without losing their better fermentation capacity under high-gravity conditions.

The complete coding region of *LEU1* was PCR amplified using genomic DNA of the laboratory strain BY4742, Expand High Fidelity polymerase, and 5' and 3' primers containing a BamHI and an XmaI site (lowercase), respectively (5' primer, CGggatccATGGTTTACACTCCATCCAAG; 3' primer, TCCccgggCTACCAATCCTGGTGGAC).

The amplified gene was cloned into pYX012 *KanMX* (39), downstream of the constitutive *TPII* promoter, by use of these sites. The construct was linearized with PacI for integration in the *TPII* genomic locus in the industrial strain CMBS33. The corresponding empty plasmid was used as a control. Correct insertion was checked by PCR analysis.

For all gene cloning experiments, standard techniques were used (35, 37). Bacterial transformations were carried out by the CaCl₂ method (35). Yeast transformations were carried out according to the LiAc-polyethylene glycol method (14). Sequencing of the constructs was performed by the dideoxy-chain-termination method with a 3100 Avant genetic analyzer (ABI) according to the supplier's instructions.

Fermentation experiments. (i) **Wort production.** All-malt wort was prepared from blond malt extract powder (8.8 European Brewery Convention [EBC]; Pharma Import, Belgium). This wort contains 90% carbohydrates, of which 1% to 2% is fructose, 5.5% to 10% is glucose, 1.5% to 2.5% is sucrose, 40% to 55% is maltose, and 8% to 13% is maltotriose. The wort also contains 4.9% to 6.3% proteins. The unit of gravity generally used in brewing is degree Plato. One degree P corresponds to 1 g extract per 100 g liquid solution. Apart from

TABLE 1. Industrial and laboratory yeast strains used in this study

Strain	Description or genotype	Source
CMBS33	Commercial lager strain	KU Leuven CMBS collection ^a
GT336	Spontaneous variant derived from CMBS33	This study
GT344	UV-induced variant derived from CMBS33	This study
CMBS33/ <i>TPII</i> p	CMBS33/pYX012 <i>KanMX</i>	This study
CMBS33/ <i>TPII</i> p <i>LEU1</i> (BY4742)	CMBS33/pYX012 <i>KanMX</i> <i>LEU1</i> (BY4742)	This study

^a KU, Katholieke Universiteit; CMBS, Centre for Malting and Brewing Science.

fermentable sugars this extract also contains nonfermentable carbon sources such as dextrans, starch, and β -glucans. Zinc ions were added to the medium (0.1 ppm Zn, added as ZnSO₄·7H₂O). After autoclaving, the wort was oxygenated (at 12°C, 8 ppm; at between 12°C and 20°C, 10 ppm; above 20°C, 12.5 ppm) before inoculation of the cells. The normal all-malt wort (12.9°C) was adjusted to very-high-gravity wort by addition of sucrose (Grant Pont, Belgium).

(ii) **Inoculation of yeast in wort.** Early-stationary-phase cells grown on YPMal (1% yeast extract, 2% peptone, 2% maltose) were collected by centrifugation (5 min, 1,300 × g) and counted with a hemocytometer. Yeast was inoculated in oxygenated wort with a pitching rate of 10⁶ cells/ml/°P. Fermentations were carried out in unstirred 250-ml small tubes (height, 30 cm; diameter, 4 cm), tall 2-liter EBC tubes (height, 72 cm; diameter, 7.5 cm), or 3.5-liter stirred fermenters (type Chemap [Heineken facility, Zoeterwoude, The Netherlands]; height, 21 cm; diameter, 14.5 cm) at the indicated temperature until the wort attenuated.

(iii) **UV mutagenesis.** For UV treatment, stationary-phase cells were spread on several YEPD plates and a UV dose (5, 10, or 15 mJ per cm²) was applied (GS Gene Linker from Bio-Rad) (254 nm). The plates were then incubated at 30°C in the dark for 3 days to allow recovery and growth.

(iv) **Selection experiment.** To start the selection experiment, the pool of variants and the control strain were inoculated in very-high-gravity wort (24.2°C) in tall 2-liter tubes and the fermentation was carried out at 20°C. Density of the wort was measured at regular time points. The starting densities of the worts in the successive fermentations were 26.7°C, 25.19°C, 27.6°C, and 22.14°C. The yeast was cropped at room temperature at the end of each fermentation round. At that time point, the yeast that was left in suspension as well as the yeast at the top of the sediment was harvested by centrifugation (5 min, 1,300 × g). The relatively high temperature and high ethanol content caused some autolysis, which resulted in a loose top of the yeast pellet. Approximately half of the total yeast in the tube was used again. The yeast was washed twice with YEPD, and all the yeast was used in the subsequent round except in the last round of the experiment, where the same amount of viable cells (18 × 10⁶/ml) was inoculated. At the end of the last fermentation round, samples were spread on yeast extract-peptone-maltose plates for the isolation of individual colonies. Several hundred colonies were screened for colony size, stress tolerance, and growth under normal and anaerobic conditions. Sixteen fast-growing colonies were then individually tested under high-gravity conditions.

Determination of different parameters during the fermentation. (i) **Viability after methylene blue staining.** Viability was determined using methylene blue staining according to the EBC Analytica method (17) and a hemocytometer.

(ii) **Wort density.** The density of the wort was measured using an A. Paar DMA 35 N apparatus and expressed in degrees Plato.

(iii) **Attenuation limit of the wort.** To determine the attenuation limit of the wort corresponding to the unfermentable fraction, 12 g pressed yeast (Bruggeman) was added to a 200-ml wort sample collected at the end of the fermentation. The flask with waterlock was placed on a stirring plate at room temperature. Density of the wort was determined after 24 h and 48 h of stirring and named "apparent extract after final attenuation." The difference in apparent extract (delta apparent extract) is calculated by subtracting "apparent extract after final attenuation" from "apparent extract at the end of the fermentation"; this gives an indication of how much of the fermentable sugars remained in the beer at the end of the fermentation.

(iv) **Ethanol content.** Ethanol production was determined enzymatically with an ethanol kit (Boehringer Mannheim).

(v) **Trehalose and glycogen content of the cells.** Trehalose and glycogen levels and trehalase activity were determined as previously described (8, 10, 25).

(vi) **Headspace analysis of esters and higher alcohols.** Five-milliliter samples were collected in 15-ml precooled glass tubes, which were immediately closed with a cap and stored at -20°C . The formation of the volatile compounds was monitored using a calibrated Perkin-Elmer Headspace Sampler HS40 Autosystem XL equipped with a Chrompack-Wax 52 CB column (length, 50 m; internal diameter, 0.32 mm; layer thickness, 1.2 μm). The injection block and flame ionization detector temperatures were kept constant at 180°C and 250°C , respectively, and helium was used as the carrier gas. Results were analyzed using Perkin-Elmer Turbochrom Navigator software.

(vii) **Genome-wide gene expression analysis.** Samples were taken 22 h after the beginning of a high-gravity fermentation in tall 2-liter tubes at 20°C and immediately added to ice-cold water. Total RNA was extracted using RNeasy (Qiagen) or TRIzol reagent (Invitrogen) according to the instructions of the manufacturers. Those samples were handled by the VIB microarray facility at Katholieke Universiteit Leuven (Belgium). cDNA was made from the mRNA, the samples were fluorescently labeled with Cy3 or Cy5, and hybridization took place on 70-mer oligochips representing 6,307 yeast open reading frames. The oligonucleotides were spotted twice per array (for protocols, see <http://www.microarrays.be/service.htm>). Fluorescence imaging of the arrays was performed with a GenIII confocal laser scanner (Amersham Biosciences). The data were normalized, and genes were judged differentially expressed with a ratio of 1.8 or more.

Reproducibility of the results. All experiments described were repeated at least two times. The results always showed consistent trends; i.e., differences between the control strain and variants were highly reproducible. The tall-tube fermentations were repeated at least six times with all strains. A paired t test with data obtained from the fermentations after 1 day resulted in a P of <0.005 between strain CMBS33 and both strains GT336 and GT344. On the other hand, a P value of 0.25 was obtained when strains GT336 and GT344 were compared. Representative results are shown for comparisons between collections of interdependent datum points (e.g., time course measurements). The error bars shown here indicate a standard deviation of at least two independent measurements (e.g., fermentations at a 3.5-liter scale).

RESULTS

Isolation of better-performing variants under very-high-gravity conditions. A commercial lager brewing strain, CMBS33, was used as the starting strain for selection of better-performing variants under very-high-gravity conditions. The strain was mutagenized by UV irradiation to create a pool of variants. This pool was subsequently used to inoculate ultra-high-gravity wort. Since gravities up to 32.5°P were not high enough to eliminate the weaker variants rapidly (results not shown), we increased the stress applied on the yeast by adding 10% ethanol either 1 day or 8 days after the start of the fermentation. Under these conditions there was very poor survival of the yeast cells. From 10^7 cells/ml at the start of the fermentation, we only recovered fewer than 1,000 colonies/ml after 9 days of fermentation. Several hundred of the last-surviving variants thus obtained were then tested individually for better growth under high-gravity conditions. However, all variant isolates actually performed worse than the starting wild-type strain. Further analysis indicated that most of these isolates were petite mutants. The modified procedure in which the pool of UV-induced variants and the control strain were repitched several times in ultra-high-gravity (between 22 and 28°P) wort in tall 2-liter tubes at 20°C turned out to be more successful. In the fifth fermentation round, the pools of UV mutagenized as well as nonmutagenized cells and started to ferment the high-gravity medium faster and more completely than the parent CMBS33 strain in a parallel first fermentation round (data not shown). This suggests that in the nonmutagenized culture spontaneous variants were selected by the high-gravity conditions. At the end of the last round of the selection experiment, samples were spread on yeast extract-peptone-maltose plates

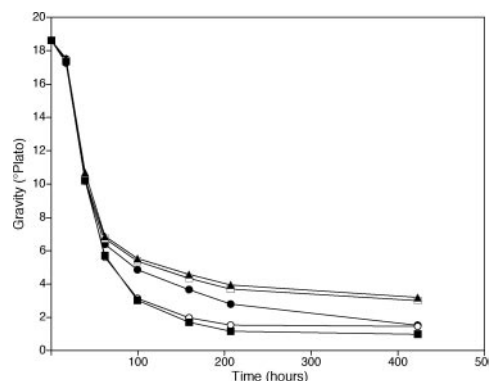


FIG. 1. Fermentation profiles in tall 2-liter tubes under high-gravity conditions at 20°C of different variants isolated from the repitching screen and compared to the wild-type control strain. Control strain, ●; GT336, ○; GT338, □; GT344, ■; GT343, ▲. Paired Student t tests were performed using data obtained from six independent fermentations. Comparisons at days 1, 2, 4, and 7 between CMBS33 and either GT344 or GT336 gave P values of 0.005, 0.0005, 0.05, and 0.05, respectively, in both cases.

for the isolation of individual colonies. In total, a few hundred variants (either induced by UV or spontaneous) were isolated for further characterization. These variants were initially screened for large colony size, growth capacity under anaerobic conditions, and growth on plates containing high ethanol concentrations in order to eliminate uninteresting slow-growing variants.

Characterization of the variants GT336 and GT344 and the control strain under normal, high-gravity, and very-high-gravity conditions in tall 2-liter tubes at 20°C . (i) **Fermentation behavior, viability, and ethanol production.** Sixteen colonies (8 derived from the UV-mutagenized starting culture and 8 derived from the wild-type inoculum) with a normal growth phenotype were tested individually for their fermentation characteristics under high-gravity (between 18 and 20°P) conditions in 2-liter fermentation tubes at 20°C . Fourteen variants displayed fermentation behavior similar to or worse than that seen with the control strain under these conditions, but two variants (GT336 and GT344) displayed better fermentation characteristics than those seen with the control strain. Figure 1 shows the results obtained with the wild type, two poorly performing variants (GT338 and GT343), and the two variants with better fermentation characteristics (GT336 and GT344).

To test whether the better fermentation capacity of GT336 (variant enriched from the nonmutagenized cells) and GT344 (variant enriched from the UV-mutagenized cells) was specific for high-gravity fermentations we compared them to the control strain in normal (13°P), high-gravity (17.1°P), and very-high-gravity (23°P) wort in tall 2-liter tubes (Fig. 2A to C). The improvement was more pronounced under the most stringent conditions (Fig. 2C). The fermentations with these strains have been repeated at least six times under very-high-gravity conditions; on the basis of the results of a paired t test, the fermentation with the two variants was in all experiments much faster than the fermentation with the wild-type strain ($P < 0.005$, measured after 1 day of fermentation). While both strains showed improved fermentation rates, under these conditions only variant GT344 showed an improved final attenuation in

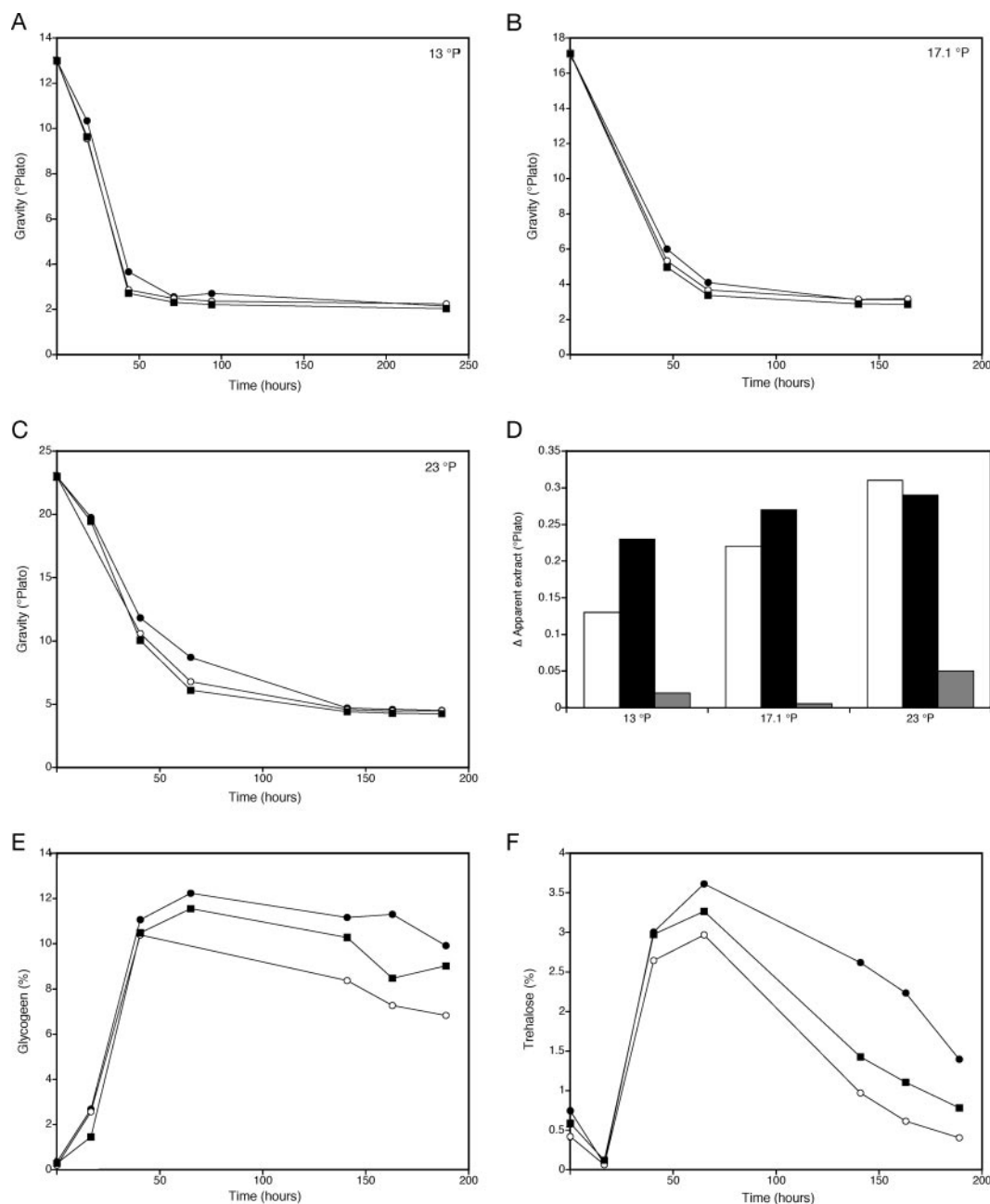


FIG. 2. (A, B, and C) Fermentation profiles of the control strain CMBS33 (●), GT336 (○), and GT344 (■) during normal (A), high-gravity (B), and very-high-gravity (C) fermentations in tall 2-liter tubes at 20°C. The initial wort densities are indicated. Yeast was pitched in wort with a pitching rate of 10^6 cells/ml/°P. (D) Delta apparent extract of CMBS33 (white bar), GT336 (black bar), and GT344 (gray bar) in normal, high-gravity, and very-high-gravity fermentations in tall 2-liter tubes at 20°C. (E and F) Glycogen (E) and trehalose (F) levels for the different strains during very-high-gravity fermentation.

the three types of fermentations (Fig. 2D). Here also, this difference was statistically significant for GT344 compared to the wild-type strain results ($P < 0.05$; see Fig. 1 also). The delta apparent extract for the differed very much (from close to 0°P to 1.5°P) from experiment to experiment, depending on the initial gravity. Use of pulsed-field gel electrophoresis for karyotyping of the variants did not reveal any difference in the chromosomal banding pattern compared to that of the wild-type strain (data not shown). The viability of all strains at the

end of the fermentation was higher than 90% (results not shown). Ethanol production correlated very well with the fermentation rate and was higher for the two variants. However, at the end of the fermentation ethanol levels were varying from one experiment to the other and especially in the variants started to drop again (data not shown). The reason for the variation at the end of the fermentation is not clear but may indicate higher postdiauxic metabolism in the variants under these conditions (see below).

(ii) **Glycogen and trehalose.** Glycogen and trehalose are known to provide higher stress tolerance (4, 12), and we therefore investigated whether the variants were affected in the levels of these metabolites. The trehalose and glycogen contents of the control strain and the two variants (GT336 and GT344) were determined during fermentation in tall 2-liter tubes at 20°C in normal, high-gravity, and very-high-gravity wort. All strains showed the same glycogen accumulation pattern in the three types of fermentation (see, e.g., Fig. 2E for 23°P results), but the variants seemed to use the glycogen somewhat faster than the wild-type control strain.

Trehalose accumulation patterns of the two variants (GT336 and GT344) and the control strain during very-high-gravity fermentations in tall 2-liter tubes at 20°C are shown in Fig. 2F. The amounts of trehalose in the cells at the moment of inoculation were in most cases approximately the same. As soon as the yeast was inoculated into fresh wort, trehalose was rapidly dissimilated. This is a known consequence of the initial concentration of glucose and fructose, which activate the cyclic AMP-protein kinase A pathway and trigger trehalose breakdown (42). Trehalose levels started to increase again after 24 h. The amount of trehalose built up when the yeast was pitched in very-high-gravity wort was higher the amounts seen with normal and high-gravity wort (data not shown). No differences in maximum levels were observed for the different strains tested. Trehalose levels declined gradually at the end of the fermentation. Surprisingly, the variants GT336 and GT344 had a smaller amount of trehalose than the CMBS33 control strain towards the end of the fermentation. This effect was especially pronounced in very-high-gravity wort (Fig. 2F) and was always seen in the tall-tube fermentations under these conditions; the latter observation could not be explained by corresponding changes in intrinsic activity of the enzymes responsible for the synthesis (Tps1 and Tps2) and hydrolysis of trehalose (Nth1) as determined with cell extracts (results not shown). In combination with the ethanol and glycogen level results, this seems to indicate that in the tall-tube fermentations, the variants are able to sustain their metabolism under conditions in which the wild type cannot.

Characterization of the variants GT336 and GT344 under high-gravity conditions in 3.5-liter fermenters at 11°C. (i) **Fermentation behavior, viability, and ethanol production.** To further evaluate the fermentation behavior of the two variants under high-gravity conditions, we tested their fermentation capacity in a 3.5-liter stirred fermenter at 11°C. Cells of the two variants and the control strain were inoculated in 18°P wort, and this experiment was performed in duplicate. The starting temperature was 8°C, and the temperature was increased within 48 h to 11°C. When all oxygen was consumed (after 8 h) the stirring speed was increased from 50 rpm to 250 rpm.

In similarity to the fermentation results seen with tall 2-liter tubes, the two variants GT336 and GT344 fermented faster than the control strain (Fig. 3A). In addition, both variants showed a lower apparent extract level at final attenuation than the control strain, indicating that their fermentation was more complete (more sugars converted into ethanol; Fig. 3G). This is also reflected in the final ethanol levels obtained with the respective variants and the wild type (Fig. 3F). The control strain CMBS33 and the variants GT336 and GT344 produced respectively 7.75% (vol/vol), 8.14% (vol/vol), and 8.10% (vol/vol)

ethanol. This increase in ethanol corresponds to the lower delta apparent extract (Fig. 3G).

The viability measured using methylene blue staining was in all fermentations and with all strains higher than 99% (results not shown). Hence, the yeast in suspension at the end of the first fermentation was suitable for recycling in consecutive rounds of fermentation. The biomass content and cell count were increasing faster for variant GT336 than for GT344 and the wild type (Fig. 3B and C). Under these conditions (11°C, stirred fermentation), no differences in trehalose and glycogen accumulation patterns between the different strains tested could be observed (Fig. 3D and E).

(ii) **Flavor compound determination.** Apart from the reduced viability and fermentation performance of the yeast in very-high-gravity fermentations, another frequently encountered problem is a change in the flavor of the beer produced by high-gravity brewing. More specifically, high-gravity brewing can result in an undesirable increase in ester production (41). This can also be seen in Table 2, where values are given for flavor volatiles of the fermentation by the CMBS33 strain in normal wort (12°P) and high-gravity wort (18°P). The volatile amounts were determined in the green beer taken at the end of the fermentation. The amounts of volatiles produced by each of the two variants were more or less the same as that seen with the control strain CMBS33. However, small differences could be found in the results of the 18°P fermentation (Table 2). Smaller amounts of acetaldehyde and ethyl acetate were formed in the green beer obtained with the variants compared to the control strain results at the end of the 18°P fermentation, and the level was close to that of the CMBS33 at 12°P. Similar amounts of isoamyl acetate were formed by the variants and the control strain. However, larger amounts of iso-butanol, iso-amylalcohol, diacetyl, and 2,3-pentanedione were produced by the variants.

On the other hand, when we compare the volatiles of the 12°P fermentation and 18°P fermentation, which was diluted to an alcohol content of 5%, greater differences were detected (Table 2). It is clear that larger amounts of acetate esters and acetaldehyde and smaller amounts of iso-amyl alcohol, diacetyl, and 2,3-pentanedione were formed in high-gravity fermentation than in normal fermentation. This fits with the results of a previous study (43). Only small differences were detected in the amount of iso-butanol. Taking these results together, it is most likely that after dilution the taste of the beer brewed under high-gravity conditions will be different from that of the original beer but that the differences will not be greater for the variants GT344 and GT336.

(iii) **Genome-wide gene expression analysis in the initial phase of a high-gravity fermentation.** In order to identify the molecular determinants responsible for the improved fermentation behavior in the variants, we used 70-mer oligonucleotide DNA arrays representing 6,307 yeast open reading frames to compare the global gene expression pattern seen under high-gravity conditions with the control strain CMBS33 to those seen with the two variants (GT336 and GT344). The global gene expression patterns of these strains were compared 22 h after the start of a tall-tube high-gravity fermentation (CMBS33, 11.2°P; GT336, 8.9°P; GT344, 9.3°P) (initial gravity = 19.1°P). This experiment was repeated with samples taken at 13.5°P (27 h for the wild type and 24 h for the variants), and each time a

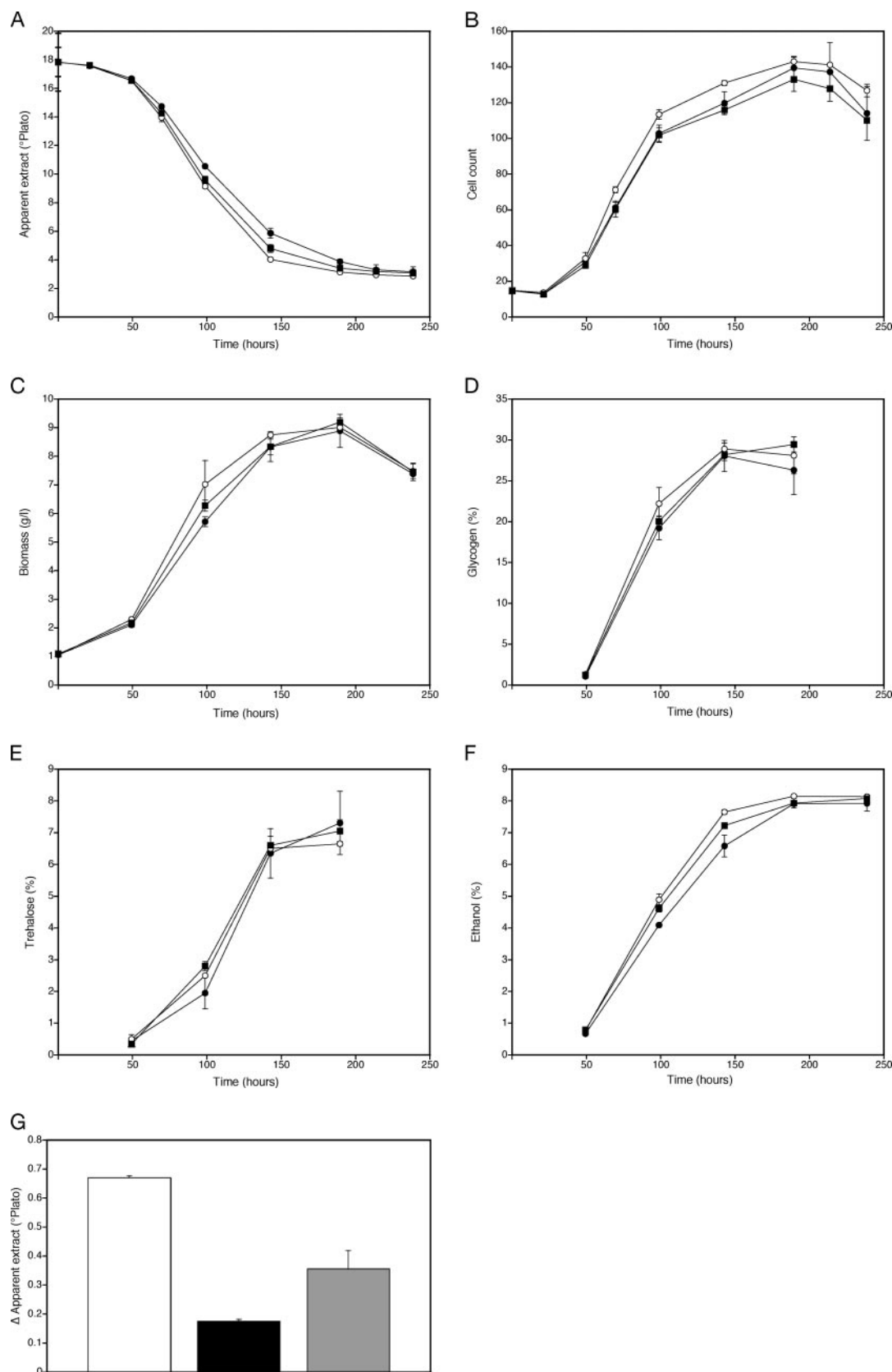


FIG. 3. (A) Fermentation profiles of the control strain CMBS33 (●), GT336 (○), and GT344 (■) during 18°P stirred fermentation in 3.5-liter fermenters at 11°C. Yeast was pitched in wort with a pitching rate of 10⁶ cells/ml/°P. (B to F) Cell count (× 10⁶ cells/ml) (B), biomass (grams per liter) (C), glycogen (%) (D), trehalose (%) (E), and ethanol (%) (F) levels for the different strains (same symbols as described for panel A) during high-gravity fermentation. (G) Delta apparent extract of CMBS33 (white bar), GT336 (black bar), and GT344 (gray bar) in high-gravity fermentation in 3.5-liter fermenters at 11°C. The delta apparent extract was determined after 238 h of fermentation. All datum points represent the means of two independent experiments. Error bars indicate standard deviations obtained from the two independent experiments.

TABLE 2. Volatile analysis of samples taken from green beer at the end of 12°P fermentation with the control strain CMBS33 and at the end of 18°P fermentation with control strain CMBS33 and strains GT336 and GT344 in 3.5-liter stirred fermenters at 11°C

Volatile	Value for strain under indicated conditions				Threshold value(s)
	CMBS33 (12°P, 5% EtOH)	18°P, beer diluted to 5% EtOH			
		CMBS33	GT 336	GT 344	
Acetaldehyde (mg/liter)	0.85	2.0	1.2	1.3	10
Ethyl acetate (mg/liter)	15.0	28.7	24.4	26.3	21–30
Isoamyl acetate (mg/liter)	2.0	3.0	3.0	3.0	1.4
Isobutanol (mg/liter)	22.6	18.6	22.3	20.8	200
Isoamylalcohol (mg/liter)	60.3	44.4	46.1	45.7	70
Diacetyl (μg/liter)	52.8	12.8	16.0	15.7	80
2,3-Pentanedione (μg/liter)	60.7	26.7	35.2	37.3	900

color flip hybridization, in which each of the RNA samples was labeled with the dye previously used for the other sample, was included.

The expression analysis revealed 13 genes with expression levels that differed (at a ratio of 1.8 or higher) between the control strain and both variants. Among those, 10 genes showed lower and 3 genes showed higher expression in one or both variants with improved fermentation behavior under high-gravity conditions (Table 3 and Table 4). The most conspicuous genes were *HXK2*, whose expression under high-gravity conditions was reduced twofold, and *LEU1* and *ARG1*, whose expression was reduced two- to sixfold.

From previous experience with industrial baker's yeast, we know that it is extremely difficult to obtain deletion strains in aneuploid industrial strains. Hence, we could not validate the genes that were down regulated in the two variants in the original strain background. We were able to use the corresponding deletion mutants in a laboratory strain background but only with YEPD instead of wort medium. We tested deletion mutants in *RPL8A*, *RPL22A*, *PUT1*, *YLR164w*, *HMG1*, *NDE1*, *PDR16*, *ALD6*, and *HXK2*, but a weak improvement of the fermentation rate was observed only with the *hvk2Δ* strain (data not shown).

Overexpression of *LEU1* in the industrial strain CMBS33 improves fermentation rate in high-gravity conditions. Genome-wide gene expression analysis revealed that the genes *LEU1*, *ARG1*, and *ICY2* were expressed more strongly in one or both variants compared to the control strain results in the initial phase of high-gravity fermentation. The effect of overexpression of these genes in the wild-type strain on the fermentation behavior in high-gravity wort was tested in small tubes (250 ml) at 20°C and, in cases in which the fermentation rate improved subsequently, also in tall 2-liter tubes under the same conditions. Overexpression of *ARG1* and *ICY2* did not affect the fermentation behavior under high-gravity conditions compared to results seen with the control strain with the empty plasmid (results not shown). Overexpression of both genes during the high-gravity fermentation was confirmed by real-time PCR (results not shown). Hence, we conclude that these genes are probably not involved in improvement of the fermentation rate under high-gravity conditions.

TABLE 3. Genes with lower expression (ratio of 1.8 or more; $P < 0.01$) in one or both variants with better fermentation behavior under high-gravity conditions compared to the control strain^a

Variant gene (genotype or description), collection date, and gravity value	Ratio for variant:	
	GT336	GT344
<i>HXK2</i> (hexokinase II)		
Day 1	−2.18	−1.70
13.5°P	−1.72	1.05
<i>RPL8A</i> (ribosomal protein L8)		
Day 1	−2.13	−1.55
13.5°P	−2.24	−1.14
<i>RPL22A</i> (ribosomal protein 22A)		
Day 1	−2.05	−1.52
13.5°P	−2.22	−1.13
<i>PUT1</i> (proline oxidase)		
Day 1	1.28	−1.85
13.5°P	−2.18	−2.70
<i>YLR164w</i> (member of the cytochrome B small-subunit mitochondrial precursor (CyB) family)		
Day 1	−1.86	−1.59
13.5°P	−1.49	−1.83
<i>HMG1</i> (3-hydroxyl-3-methylglutaryl- coenzyme A reductase 1)		
Day 1	−2.40	−2.30
13.5°P	−1.41	−1.69
<i>NDE1</i> (mitochondrial NADH dehydrogenase)		
Day 1	−2.16	−2.02
13.5°P	−1.57	−1.90
<i>PDR16</i> (phosphatidylinositol transfer protein)		
Day 1	−2.64	−2.45
13.5°P	−1.52	−1.77
<i>ALD6</i> (acetaldehyde dehydrogenase)		
Day 1	−5.03	−3.88
13.5°P	−1.25	−1.50
<i>RPL5</i> (ribosomal protein L5)		
Day 1	−1.96	−1.37
13.5°P	−2.26	−1.14

^a Samples were taken in the beginning of the fermentation at a fixed time point (day 1) or at a fixed density (13.5°P). The ratios were calculated based on the fluorescence obtained from two independent hybridizations (using a color flip) and from two independent spots using Student's *t* test.

Overexpression of *LEU1* did result in better fermentation behavior under high-gravity conditions. The overexpression strains fermented faster, but the final attenuation of all the strains was the same. The effect was detected with both small-scale 250-ml fermentations (results not shown) and tall 2-liter tubes (Fig. 4). The viability measured after methylene blue staining remained higher than 95% at the end of the fermentation (results not shown). The overexpression of *LEU1* in the transformant CMBS33 + *TPII*p *LEU1* during this high-gravity fermentation was confirmed by real-time PCR (data not shown).

TABLE 4. Genes with higher expression (ratio of 1.8 or more) in one or both variants with better fermentation behavior under high-gravity conditions compared to the control strain^a

Variant gene (genotype or description), collection date, and gravity value	Ratio for variant:	
	GT336	GT344
<i>LEU1</i> (3-isopropyl malate isomerase)		
Day 1	2.44	2.13
13.5°P	6.48	3.11
<i>ARG1</i> (argino-succinate synthetase)		
Day 1	2.40	2.27
13.5°P	2.73	1.35
<i>ICY2</i> (unclassified protein)		
Day 1	2.45	2.19
13.5°P	1.71	1.49

^a Samples were taken in the beginning of the fermentation at a fixed time point (day 1) or a fixed density (13.5°P).

DISCUSSION

Selection of better-performing variants in an industrial lager yeast strain. Our results show that it is possible to isolate variants of industrial brewer's yeast with better performance in high-gravity fermentation conditions. This is not trivial, as fermentation performance is a trait that is not readily selectable (i.e., poorly fermenting cells may survive just as well as superior mutants). The direct isolation of variants of the industrial strain (as opposed to studies with laboratory strains) enhances the chance that the identified changes are also relevant for large-scale industrial conditions. The use of laboratory strains that are far from optimal with respect to industrial performance increases the risk of identifying mutations that merely reverse the effect of domestication of these laboratory strains rather than further improving the already optimized performance of industrial strains. Furthermore, selection directly in brewer's yeast and under conditions that closely mimic the conditions of the industrial brewing process minimizes the risk that other parameters important for brewing are negatively affected. This was confirmed by further characterization of the variants in tall 2-liter tube and pilot-scale (3.5-liter) fermentations. The growth rate was not affected, the ester profile was quite similar to that seen with the control strain, and the level of isoamylacetate, a flavor ester of major importance, was not changed. A second important advantage of new strains created in this way is that they are not genetically modified by recombinant DNA technology (i.e., they are not GMOs, or genetically modified organisms) and can therefore be introduced immediately on the market without regulatory restrictions.

The method we developed for the isolation of these variants has since been applied successfully in our laboratory, with different strain backgrounds demonstrating the general applicability of this methodology. Two industrial strains were UV mutagenized and used in successive rounds in very-high-gravity fermentations (25.5°P, 16 ppm O₂, 3×10^7 cells/ml, and 20°C). After the first fermentation a survival rate of about 65% was measured; after the second fermentation, this had already dropped to 15% survival. After four fermentations, individual colonies were checked and a percentage of variants similar to that seen in our original experiment showed improved fermenta-

tion behavior (F. Dumortier, P. Van Dijck, and J. M. Thevelein, unpublished data). This indicates that repeated challenge by the combined stress factors present during a high-gravity fermentation, in combination with a high temperature, is enough to enrich for better-fermenting cells. During normal fermentations, the viability is very high and the yeast only divides two to three times during one fermentation round. Under these conditions, it would be impossible to obtain better-fermenting variants in five rounds of fermentations. Under the conditions we used (high temperature and very-high-gravity wort), the survival rate of the yeast cells is much lower. As mentioned above, in similar experiments using the same conditions we obtained a viability rate of 65% after the first round, with the rate going down to about 35% in the second round and dropping further in the third round. In the fourth round, this was increasing again to above 10%, probably because of enrichment of more-tolerant strains. The high degree of mortality is probably caused by the fact that the cells stay in the tall tubes under very-high-ethanol conditions for prolonged times. The result is that after pitching, the cells that survived the previous fermentation must bud many more times in order to complete the fermentation. In this way, a very strong enrichment for strains that ferment better under very-high-gravity conditions may occur during the five successive rounds of fermentation. This is probably the reason that of 16 individual colonies tested at the end of the fifth fermentation, 2 showed consistently better fermentation capacity.

Characterization of the better-performing variants GT336 and GT344. As we have noticed in the past for baker's yeast (40), the behavior characteristics of variants can be quite different under laboratory and pilot-scale conditions. Under laboratory conditions, variant GT344 seemed most suitable for high-gravity brewing, as it showed the best final attenuation of all the strains. However, in pilot-scale conditions at the Heineken facility (using completely different parameters), GT336 seemed to be the best variant. The fact that this variant ferments faster and more completely under high-gravity conditions and has high crop viability at the end of the fermentation provides a significant economical benefit for the brewer. It is important to note that, while the absolute differences between the variants and the control strain may seem small to nonsp-

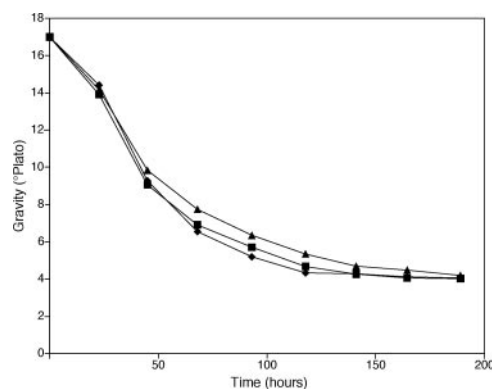


FIG. 4. Fermentation behavior of CMBS33/TPIp (▲), CMBS33/TPIp *LEU1* (◆), and CMBS33/TPIp *YOR387c* (■) in 17°P wort in tall 2-liter tubes at 17°C. Yeast was inoculated in wort with a pitching rate of 10^6 cells/ml/1°P.

cialists, they are in fact quite important for the brewer. Suppose we can finish a fermentation with 0.5°P-lower apparent extract using such a strain. When we start a fermentation with a gravity of 20°P and end up with an apparent final attenuation with the wild type of 5.9 and with the variant of 5.4, the benefit is $(14.6 - 14.1)/14.1 \times 100\% = 3.54\%$. A fermentation vessel with a content of 3,000 hl produces 3,106 hl of beer, which is 106 hl more with the superior variant. Hence, a better attenuation of 0.5°P results in an increase of about 32,000 beer bottles of 33 cl each per fermentation tank. It is interesting that variants of already optimized industrial strains showing a further increase in attenuation and in fermentation rate could be isolated. Due to the large-scale production of today's fermentation industry, even a 1% improvement in production efficiency represents a highly significant advantage.

Trehalose and glycogen levels are important parameters in brewer's yeast usage. Glycogen provides energy for cellular maintenance during storage (34). Trehalose functions not only as a storage carbohydrate but also as a stress protectant (4). Its protective effect against osmotic stress (18, 38) and ethanol stress (27, 36) has been well documented. In the pilot-scale fermentations, the levels of trehalose and glycogen were very similar in all strains, but in the tall tubes the levels were considerably lower in the variants toward the end of the fermentation. The reason for this difference is unclear, but the explanation can probably be found in the conditions (temperature, stirring, oxygen availability) that differ between the two types of fermentations. Apparently, metabolism in the variants is still ongoing at the end of the tall-tube fermentation, whereas this is no longer the case in the fermenter. Whether the lower expression of Hxk2 in the variants has something to do with this phenotype is not clear, as no glucose is present in the final stages of the fermentation and therefore a reduction in glucose repression possibly caused by the reduced expression of Hxk2 should normally have no relevance.

Genome-wide expression analysis in the beginning of a high-gravity fermentation. To date no genes have been identified that are relevant for high-gravity brewing. Also, little is known about the stress response during high-gravity brewing, in which the yeast encounters multiple stress conditions at the same time. Genome-wide expression analysis has previously shown that there is a strong response of genes involved in the biosynthesis of ergosterol and oxidative stress protection during the first stages of the industrial lager fermentation (16, 20, 28). In addition, it has been noticed that all heat shock genes are repressed as the fermentation continues (5, 20). Much more information has been accumulated on general and specific stress response mechanisms in laboratory yeast strains of *S. cerevisiae* (11).

In this study, genome-wide gene expression analysis was performed at the beginning of high-gravity fermentation of the control strain CMBS33 and two variants (GT336 and GT344) in order to get better insight into the molecular determinants important for good performance under high-gravity conditions.

Of the 10 differentially expressed genes that showed lower expression in the variants, deletion of only one gene, *HXK2*, from a laboratory strain had a weak positive effect on the fermentation rate in high-glucose YEPD medium. We hypothesize that the lower expression of this gene may result in

weaker catabolite repression and an increase in the transport and fermentation of maltose and maltotriose.

Overexpression of *LEU1* also resulted in fermentation behavior faster than that seen with the control strain CMBS33 under high-gravity conditions, but in this case no difference in attenuation was found. *LEU1* encodes isopropylmalate isomerase and catalyzes the second step in the leucine biosynthesis pathway. At present, there is no obvious explanation for the positive effect of overexpression of *LEU1*. Leucine belongs to the group of amino acids that are taken up gradually during fermentation (32). It has been shown that under normal gravity conditions, addition of leucine to the wort or overexpression of *BAP2*, a leucine transporter, results in faster assimilation of this amino acid by the yeast cells (23). Whether the result is similar in very-high-gravity fermentations is not known. It has also been shown that yeast strains with a wild-type copy of *LEU2* grow faster than yeast cells with a *leu2* mutation in the presence of extracellular leucine (33). This indicates that uptake of leucine requires more energy than biosynthesis of this amino acid. Therefore, under high-gravity fermentations, stimulation of leucine biosynthesis could relieve bottlenecks in amino acid metabolism or protein synthesis. However, the possibility of an additional function of Leu1 beyond its classical function also cannot be excluded at present.

In summary, our report is the first to identify variants of a brewer's yeast strain that allow faster and more complete fermentation ("attenuation") under high-gravity brewing conditions. A recent meeting report also mentions a new selection procedure for brewer's yeast mutants suitable for very-high-gravity fermentation (A. Huuskonen and J. Londesborough, Abstr. 30th European Brewery Convention Congress, Prague, Czech Republic, abstr. 35, 2005).

Many parameters important for beer brewing seem to be unaltered in the two isolated variants. As these variants were isolated directly from an industrial strain, they can be used to produce beer in industrial high-gravity brewing. Moreover, our study also serves as an example for the development of methodologies to isolate yeast strains with improved performance in other fermentation processes where the stress resistance of the yeast is a limiting factor, such as the production of bioethanol.

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