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A Selection from Industrial Lager Yeast Strains of Variants with Improved Fermentation Performance in Very High Gravity Worts.

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Running title: Selection of brewer's yeast variants for VHG fermentation

Section: Physiology and Biotechnology

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ABSTRACT

There are economic and other advantages if the fermentable sugar concentration in industrial brewery fermentations can be increased from that of currently used high gravity (ca. 14-17 °P) worts into the very high gravity (VHG; 18 - 25 °P) range. Many industrial strains of brewer's yeasts perform poorly in VHG worts, exhibiting decreased growth, slow and incomplete fermentations and low viability of the yeast cropped for recycling into subsequent fermentations. A new and efficient method for selecting variant cells with improved performance in VHG worts is described. In the method, mutagenized industrial yeast was put through a VHG wort fermentation and then incubated anaerobically in the resulting beer whilst maintaining the α-glucoside concentration at about 10 to 20 g·l^{-1} by slow feeds of maltose or maltotriose until most of the cells had died. When survival rates fell to one to ten cells per million original cells, a high proportion (up to 30 %) of survivors fermented VHG worts 10 - 30 % faster and more completely (residual sugars lower by 2 - 8 g.l^{-1}) compared to the parent strains, but exhibited similar sedimentation behaviour and profiles of yeast-derived flavour compounds.

Beer is traditionally produced by fermentation of brewer's wort with an original extract of about 10 - 12 ^{o}P (degrees Plato; see Materials and Methods for definition of brewery terms). A 12 ^{o}P wort contains about 90 $g \cdot l^{-1}$ of fermentable sugars (mainly maltose, maltotriose and glucose) and 25 $g \cdot l^{-1}$ of non-fermentable polysaccharides (dextrins etc). Fermentation of a 12 ^{o}P wort yields about 35-40 g of $ethanol \cdot l^{-1}$, which is normal drinking strength. Since the 1960s, many large breweries use so-called high gravity worts (about 16 ^{o}P), producing beers containing higher concentrations of ethanol, which are then diluted to drinking strength. These high gravity worts often contain adjunct carbohydrates in addition to those from the barley malt. Because the adjunct carbohydrates contain a higher proportion of fermentable sugars, relatively more ethanol is produced from each ^{o}P of extract. Fermenting at high gravity provides increased production capacity from the same size brewhouse and fermentation facilities and thus decreases capital investment costs. Other advantages are decreases in energy consumption and in labour, cleaning and effluent costs (26). These advantages would be greater if very high gravity (VHG) worts could be used. Above about 18 ^{o}P, however, fermentation rates decrease disproportionately (i.e., it takes longer to ferment the same mass of sugar in the more concentrated wort), fermentations do not go to completion (i.e., unacceptably large concentrations of sugars, especially maltotriose, remain in the final beers, causing low ethanol yields and undesirable flavour) and the physiological condition of the yeast at the end of fermentation is poor (4, 7, 17, 27, 35). It is standard practice to crop (harvest) yeast at the end of brewery fermentations and to use this cropped yeast to pitch (inoculate) subsequent fermentations. Thus, it is important that the viability of the cropped yeast is high (> 90 %). Other problems in high gravity and VHG wort fermentations include poor foam stability, possibly caused by the release of proteolytic enzymes from highly stressed yeast cells (6), haze or turbidity caused by release of glycogen from lysed yeast (18) and alterations in aroma profile. In particular, the concentration of some aroma esters in beers depends on the original gravity of the wort (20) as well

1 as on the sugar composition (16, 33) and the ratio of total sugars to free amino nitrogen (FAN) (1,
2 14, 22).
3
4 The efficiency of VHG fermentations can be improved by changing process conditions, e.g., by
5 raising fermentation temperature or using more yeast. However, levels of aroma compounds,
6 especially esters, in beer vary with fermentation temperature (20, 30). Increasing the yeast inoculum
7 also alters the aroma profile and only increases fermentation rate up to a limit (22, 28, 29).
8
9 Traditionally, brewers recycled yeast indefinitely from fermentation to fermentation, so that present
10 brewer's yeast strains are the result of hundreds of years of selection in traditional brewer's worts.
11 However, this selection stopped before the introduction of high gravity fermentation some forty
12 years ago, because brewers had started to store their strains as pure cultures. Now-a-days, yeast is
13 usually recycled 5-20 times, depending on the particular brewery. A sample of stored pure culture is
14 then propagated (i.e., cultivated through a series of steps with increasing scale) to produce the large
15 yeast mass (often a ton) required to pitch an industrial fermentation. Thus, present day strains have
16 evolved at about 11 ^{o}P but are already used at about 16 ^{o}P and are required to perform well at 18 -
17 25 ^{o}P in VHG fermentations. Because industrial selection stopped before high gravity worts were
18 introduced, an evolutionary engineering approach is likely to be successful. Each brewery has its
19 own yeast strains, which are considered to have a major impact on brand character. As well as
20 fermenting maltose and maltotriose (the sugars remaining towards the end of wort fermentations)
21 rapidly and completely and maintaining high viability in beers containing at least 90 g ethanol$\cdot$l^{-1}, a
22 new strain must also produce the same aroma profile as a brewery's present strain. This suggests the
23 approach of improving the performance of individual brewer's strains by searching for VHG-
24 tolerant variants. Evolutionary engineering (23) has proved to be useful both as a complementrary
25 strategy to genetic engineering and in cases, such as the food and beverage industry, where

1 genetically modified organisms (GMOs) are not desired. However, evolutionary engineering
2 usually relies upon the ability of more suitable genetic variants to grow faster than the parent strain
3 under specified conditions. The conditions (e.g. high ethanol and the absence of oxygen and some
4 other nutrients) during the second half of an industrial wort fermentation do not permit yeast
5 growth, so that any selection procedure based upon growth is probably selecting for the wrong
6 character and under the wrong conditions. The physiological condition of yeast cropped from
7 brewery fermentations, in particular its lipid composition (2, 9), differs markedly from that of yeast
8 propagated with even limited access to oxygen. Lipid composition is known to influence ethanol
9 tolerance (34) and sugar transport (12). We found that the ability of yeast cells to remain alive in
10 strong beers could be increased by feeding them maltose or maltotriose. We designed a selection
11 procedure based on this observation and applied it to yeast cells that had just completed a VHG
12 fermentation. The objective was to find variants with improved ability to ferment α-glucosides
13 under these harsh conditions, but the same organoleptic properties as the parent strain. Such variants
14 have immediate application for commercial beer production using VHG fermentation. Some of this
15 work has been previously presented at a meeting (15, 32)
16
17 **MATERIALS AND METHODS**
18
19 **Materials**
20 Barley syrup (Ohrasiirappi OS80) and maltose syrup (Cerestar C Sweet M 015S8) were both from
21 Suomen Sokeri (Jokioinen, Finland). Nucleotides, enzymes, antimycin A and ethyl
22 methanesulphonate (EMS) were from Sigma-Aldrich (Helsinki, Finland) or Roche (Espoo,
23 Finland).
24
25 **Definition of brewery fermentation terms**

1 Brewers monitor wort fermentations by measuring the decrease in specific gravity as fermentable
2 sugars are converted into ethanol. Specific gravities can be converted into “extracts” and “apparent
3 extracts” by, e.g., Analytica-EBC, Method 9.4, (11). The extract is a measure of the sum of
4 fermentable sugars and non-fermentable carbohydrate (mainly dextrins): a solution with an extract
5 of x ^{o}P has the same specific gravity as a solution containing x g of sucrose in 100 g of solution. For
6 the worts in Table 1, fermentable sugars accounted for 67 % of the extract of the 18 ^{o}P all-malt wort
7 but 78 % of the extract of the 25 ^{o}P worts, which contained adjuncts with relatively low contents of
8 non-fermentable polysaccharides. The “apparent extracts” that are routinely measured during
9 fermentation have not been corrected for the effect of ethanol on the specific gravity. They can be
10 corrected to so-called “real extracts” if the ethanol concentration is separately determined.
11 “Attenuation” measures the proportion of carbohydrate that has been consumed: apparent
12 attenuations are the difference between the original extract of the wort (OE) and the current
13 apparent extract (CE) divided by the original extract ([OE-CE]/OE). “Limit attenuation” is an
14 estimate of the maximum possible fermentative conversion of wort carbohydrate into ethanol
15 determined by exhaustive fermentation of a wort sample with a large excess of yeast (Analytica-
16 EBC, Method 8.6.1, (11)). Limit apparent attenuations are typically 80 - 90 %, depending on the
17 concentration of non-fermentable carbohydrate in the wort.
18
19 **Worts**
20 High and low glucose, 25 ^{o}P worts were made at VTT (the Technical Research Centre of Finland)
21 from malt with, respectively, barley syrup or high maltose syrup as adjunct (accounting for 40 % of
22 the total extract). The all-malt 18 ^{o}P wort was made at Heineken Supply Chain. Compositions of
23 these worts are shown in Table 1. About 16 ^{o}P wort (15 - 17 ^{o}P) was made at VTT by dilution of 25
24 ^{o}P wort with water containing 0.2 ppm Zn added as $ZnSO_4$. The 28 ^{o}P worts used in some
25 experiments (see Table 5) were made by addition of solid maltose or glucose, respectively, to 25 ^{o}P

1 worts. Before fermentation, the Zn content of 25 °P worts was usually increased by 0.1 ppm and on
2 one occassion by 2 ppm. Zn concentrations in worts are often suboptimal (8) and adding more Zn is
3 standard industrial practice in countries where this is allowed (not Germany). Worts for laboratory
4 scale experiments at VTT were collected hot (> 90 °C) in stainless steel kegs and stored at 0 °C for
5 at least 1 day and sometimes up to 3 months. Microbiological contamination was never observed.
6 Stored worts were mixed before use to resuspend settled solids evenly.

7

8 **Yeasts**

9 VTT-A63015 (herein called A15) is a production lager strain from the VTT Culture Collection.
10 GT344 is a UV-induced variant with improved VHG performance derived from the former
11 production lager strain CMBS33 and described by Bliek *et al*. (3).

12

13 **Yeast viabilities**

14 Viabilities above 10 % were routinely determined by staining with methylene blue in phosphate
15 buffer, pH 4.6 (10). Staining with fluoresceine diacetate (FDA (19)) at pH 7.2 was used where
16 stated. These methods record as alive metabolically active cells with intact membranes, which
17 remain unstained by methylene blue and are stained green by FDA. Viabilities below 10 % were
18 determined by spreading suitably diluted cell suspensions onto agar plates containing YP (10 g
19 yeast extract and 20 g peptone$\cdot l^{-1}$) and 20 g maltose$\cdot l^{-1}$. Colonies were counted after 3 days
20 incubation at 24 °C.

21

22 **Mutagenesis and selection**

23 A15 yeast was grown into stationary phase in YP-20 g maltose$\cdot l^{-1}$. The yeast was harvested by
24 centrifugation, washed, suspended in 0.1 M sodium phosphate, pH 7.0, to 25 mg fresh yeast$\cdot ml^{-1}$
25 and mutagenised with ethyl methanesulphonate (EMS) essentially as described (25). EMS (60 µl)

was added to 3.0 ml of the yeast suspension (about 3 x 10^8 cells) and the mixture shaken at room temperature (ca 20 ^oC) for 60 min. The EMS reaction was quenched by adding 20 ml of sodium thiosulphate (50 $g \cdot l^{-1}$). Mutagenised yeast was collected by centrifugation, washed twice with sodium thiosulphate (50 $g \cdot l^{-1}$) and suspended in sterile saline (9 g $NaCl \cdot l^{-1}$). Dilutions were spread onto agar plates containing YP-20 g $maltose \cdot l^{-1}$ to determine the proportion of dead cells, which was close to zero (0 ± 15 %). The remaining yeast, a pool of about 3 x 10^8 mutagenised cells, was inoculated into 1 l of YP-40 g $maltose \cdot l^{-1}$ and grown into stationary phase, giving about 25 g fresh yeast.

This yeast was pitched at 8.0 $g \cdot l^{-1}$ into 200 ml portions of high glucose 25 ^oP wort (Table 1) in 1 l bottles fitted with stirring magnets, glycerol-filled air locks and tubing for withdrawing samples anaerobically and for supplying liquid feeds through a capillary (1 mm) tube. The wort contained 3 mg antimycin $A \cdot l^{-1}$, to prevent respiration of adventitious oxygen. Fermentation at 20 ^oC was followed by mass loss. On day 3, when the fermentation was complete, the yeast concentration and viability and combined concentration of maltose and maltotriose (13) were measured. The bottle was then fed intermittently with 180 $g \cdot l^{-1}$ maltose *via* a peristaltic pump at about 0.2 $ml.h^{-1}$ in an attempt to maintain maltose + maltotriose at a concentration equivalent to 100 mM hexose. (This procedure was also used to study the effect of feeding maltose or maltotriose on the viability of yeast under these conditions; see Fig 1). On day 17 the composition of the feed was changed to 60 g maltose *plus* 390 g $ethanol \cdot l^{-1}$ in order to increase the ethanol stress. Every one or two days, samples were plated onto YP-20 g $maltose \cdot l^{-1}$ and the number of colony-forming units was compared to the concentration of live cells on day 3 to give the survival rate. When the survival rate was below 10^{-4} (i.e., >99.99 % of the original cells were dead), colonies were picked and grown into stationary phase in YP-20 g $maltose \cdot l^{-1}$. The yeasts (potential variants) were harvested, suspended in 30 % glycerol and stored at -80 ^oC.

1

2 For GT344 the procedure was modified as follows. After mutagenesis with EMS (killing about 20
3 % of the cells) and growth of the mutagenised pool in YP-40 g maltose·l^{-1}, the yeast was further
4 grown anaerobically in 16 ^{o}P wort, so that it more closely resembled yeast cropped from an
5 industrial fermentation. The selection was carried out in three 2 l bottles each containing 700 ml 25
6 ^{o}P wort. Low glucose 25 ^{o}P wort (Table 1; the sugar composition of industrial worts depends on the
7 nature of adjunct carbohydrates) was used. When the fermentations were almost complete, one
8 bottle was intermitently fed 180 g·l^{-1} maltose, another 180 g·l^{-1} maltotriose and the third bottle was
9 not fed. Between days 18 and 26, the bottles were intermittently fed a solution containing 390 g
10 ethanol·l^{-1}.

11

12 **Tall-tube fermentations**

13 Pure cultures of each strain were propagated to obtain enough fresh yeast mass to pitch 2 l of wort
14 in tall tube fermentors essentially as earlier described (21, 31). Stored yeast suspensions in 30 %
15 glycerol were thawed and 500 µl portions inoculated into 100 ml autoclaved YP-40 g maltose·l^{-1} in
16 250 ml Erlenmeyer flasks and grown overnight at 24 ^{o}C to an OD_{600} between 6 and 10. From these
17 precultures, 50 ml portions were inoculated into 3 l lots of 16 ^{o}P wort in 5 l Erlenmeyer flasks and
18 shaken on an orbital shaker at 24 ^{o}C for 2 days. The flasks were then stood at 0 ^{o}C for 16 - 24 h.
19 Most supernatant was decanted from each flask. Settled yeast was mixed into a smooth slurry.
20 About 5 ml samples of slurry were weighed and then centrifuged (10 min at 9000 x g). The pellets
21 were weighed and the slurry was diluted with decanted supernatant to 20 g centrifuged yeast
22 mass/100 g slurry.

23

24 Static fermentations were carried out in the stainless steel tall tubes (6 cm diam. x 100 cm height)
25 previously described (21, 31). Worts were oxygenated immediately before use to 11-13 mg.l^{-1} for

1 25 ^{o}P worts and 9-11 mg.l^{-1} for the 16 ^{o}P worts (oxygen was measured with a model 26073 Oxygen
2 Indicator from Orbisphere Laboratories, Geneva, Switzerland). Well-mixed yeast slurries were
3 pitched by mass into weighed, about 2 l portions of 24 and 16 ^{o}P worts to give, respectively, 8.0 and
4 5.0 g centrifuged yeast mass·l^{-1}, equivalent to about 32 and 20 million cells·ml^{-1}. Fermentations
5 were started at 10 - 13 ^{o}C. For 25 ^{o}P worts the temperature was raised to between 15 and 21 ^{o}C after
6 20-24 h (see Results). Samples (about 30 ml) were withdrawn daily and centrifuged. The pellets
7 were washed with water and their dry masses determined after drying overnight at 105 ^{o}C. The
8 densities of the supernatants were determined using an Anton Paar DMA58 density meter.
9
10 At the end of fermentations residual sugars in beers were determined by high-performance liquid
11 chromatography (HPLC; Waters, Milford, U.S.A.) and ethanol was determined either by gas
12 chromatography (GC) or by quantitative distillation according to Analytica EBC method 9.2.1 (11).
13
14 **Pilot scale fermentations**
15 The four yeast strains for pilot scale experiments (A15 and the variants T24.1, T24.9 and T24.201)
16 were each serially propagated in duplicate from slants through 50 ml, 1 l and 10 l scale growths in
17 15 ^{o}P all-malt wort containing 0.5 mg Zn·l^{-1} and then pitched into 200 l of freshly prepared (same
18 day) 18 ^{o}P all-malt wort containing 0.5 mg Zn and 12 mg oxygen·l^{-1} in a 250 l cylindroconical
19 fermentor. Fermentations were pitched at 10 million cells per ml and 9 ^{o}C. The temperature was
20 then allowed to rise to 14 ^{o}C. The fermentation tanks were purged of sedimented dead yeast cells
21 and wort particulates 24 h after pitching by removing 5 l from the bottom of the cylindroconical
22 vessel. Fermentations were monitored by daily assays for wort attenuation, cell concentration (using
23 a Coulter Counter, Model Z1, Beckman Coulter Nederland, BV), yeast viability, pH and haze (see
24 below). When the fermentations approached completion (after 10 days for both lots of T24.1 and
25 T24.9 and after 11 days for both lots of A15 and T24.201) the sedimented yeast was harvested from

1 the fermentations by removing 20 l of sedimented yeast slurry from the bottom of the
2 cylindroconical vessel. This yeast was then used to pitch 500 l lots of freshly prepared (same day)
3 18 ^{o}P all-malt wort (0.5 mg Zn and 12 mg oxygen·l^{-1}) in 800 l cylindroconical fermentors. The
4 fermentations were conducted and daily assays made as described above. When these 500 l primary
5 fermentations were completely attenuated, all eight beers were lagered, filtered, pasteurized and
6 bottled using standard brewery procedures.
7
8 **Turbidity (haze) measurements**
9 This assay mimics an industrial scale beer clarification procedure. Yeast cells and other large
10 particles were removed from 300 ml samples of fermenting wort or green beer by mixing with 1 g
11 of diatomaceous earth (Dicalite 231, Dicalite NV, Ghent, Belgium) and filtration through a 3 µm
12 membrane filter under vacuum. The 90° light scattering of the filtrate was measured in a Vos 4010
13 meter (Vos Instruments B.V., Zaltbommel, The Netherlands) and is a measure of the presence of
14 small particles, including glycogen, that can cause haze in the final beer (18).
15
16 **Aroma analyses**
17 Samples of final beers for aroma analyses were clarified by centrifugation. Sulfur dioxide was
18 measured by the ρ-rosanaline method (Analytica-EBC method 9.25.3; (11)). Dimethyl sulphide,
19 alcohols, esters and acetaldehyde were determined by head-space gas-chromatography with a 60 m
20 DBWaxETR column (ID 0.32 mm), hydrogen carrier gas and flame-ionization detection. Samples
21 were injected at 150 ^{o}C and the temperature programme was 14 min at 60 ^{o}C, 10 ^{o}C/min to 85 ^{o}C,
22 10 min hold and 60 ^{o}C/min to 150 ^{o}C.
23
24 **Sensory analysis**

1 All 8 bottled beers (4 yeast strains in duplicate) from the pilot scale fermentations were evaluated
2 by a taste panel of nine Heineken staff trained to evaluate beers. Each panel member gave a
3 numerical score for each of 58 descriptors for each of the 8 beers. The products were evaluated in
4 replicate in two sessions and were offered one-by-one (semi-monadic) using a balanced design.
5
6 **Fatty acid analyses**
7 Yeast samples for lipid analyses were collected by centrifugation and then handled as previously
8 described (12). Briefly, the yeast was washed with ice-cold water, saponified with 3 M NaOH and
9 methylated and the methyl esters were extracted into hexane/methyl-t-butyl ester and analysed by
10 gas chromatography.
11
12 **Statistical analyses**
13 Statistical significances (p values) were calculated by Student's t-test (2-tailed, unpaired, equal
14 variance assumed).
15
16 **RESULTS**
17 **Effect of maltose and maltotriose on yeast survival at the end of VHG fermentations.**
18 We tested whether the survival of yeast cells at the end of a VHG wort fermentation could be
19 prolonged by feeding maltose or maltotriose to maintain the concentration of fermentable α-
20 glucosides at about 10-20 $g \cdot l^{-1}$. A 25 ^{o}P wort was pitched with lager yeast at 8 g fresh $yeast \cdot l^{-1}$ and
21 fermented anaerobically in 3 identical stirred bottles. Mass losses showed that after 3 days the
22 fermentations were nearly complete. Residual α-glucosides (maltose + maltotriose) were then kept
23 between 40 and 120 mM hexose equivalents by feeding maltose or maltotriose, respectively, to each
24 of 2 bottles. The viability of lager strain GT344 in fed and unfed bottles is shown in Fig 1. The
25 proportion of yeast cells still alive on day 18 was <1% with no sugar feed and 27 and 43 %,

1 respectively, when maltose or maltotriose were fed. A similar protective effect of maltose has been
2 reported during anaerobic incubation of lager strain A15 in a VHG beer ((15); maltotriose was not
3 tested).
4
5 These results showed that survival of yeast at the end of anaerobic VHG wort fermentations was
6 promoted by maltose and maltotriose. If these sugars promote survival because they can be
7 fermented, then in a pool of mutagenised cells, those with the best ability to ferment maltose or
8 maltotriose under these conditions may survive the longest. This suggested a method to select for
9 variants with improved ability to ferment α-glucosides under VHG conditions.
10
11 **Selection of tolerant variants.**
12 During the selection from EMS-mutagenised lager strain A15, α-glucosides were kept between 40
13 and 160 mM hexose equivalents by feeding maltose. Yeast viability fell to 10 % on day 11 and 0.1
14 % on day 18. Selection pressure was then increased by raising the ethanol concentration to 106 $g{\cdot}l^{-1}$
15 during day 18 and to 160 $g{\cdot}l^{-1}$ during day 23. After the second increase, the viability fell to 6 (day
16 24) and 1.5 (day 37) live cells per million original live cells.
17
18 During the selection from EMS-mutagenised lager strain GT344, α-glucosides were kept between
19 40 and 120 mM hexose equivalents from day 5 until day 38, when the feeds were stopped, and
20 were 20 and 50 mM, respectively, in the maltose and maltotriose-fed bottles on day 59. Viabilities
21 during the first 18 days are shown in Fig 1. Viability in the maltose-fed bottle then fell to 20 % by
22 day 27, when the ethanol concentration was 120 $g{\cdot}l^{-1}$. Viability in the maltotriose-fed bottle,
23 however, fell to below 2 % on day 27 at an ethanol concentration of 106 $g{\cdot}l^{-1}$. We wanted to avoid
24 exposing this yeast strain to ethanol concentrations much greater than might be reached in an
25 industrial VHG wort fermentation. Therefore, no more ethanol was added and the cells died more

slowly than in the experiment with A15 (there may also be a strain difference). By day 41 both bottles contained 400 live cells per million original cells. Viabilities then fell to 5 live cells/million by day 47 in the maltose-fed bottle and by day 51 in the maltotriose-fed bottle. Viabilities then fluctuated between 0.1 and 5 live cells/million until the end of the experiment (day 86), being usually lower in the maltose-fed bottle.

Screening for improved fermentation characteristics. Single cell colonies (potential variants) isolated from strain A15 after 24 or 37 days selection (at viabilities of, respectively, about 6 and 1.5/million original cells) and from strain GT344 after 47 and 59 days selection (at viabilities of, respectively, about 5 and 0.05/million in the maltose-fed bottle and 200 and 0.1/million in the maltotriose-fed bottle) were screened for improved VHG fermentation in tall tubes. For each screening test, several potential variants were propagated from pure cultures together with independent duplicate lots of the corresponding parent strain (A15 or GT344). The propagated yeasts were pitched into 16 or 25 ^{o}P worts in tall tubes and the resulting fermentations were monitored. A typical set of screening fermentations is shown in Fig 2. When the different yeasts were pitched at the standard concentration of 8.0 g fresh yeast·l^{-1}, their growth in early fermentation and subsequent flocculation and sedimentation in late fermentation (indicated by the profiles of dry mass in suspension shown in the upper panel of Fig 2) were similar, except that T24.5 sedimented faster than A15. The middle panel shows that also the attenuation profiles were similar throughout most of the fermentation. However, fermentations by the variants T24.1, T24.2, T24.5 and T37.2 reached lower final apparent extracts than those by the parent strain (Fig 2, lower panel). For fermentations pitched with 8.0 g fresh yeast·l^{-1}, the final apparent extracts were between 3.87 and 4.05 ^{o}P for the variants, compared to 4.36 and 4.50 for the duplicate fermentations by parent A15 (Table 2). Increasing the pitch rate of the parent yeast to 10.0 g·l^{-1} increased the early fermentation rate, but did not drive the final apparent extract down to the values observed with variants.

1 Decreasing the pitch rate of variant T24.1 to 6.0 g.·l^{-1} decreased the early fermentation rate, but the
2 final apparent extract (4.06 ^{o}P) was still lower than that observed with even 10.0 g·l^{-1} of A15 (4.23
3 ^{o}P; Table 2).
4
5 When pitched at the same rate as A15 (8.0 g·l^{-1}), the variants caused the consumption during
6 fermentation of an extra 0.38 to 0.56 ^{o}P, corresponding to increases in apparent attenuation from
7 81.2 ± 0.3 % (mean ± range) for A15 to between 83.4 and 83.6 % for variants T24.1, T24.2 and
8 T24.5 and 82.8 % for variant T37.2. Thus, the variants approached closer to the limit apparent
9 attenuation (~86 %) of this wort. For variants T24.1, T24.2 and T24.5, the increases in apparent
10 attenuation were accompanied by increases in final ethanol concentration from about 105 - 108 g·l^{-1}
11 for A15 to 109 - 113 g·l^{-1} (Table 2).
12
13 In normal brewing practice, the fermentations of Fig 2 would be chilled to below 5 ^{o}C and
14 sedimented yeast cropped at about 168 h, when the decreases in apparent extract were essentially
15 complete. We kept these fermentations at 20 ^{o}C for a further 4 days. During this time, little or no
16 further changes in apparent extract occurred (Fig 2 bottom panel) although there was still yeast in
17 suspension in all fermentations (Fig 2, upper panel). The differences between the final apparent
18 extracts achieved by parent and variant strains therefore represented not just differences in
19 fermentation speed but an inability of the parent strain to continue fermenting wort sugars at low
20 concentrations that the variants could still use. When sedimented yeasts were eventually cropped
21 from the bottom of the tall tubes after 263 h of fermentation, they had been exposed to over 100 g
22 ethanol·l^{-1} at 20 ^{o}C for 4 days and their viabilities were very low. However, the viabilities of the
23 variant yeast crops (about 25 to 50 %) were higher than those of the parent crops (about 15 %)
24 (Table 2).
25

The fermentation performances of 21 potential variants of A15 were screened in high or low glucose VHG worts. Six variants (29 %) performed better than the parent strain by showing, in at least two independent tests (i.e., with yeast propagated and fermented on two different occasions), faster fermentation, more extensive fermentation and sometimes higher crop viability (data not shown). Eight potential variants of GT344 (four each from the maltose- and maltotriose-fed bottles) were screened in low glucose VHG worts. One of these (LL2-47-1, isolated from the maltotriose-fed bottle on day 47 at a viability of 200/million) showed improved VHG fermentation performance compared to the parent GT344 strain in two independent experiments. Fermentation characteristics of this variant and of the three most studied variants of A15 are shown in Table 3.

For the VHG fermentations summarised in Table 3, the parent strains completed their fermentations in about 10 days (range, 7 - 13). Conditions varied between fermentations, both because of deliberate variations (e.g., zinc concentrations between 0.2 and 2 ppm and final VHG fermentation temperatures between 15 and 21 ^{o}C were used in different fermentation sets) and because of accidental variations (e.g., fermentation sets with worts stored at 0 ^{o}C for several weeks appeared to be slower than those with freshly prepared worts). Fermentation rates were quantified in each set of fermentations by measuring the time required to reach a certain high apparent attenuation. Where possible the target apparent attenuation was 80 %, but in experiments where the parent did not reach 80 % apparent attenuation, or reached it very slowly, targets of 77 or 75 % were used. All the variants described in Table 3 consistently fermented wort faster than the corresponding parent strain, with average time savings between half a day and nearly 3 days. All these variants also fermented the worts more extensively, with average gains in apparent extract (ΔAE) of between 0.25 and 0.88 ^{o}P, depending on the variant and the wort. The increased apparent attenuations were accompanied by corresponding decreases in residual sugars of between 2.1 and 7.8 $g \cdot l^{-1}$. Differences in residual maltotriose accounted for most (at least 90 %) of the changes in residual

sugar (for the parent strains, residual glucose and maltose after VHG fermentations were usually below 0.5 $g \cdot l^{-1}$, whereas residual maltotriose was 10-20 $g \cdot l^{-1}$ for 25 ^{o}P worts and 3-4 $g \cdot l^{-1}$ for 16 ^{o}P worts). All variants produced more ethanol than the corresponding parent. At least for the most studied mutant, T24.1, the changes in fermentation time, final extract and residual sugars were statistically significant in both 25 and 16 ^{o}P worts and changes in final ethanol were significant in 16 ^{o}P wort (Table 3).

For T24.1 (Table 3) and some other variants (T24.2, T24.5 and T37.2; data not shown), the viability of yeast cropped from high glucose VHG worts was higher than that of cropped A15. This improvement in crop viability was not seen when low glucose worts were used. Variants and parent yeast were always cropped at the same time, when the parent fermentation was complete. Because the parent yeasts were usually cropped promptly when fermentations stopped, their viabilities were often ≥90%. By this time, the variant yeasts had stopped fermenting one or two days ago, and their cropped yeast viabilities were usually close to or less than those of the parent yeast (Table 3).

Pilot scale fermentations

To test whether the advantages of the variants were maintained when yeasts were recycled and used in larger scale fermentations, closer to industrial practice, A15 and three promising variants were first pitched into 200 l of 18 ^{o}P all-malt wort and then yeasts harvested from these fermentations (two independent lots per strain) were pitched into 500 l of 18 ^{o}P all-malt wort. Yeast growth and sedimentation in the 500 l scale fermentations were similar for all strains, except that the variants reached their peak concentrations 1 - 2 days earlier than the parent strain (Fig 3, upper panel). Fermentations were faster with all three variants and reached lower final apparent extracts (Fig 3 lower panel and inset). The proportion of dead cells in suspension increased during the fermentation from near zero to about 15 % for parent and variant strains, except that the percentage of dead

T24.9 cells increased sharply after 10 days in both replicate fermentations to more than 30 % at 14 days (Fig 4, upper panel). Turbidity or haze, which is often caused by the release of glycogen particles from dead cells, rose more rapidly for variants T24.9 and T24.201 than for A15, but reached a similar final value (about 1.5 EBC units) and was lower for T24.1 than the other strains (Fig 4, lower panel).

Aroma compounds

Table 4 shows the levels of several aroma compounds found in the beers produced on the 500 l pilot scale. In general, differences between the variants and the parent strain, A15, were small. Free amino nitrogen compounds (FAN) were 15 - 20 % lower in beers produced by variants, which may indicate that the variant yeasts grew more, even though the peak yeast concentrations were similar (Fig 3). Total SO_2 (i.e. free SO_2 plus carbonyl-bound SO_2) was 20-30 % lower for T24.9 and T24.201 beers. The branched chain alcohols, 2- and 3-methyl-1-butanol, were roughly 15 % higher for all variants, ethyl acetate was slightly higher and 3-methyl-1-butyl acetate (important for its fruity, banana flavour) roughly 30 % higher in beers from T24.1 and T24.9, but not T24.201. Similar increases in these compounds were previously reported (15) for 2 l-scale tall tube fermentations of 15 and 25 ^{o}P worts by laboratory-grown T24.1, T24.9 and T24.201.

Sensory analysis and foam stability of bottled beers.

The organoleptic properties and foam stability (which has aesthetic appeal) of all 8 bottled beers from the pilot scale were evaluated. There were small differences between the organoleptic properties of individual beers, but when the scores for both beers made by each strain were combined no sensory differences between the 4 yeast strains could be detected by a trained taste panel (n=9). No statistically significant differences between the 8 beers were observed in foam stability, measured as the time required to collapse the foam head by 3 cm. Beer made by strain

T24.1 exhibited the highest foam retention (269 ± 22 s compared to 245 ± 22, 245 ± 24 and 246 ± 30 for strains A15, T24.9 and T24.201, respectively).

Changed fatty acid compositions of T24.1 compared to A15

We tested whether there were differences in lipid composition between parent and several successful variant strains. We could not detect reproducible differences between the sterol contents of A15 and tested variants. In contrast, there were strain-specific differences between their fatty acid contents. Table 5 (upper portion) shows analyses of total fatty acids in A15 and two variants cropped at the end of a 25 ^{o}P fermentation. The yeasts contained considerable amounts of wort-derived linoleic (C18:2) and linolenic (C18:3) acids (*S. cerevisiae* cannot synthesise these polyunsaturated acids (24)). Fatty acid unsaturation indices and also the ratios of total C18 and C16 fatty acids were calculated both including and excluding these wort-derived C18 fatty acids. The unsaturation indices were greater for T24.1 than for A15, but smaller for T24.5. The ratio C18/C16 fatty acids was greater for T24.1 than for A15 or T24.5. T24.1 contained a higher proportion of oleic acid (C18:1/total acids) than either A15 or T24.5. When 11 independent samples of each strain from six fermentation series were analysed, only the difference in C18/C16 ratios between T24.1 and A15 was statistically significant ($p<0.01$) (Table 5, lower portion). The difference in the proportions of oleic acid between these strains approached significance ($p=0.06$), but no significant difference was found between their unsaturation indices ($p>0.2$) and no significant differences were found between T24.5 and A15.

We also examined the fatty acid compositions of A15 and three variants during shake-flask cultivations of YP-20 $g \cdot l^{-1}$ maltose or glucose, where the yeasts were not exposed to high gravity stresses (Table 6). With maltose as carbon source, the C18/C16 ratios were significantly higher for T24.1 than for A15 ($p<0.01$ in growth and stationary phases) and the unsaturation index of T24.1

was significantly higher during growth (p<0.01). The C18/C16 ratio was significantly higher also for T24.9 than A15 during growth on maltose (p<0.05) but not in stationary phase (p>0.5). Also with glucose as carbon source, the C18/C16 ratios were markedly higher for T24.1 in both growth and stationary phases and for T24.9 in growth phase. No significant differences were observed between T24.201 and A15 (p>0.1).

These results suggest that the high C18/C16 ratio of T24.1 is a specific characteristic of this variant, possibly shared by T24.9, but not by T24.201 nor T24.5. The results of Table 6 show that the different C18/C16 ratios of T24.1 and A15 are not a consequence of exposure to high gravity stress.

DISCUSSION

With the selection method described, a high proportion of the single cell colonies isolated from the industrial lager strain, A15, showed improved VHG fermentation performance compared to the parent. Six variants out of the 21 tested isolates showed faster fermentations, more complete attenuation (Table 3) and sometimes higher crop viability (15). For 3 of the variants (T24.1, T24.9 and T24.201), the improvements were observed both in both 2 l tall tube fermentations pitched with yeast propagated in the laboratory (Table 3) and in 500 l fermentors pitched with recycled yeasts (Fig 3). This is important because nearly all industrial brewery fermentations are made with yeast recycled from earlier fermentations, and this recycled (cropped) yeast differs physiologically from yeast freshly propagated from pure culture (2, 9). The improvements in fermentation time and extent were obtained without significant changes in the profile of yeast-derived aroma compounds (Table 4), in sensory perception by a trained panel or in foam stability. As well as showing improved performance in VHG worts (25 ^{o}P) containing adjunct carbohydrates, variants also performed better than the parent A15 in about 16 ^{o}P worts (Table 3) and in 18 ^{o}P all-malt wort (Fig 3). Variant T24.9 exhibited poor viability after the tenth day of the 18 ^{o}P all-malt pilot

fermentations (Fig 4). This might cause problems with this strain, although on this occasion it did not affect the organoleptic properties of the beer and sedimented yeast might be cropped earlier at acceptable viability, as is standard practice in many breweries. Some of the other variants, especially T24.1, seem to be suitable for industrial use under current industrial high gravity conditions and to offer potential to increase the gravity of industrially used worts into the VHG region of 18 - 25 ^{o}P.

The selection method also yielded at least one improved variant (LL2-47-1) from strain GT344 (Table 3) out of the 8 tested isolates from this strain. GT344 is itself a variant with improved VHG performance, isolated from the former production lager strain, CMBS33 (3). Thus, compared to CMBS33, LL2-47-1 presumably contains at least two genetic changes that promote VHG performance. It is no surprise that VHG performance depends on more than one genetic locus.

The selection procedure involves first a VHG fermentation and then a long incubation of the resulting yeast in its own beer, during which the concentration of α-glucosides is maintained (with the objective of prolonging the life of variants best able to ferment these sugars) and the concentration of ethanol is increased (with the objective of increasing stress). Continuous monitoring of α-glucosides, ethanol and cell viability is required and the exact protocol is strain-dependent because different brewer's yeast strains have different tolerances towards VHG stresses. One of the strains in the current work (GT344) exhibited large oscillations in viability towards the end of the selection (days 47-86). This yeast formed films on the surfaces of the bottles, so a reason could be that occassional detachment of pieces of these films released viable cells into suspension. Another possibility is that nutrients released from the mass of dead yeast may have promoted growth of some of the very rare survivors. Such release of nutrients means that survivors were selected in a medium that is nutritionally richer than the beer at the end of a VHG fermentation.

1 Some of the isolated colonies that did not perform well in VHG-fermentations may be cells that
2 exploited these nutrients.
3
4 The decreases in total fermentation time were about 1 day in fermentations that lasted about 10
5 days. This represents a significant (*ca* 10 %) increase in brewery fermentation capacity. The more
6 complete apparent attenuation is still more important. The gains (0.3-0.9 ^{o}P lower final apparent
7 extract in fermentations where the total changes in apparent extract were about 12 or 20 ^{o}P for high
8 gravity and VHG worts, respectively) correspond to 3 to 8 $g \cdot l^{-1}$ less residual sugar (Table 3), and up
9 to 4 $g \cdot l^{-1}$ more ethanol. This has considerable economic impact when beers are adjusted to a
10 standard final ethanol concentration and also lessens the possibility of undesired flavour changes
11 caused by residual sugar. The extra apparent attenuation reached by the A15 variants was not
12 reached by the parent strain when fermentation time was prolonged or fermentations were pitched
13 with more yeast (Fig 2). It is not yet known why the parent yeast (and other current industrial
14 strains) stop fermenting VHG worts before all fermentable sugar (mostly maltotriose) is consumed.
15
16 The success, and limitations, of the present method are related to the selection conditions used. We
17 looked for cells that could stay alive when supplied with maltose or maltotriose under the adverse
18 conditions (high ethanol concentrations and low nutrient concentrations including a complete lack
19 of oxygen) at the end of VHG fermentations. The selection procedure was applied to yeast cells that
20 were in the same physiological condition as yeast at the end of an anaerobic VHG fermentation. We
21 obtained variants that showed improved performance at the end of high gravity and VHG
22 fermentations (Figs 3 and 4). None of the variants obtained by this method fermented faster than the
23 parent strain during the early stages of wort fermentation, presumably because the method applied
24 no selection pressure for such a character. It follows that to obtain variants with improved
25 performance throughout the fermentation, it may be necessary to apply different selection pressures

sequentially. Variants isolated at each step can be subjected to further mutagenesis and selection under different conditions, as was done here to obtain variant LL2-47-1 from the already improved (3) GT344 strain.

The physiological condition of *Saccharomyces* yeasts varies greatly with growth conditions. It is known, e.g., that the sterol and unsaturated fatty acid contents of brewer's yeasts cropped from brewery fermentations are much lower than those of yeast grown under normal laboratory conditions or with aeration during propagation in a brewery (2, 9), whereas the correct function of maltose transporters is dependent on the lipid composition of the yeast (12). The variants with improved survival under our selection conditions may have genetic alterations that increase the ability of anaerobically grown, sterol- and unsaturated fatty acid-deficient cells to ferment α-glucosides in the presence of high ethanol concentrations and other VHG stresses. The VHG-fermentation performance of one of the parent lager strains (A15) used in the present work is known to be limited by its α-glucoside transport activity and to be increased by genetic engineering of the *AGT1* gene that encodes a transporter of maltose and maltotriose (31).

We have not identified the mutations responsible for the improved performance of the variants. Such identification is of scientific interest and would also facilitate the directed improvement of strains by genetic engineering or directed evolution and facilitate the combination of different positive mutations identified in different variants. All 21 tested isolates from A15 were karyotyped using pulsed field gel electrophoresis and no alterations of chromosome structure were detected (L. Mulder and M. Walsh, unpublished data). At least one successful variant (T24.1; possibly also T24.9, but probably not T24.5 nor T24.201) had a greater average length of fatty acid carbon chains (higher ratios of C18 fatty acids to C16 fatty acids) than A15 under a variety of conditions, including conditions (YP-20 $g \cdot l^{-1}$ glucose or maltose) where the yeast was not exposed to high

gravity stress (Tables 5 and 6). This change in the fatty acid composition of T24.1 compared to its parent, A15, was not, therefore, a result of its improved performance in high gravity fermentations. However, we do not know whether the change is a cause of the improved VHG-performance of T24.1. Brewer's yeasts usually contain about twice as much palmitoleic acid (C16:1) as oleic acid (C18:1) (5), but there is evidence that increase in, specifically, oleic acid, rather than palmitoleic acid, increases the ethanol tolerance of *Saccharomyces cerevisiae* (34). Identifying the mutations responsible for the improved performance presents considerable problems. Blieck *et al* (3) isolated variants with improved VHG-fermentation performance from the lager strain, CMBS33 and detected differences in gene expression levels between CMBS33 and two variants, GT336 and GT344 (lower expression of *HXK2* and increased expression of *LEU1*). They presented evidence that these differences might be causative of the improved performance of the variants. However, there is, in general, no reason to suppose that the causative mutations will change the expression levels of the responsible genes. It is at least as likely that these mutations will be changes in coding sequences that alter the functional properties of enzymes or other proteins. A genomic library of variant T24.1 is now available (J. Dietvorst and Y. Steensma, unpublished work), which provides a possible route to identifying the causative mutation(s) in this variant.

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1 **Figure legends**

2 Fig 1. Stabilization of yeast by maltose and maltotriose after a VHG fermentation. A 25 ^{o}P wort was
3 fermented with lager strain GT344 at 18 ^{o}C in stirred anaerobic bottles. When the fermentation was
4 completed (day 3), stirring was continued and the sum of maltose and maltotriose was kept between
5 40 and 120 mM hexose equivalents in the maltose-fed bottle and between 80 and 120 mM hexose
6 equivalents in the maltotriose-fed bottle by feeding maltose or maltotriose. Viabilities are average
7 values from two or more analyses with methylene blue or (<10 % viability) two or more agar plates.

8

9 Fig 2. A set of screening fermentations in 2 l tall tubes. Duplicate growths of the parent A15 strain
10 (A15 (A) and (B)) and single growths of variants (T24.1, T24.2, T24.5 and T37.2) were pitched at
11 10 ^{o}C into 24 ^{o}P wort at 8.0 g fresh yeast$\cdot$l^{-1}. A15 and T24.1 were also pitched at, respectively 10.0
12 (A15(10)) and 6.0 (T24.1(6)) g fresh yeast$\cdot$l^{-1}. The temperature was raised to 20 ^{o}C after 24 h. The
13 upper panel shows the dry mass (mainly yeast) in suspension, the middle panel shows changes in
14 apparent extract throughout the fermentations and the lower panel shows the changes in apparent
15 extract in the final stages.

16

17 Fig 3. Pilot scale (500 l) fermentations of 18 ^{o}P all-malt wort by A15 and variants T24.1, T24.9 and
18 T24.201. Worts were pitched with yeasts harvested from 200 l 18 ^{o}P all-malt fermentations. The
19 upper panel shows the dry yeast in suspension, the lower panel the overall progress of the
20 fermentation and the inset shows changes of apparent extract in the final stages. Results are
21 averages ± ranges of independent duplicate fermentations.

22

23 Fig 4. The percentage of dead cells in suspension (upper panel) and turbidity (haze) of filtered beer
24 samples (lower panel) during the 500 l fermentations of Fig 3. Data are averages ± ranges from
25 independent duplicate fermentations.

Table 1. Typical composition of worts

	High Glucose 25 °P	Low Glucose 25 °P	All-malt 18 °P
Glucose ($g \cdot l^{-1}$)	62	25	13
Fructose ($g \cdot l^{-1}$)	5	4	3
Maltose ($g \cdot l^{-1}$)	105	120	80
Maltotriose ($g \cdot l^{-1}$)	24	40	24
Total sugars ($g \cdot l^{-1}$)	198	193	121
Maltose/glucose ($g \cdot g^{-1}$)	1.7	4.8	6.2
Zn ($mg \cdot l^{-1}$)	0.29	0.13	0.11
Ca ($mg \cdot l^{-1}$)	82	43	NA
Mg ($mg \cdot l^{-1}$)	180	120	156
pH	4.90	5.04	4.98
FAN[a] ($mg \cdot l^{-1}$)	374	390	227
Bitterness (EBU)	62	70	44

[a] FAN, free amino nitrogen.

NA, not analysed.

Table 2. Fermentation parameters from a set of screening fermentations[a].

	A15(A)	A15(B)	A15	T24.1	T24.1	T24.2	T24.5	T37.2
Pitch rate ($g{\cdot}l^{-1}$)	8.0	8.0	10.0	6.0	8.0	8.0	8.0	8.0
Final apparent extract (^{o}P)	4.36	4.50	4.23	4.06	3.89	3.91	3.87	4.05
Final apparent attenuation (%)[b]	81.5	80.9	82.0	82.7	83.5	83.4	83.6	82.8
Viability of cropped yeast (%)[c]	15	15	NA	NA	25	50	25	50
Ethanol ($g{\cdot}l^{-1}$)[d]	105 ± 3	108 ± 3	NA	115 ± 3	109 ± 2	109 ± 3	113 ± 5	NA

[a] Data are from the fermentations of Fig 2.

[b] The limit apparent attenuation of worts prepared in the same way was 86 %.

[c] Viability was measured with FDA.

[d] Ethanol results are means ± ranges of duplicate GC assays.

NA, not analysed.

Table 3. Fermentation characteristics of selected variants[a].

	High Glucose 25 ^{o}P			Low Glucose 25 ^{o}P			Low Glucose 16 ^{o}P		
	n[b]	Av[b]	D[b]	n	Av	D	n	Av	D
Strain T24.1[c]									
Time saved (h)	2	18.3	±2.5	5	60*	±40	5	32**	±12
Δ final AE (^{o}P)	2	0.58	±0.05	5	0.88*	±0.22	5	0.59*	±0.31
Δ residual sugars (g/l)		NA		5	7.8**	±2.0	5	4.5*	±2.3
Δ final ethanol (g/l)	2	0.4	±0.8	2	3.9	±1.4	4	3.9**	±1.8
Δ viability (%)	2	14	±4	3	0.4	±0.4	4	0.3	±4.3
Strain T24.9									
Time saved (h)	1	52	-	2	65	±40	2	23	±8
Δ final AE (^{o}P)	1	0.77	-	2	0.78	±0.39	2	0.26	±0.11
Δ residual sugars (g/l)	1	4.6	-	2	7.4	±3.2	2	2.1	±0.8
Δ final ethanol (g/l)		NA		1	3.7		1	0.1	-
Δ viability (%)		NA		2	-11.4	±9.6	2	-0.4	±0.4
Strain T24.201									
Time saved (h)				2	48	±30	2	25	±7
Δ final AE (^{o}P)				2	0.57	±0.28	2	0.25	±0.1
Δ residual sugars (g/l)				2	5.6	±1.7	2	2.1	±0.6
Δ final ethanol (g/l)				2	7.0	±0.4	2	2.6	±0.3
Δ viability (%)				2	-15	±14	2	-4.2	±3.1
Strain LL2-47-1									
Time saved (h)				2	16	±6			
Δ final AE (^{o}P)				2	0.28	±0.03			
Δ residual sugars (g/l)				1	2.1	-			
Δ final ethanol (g/l)				1	3.0	-			
Δ viability (%)				2	0.45	±0.35			

[a] Sets of tall tube fermentations by the parent A15 strain and one or more of its variants T24.1, T24.9 and T24.201 or by the parent GT344 strain and its variant LL2-47-1 were carried out in high glucose or low glucose 25 ^{o}P worts or low glucose 16 ^{o}P wort . Time saved is the difference between the times for the variant and parent to reach the same high apparent attenuation (see text). Δ final AE shows how much lower was the final apparent extract reached by the variant than that reached by the parent. Δ residual sugars and Δ final ethanol are the decrease in residual sugars and increase in final ethanol for variant compared to parent. Δ viability is the increase in viability of variant compared to parent strain for yeast samples cropped from the bottom of the fermentation tubes at the same time near the end of fermentation.

[b] n = the number of independent fermentation sets, Av = the average value of the difference between variant and parent and D = the range for duplicate sets or standard deviation when n ≥ 3.

[c] The statistical significance of differences between T24.1 and A15 is indicated: ** $p<0.01$, * $p<0.05$.

NA = not analysed.

Table 4. Aroma compounds in beers produced by A15 and variants in 500 l fermentations[a].

	A15	T24.1	T24.9	T24.201
FAN[b] (mg/l)	110 ± 1	95 ± 1	89 ± 1	92 ± 1
Total SO_2 (mg/l)	10.9 ± 0.1	11.0 ± 0.5	8.6 ± 0.2	7.6 ± 0.4
Acetaldehyde (mg/l)	5.8 ± 1.0	3.9 ± 0.1	3.6 ± 0.4	4.5 ± 1.1
Dimethyl sulphide (mg/l)	21 ± 1	24 ± 0	19 ± 1	20 ± 1
n-Propanol (mg/l)	8.4 ± 0.0	8.6 ± 0.1	9.5 ± 0.2	8.9 ± 0.4
Iso-butanol (mg/l)	9.6 ± 0.1	9.0 ± 0.1	10.8 ± 0.1	9.5 ± 0.5
2-methyl-1-butanol (mg/l)	15.6 ± 0.6	17.2 ± 0.4	18.0 ± 0.3	17.1 ± 0.4
3-methyl-1-butanol (mg/l)	40.5 ± 1.3	44.8 ± 0.9	50.3 ± 0.6	46.0 ± 2.9
Ethyl acetate (mg/l)	23.6 ± 0.3	28.3 ± 0.8	25.8 ± 0.1	24.8 ± 1.3
3-methyl-1-butyl acetate (mg/l)	1.6 ± 0.1	2.3 ± 0.1	2.1 ± 0.0	1.7 ± 0.1
Ethyl propionate (mg/l)	0.07 ± 0.01	0.05 ± 0.0	0.06 ± 0.01	0.07 ± 0.01
Ethyl capronate (mg/l)	0.19 ± 0.01	0.18 ± 0.01	0.21 ± 0.02	0.18 ± 0.01
Diacetyl (µg/l)	5.8 ± 1.0	3.9 ± 0.1	3.6 ± 0.4	4.5 ± 1.1
2,3-pentanedione (µg/l)	21 ± 1	24 ± 0	19 ± 1	20 ± 1

[a] Beers from the 8 fermentations of Fig 3 were lagered and then analysed. Results were normalised to a sales strength of 5.0 % $_{(v/v)}$ ethanol and are presented as averages ± ranges for the duplicate fermentations.

[b] FAN = free amino nitrogen.

Table 5. Fatty acid composition of yeasts from tall tube fermentations.

		A15	T24.1	T24.5
Yeasts cropped at 260 h from a 25 °P fermentation [a]				
Lauric	C12:0	0.08	0.07	0.03
Myristic	C14:0	0.07	0.07	0.05
Palmitic	C16:0	3.45	2.82	3.08
Palmitoleic	C16:1	1.72	1.51	1.21
Stearic	C18:0	0.66	0.71	0.68
Oleic	C18:1	0.70	0.77	0.55
Linoleic	C18:2	0.39	0.36	0.28
Linolenic	C18:3	0.09	0.08	0.07
Total fatty acids		7.15	6.37	5.93
Unsaturation index (all acids) (%)[b]		40.5	42.5	35.5
Unsaturation index (yeast acids) (%)[b]		36.2	38.2	31.5
C18/C16 ratio (all acids)[c]		0.36	0.44	0.37
C18/C16 ratio (yeast acids)[c]		0.26	0.34	0.28
C18:1/total yeast acids (%)		10.5	12.9	9.8
Yeasts from 6 fermentations [d,e]				
Unsaturation index (yeast acids) (%)[b]		40 ± 7	43 ± 6	36 ± 10
C18/C16 ratio (yeast acids)[c]		0.34 ± 0.08	0.45 ± 0.09**	0.32 ± 0.04
C18:1/total yeast acids (%)		13.5 ± 3.8	16.7 ± 3.7	11.6 ± 2.8

[a] Fatty acid amounts (mg·g fresh yeast^{-1}) are averages of (A15) four or (T24.1 and T24.5) two replicate analyses.

[b] The unsaturation indices show (all acids) the amounts of all unsaturated C16 and C18 fatty acids as a percentage of the total amount of C12-C18 fatty acids or (yeast acids) the amounts of yeast-derivable C16:1 and C18:1 unsaturated fatty acids as a percentage of the total amount of C12-C18 yeast-derivable fatty acids (i.e., excluding C18:2 and C18:3).

[c] The C18/C16 ratios show (all acids) the sum of all C18 fatty acids divided by the sum all C16 fatty acids or (yeast acids) the sum of yeast-derivable C18:0 and C18:1 fatty acids divided by the sum of all C16 fatty acids.

[d] Six fermentations were carried out with each strain. The original extracts were 17 ^{o}P (1 fermentation), 25 ^{o}P (3 fermentations) and 28 ^{o}P (2 fermentations) and yeast samples were either collected from yeast still in suspension (9 samples for each yeast) or cropped from the bottom of the fermentation tubes (2 samples for each yeast). Data are means ± SDs (n=11).

[e] The statistical significance of the differences between variants and A15 is indicated: ** $p<0.01$.

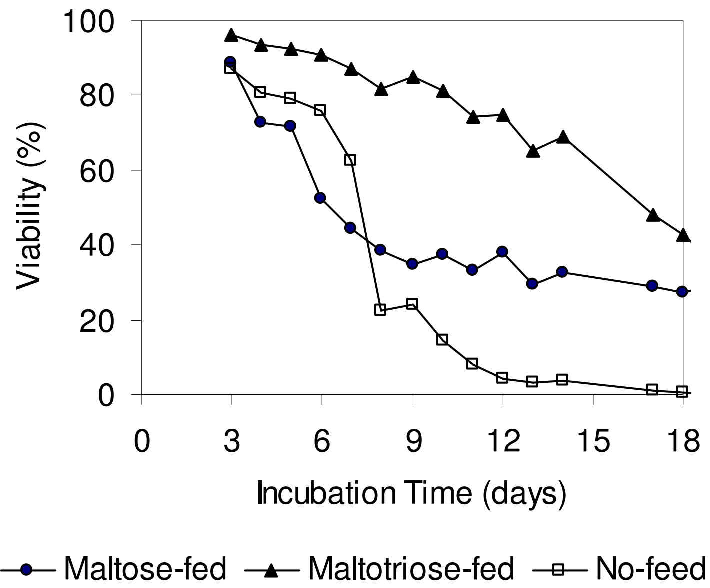
Table 6. Fatty acid composition of A15 and three mutants grown without high gravity stress.[a]

	A15	T24.1	T24.9	T24.201
Growth phase on maltose[b]				
C18/C16 ratio	0.51 ± 0.05	0.98 ± 0.03**	0.71 ± 0.05*	0.41 ± 0.03
Unsaturation index (%)	82 ± 2	89 ± 1**	85 ± 1	82 ±1
Stationary phase on maltose[b]				
C18/C16 ratio	0.64 ± 0.14	1.08 ± 0.10**	0.70 ± 0.02	0.62 ± 0.03
Unsaturation index (%)	90 ± 3	92 ± 1	91 ± 1	92 ± 1
Growth phase on glucose[c]				
C18/C16 ratio	0.43 ± 0.07	0.85 ± 0.01	0.67	0.36
Unsaturation index (%)	86 ±1	87 ± 1	86	82
Stationary phase on glucose				
C18/C16 ratio	0.81 ± 0.02	1.22 ± 0.01	0.86	0.82
Unsaturation index (%)	90 ± 1	91 ± 1	89	89

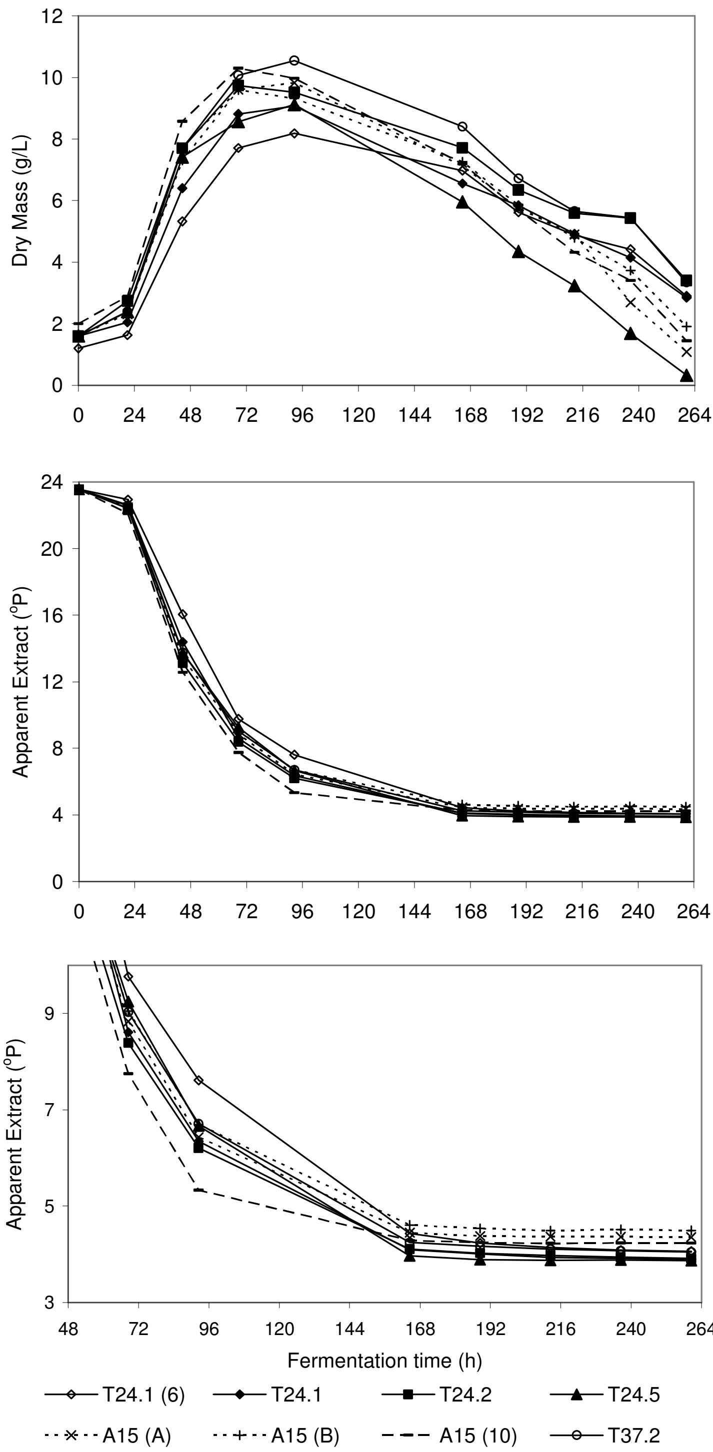
[a] Strains were grown at 24 °C in YP-20 g·l^{-1} maltose or glucose and harvested in growth phase (at about 2.7 g dry yeast·l^{-1}) or stationary phase (at about 4.8 g dry yeast·l^{-1}). C18/C16 ratios and unsaturation indices are defined as in Table 5 (yeast acids).

[b] Data on maltose are means of two to four biological replicates ± SDs for A15 (n=4) and T24.1 (n=3) or ± ranges for T24.9 and T24.201 (n=2). The statistical significance of the differences between variants and A15 is indicated: ** p<0.01, * p<0.05.

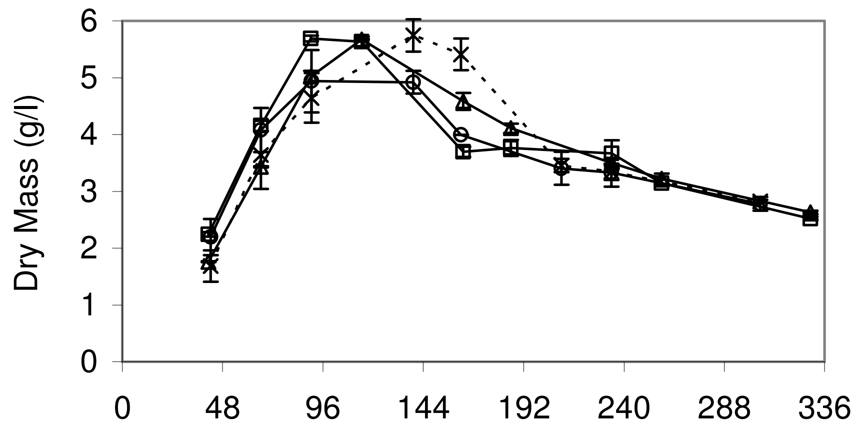
[c] Data on glucose are means of two biological replicates ± ranges for A15 and T24.1 and single results for T24.9 and T24.201.

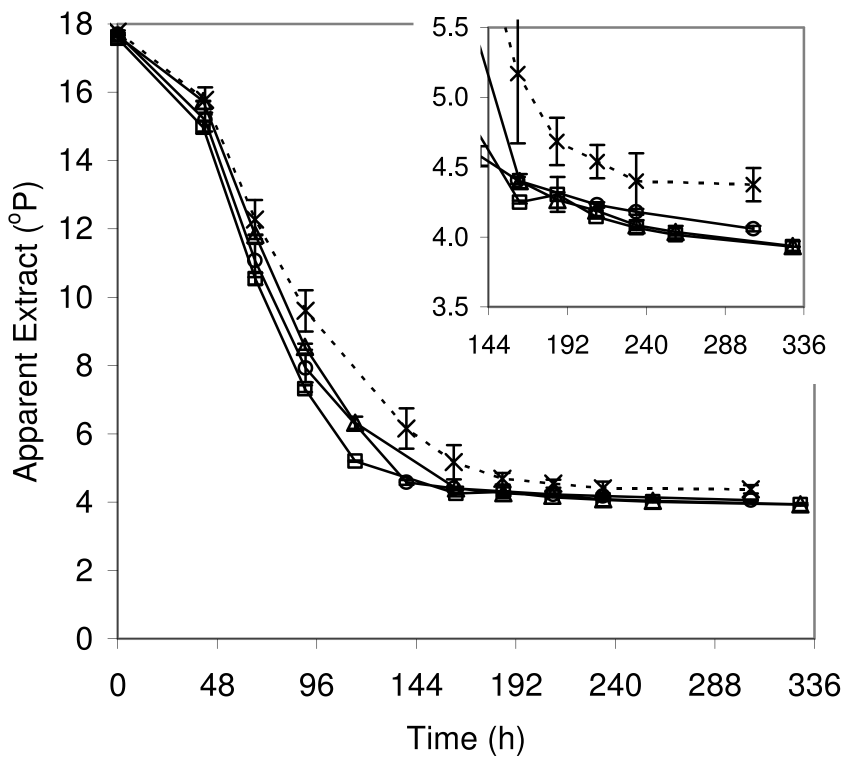


Huuskonen et al, Fig 1.

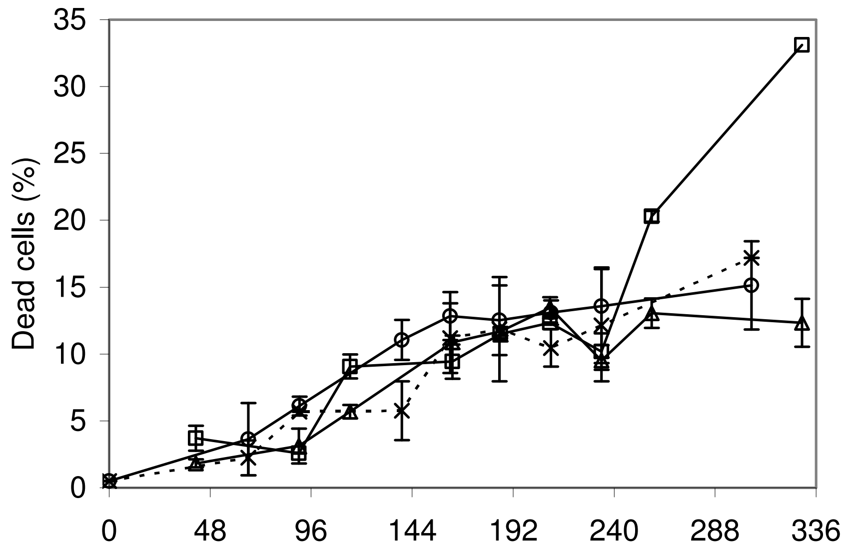


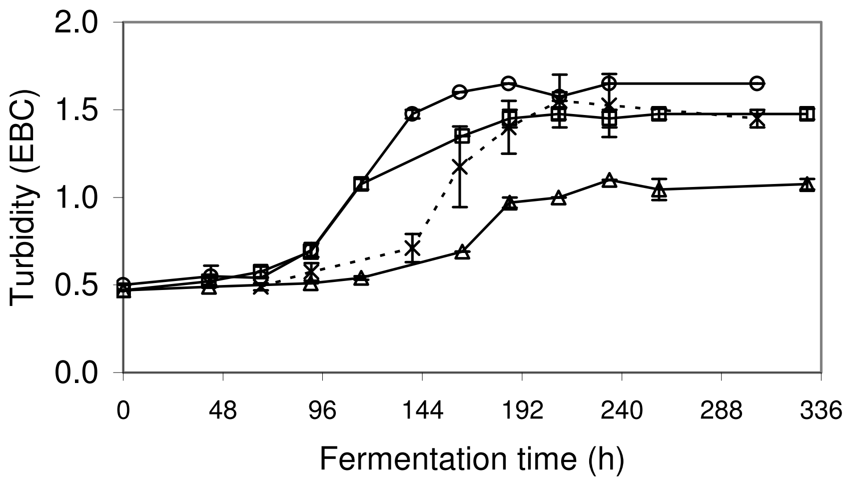
Huuskonen et al. Fig 2.





Huuskonen et al Fig 3.





A15 T24.1 T24.9 T24.201

Huuskonen et al Fig 4