Isolation and Characterization of Two New Lager Yeast Strains from the WS34/70 Population

IRINA C. BOLAT[1], MICHAEL C. WALSH[2], MARIA TURTOI[3]
[1]Heineken Supply Chain, Dept. Research & Innovation, Brewing Science Yeast, Zoeterwoude,

[2]Heineken Supply Chain, Zoeterwoude, The Netherlands
[3]Sibiu Alma Mater University, 57 Somesului, 550003 Sibiu, Romania, Phone +40 269 21 64

Abstract

WS34/70 is one of the most widely used non-proprietary lager yeasts in the brewing industry. The genetic characterization of lager yeast WS34/70, performed by electrophoretic karyotyping, has revealed that this strain is composed of a large number of variant strains within its composition. Each variant has different properties, therefore the behaviour of the lager yeast WS34/70 will be strongly influenced by those variants present in the highest composition in the original strain mixture. During serial re-pitching, however, the more flocculent variants will start to influence the fermentation characteristics. The present study focused on two variants with different flocculation characteristics selected from the commercial brewers lager yeast WS34/70, purchased from Weihenstephan, Germany as yeast agar slant. The initial mixtures WS34/70 as well as the variants selected were stored at -80°C, in liquid nitrogen.

Their behaviour in terms of fermentation performance, flavour compounds, flocculation characteristics and genetic stability was analyzed against the initial WS34/70 population. Furthermore a comparison of the two strains in terms of phenotypic behaviour with respect to the catabolism of carbon and amino acids sources was also performed.

The two variants displayed different fermentation characteristics, different flavour profile and opposite flocculation properties. While variant 1 is genetically stable, variant 2 showed a high genetic instability. The metabolic profiles of the two strains both on C-sources and N-sources showed similar patterns.

Keywords: yeast selection, flocculation, genetic stability, brewer's yeast.

Introduction

In the breweries non-pure lager yeast cultures are often being used either as a starter culture or as a result of genetic divergence during serial re-pitching. These cultures are usually mixtures of very closely related strains. Some of these strains will perform differently with respect to certain fermentation characteristics as well as their degree of tolerance to stresses experienced during the brewing process.

Selecting the best yeast strain with the desired brewing characteristics has always been brewer's dream. The desired characteristics for an ideal yeast strain comprise a fast fermentation rate, balanced flavour compounds, appropriate flocculation avoiding incomplete attenuation, efficient conversion of wort sugars to alcohol and genetic stability [1]. High fermentation rates increase the brewery capacity without further investments, an appropriate flocculation of the yeast avoids extract losses, and balanced and reproducible flavours compounds assure consistent product quality.

In comparison with other media employed in alcoholic fermentation, wort is by far the most complex one, consisting of simple sugars, dextrins, amino acids, peptides, proteins, vitamins, ions and other constituents. Yeast cells utilize in an orderly manner the plethora of nutrients. Thus the uptake sequence of wort sugars, under normal conditions, is: sucrose, glucose, fructose, maltose and maltotriose. In order to achieve a good attenuation (efficient conversion of sugars to ethanol) the yeast must be able to use maltose and maltotriose, as they represent the major sugars in brewers' wort [1,2]. However, the production of ethanol is not the only objective of brewery fermentation. The flavour and aroma of a beer is a vital aspect of its quality [3]. Yeast metabolites that contribute to beer flavour include organic acids, medium chain-length (8-10 carbon atoms) aliphatic alcohols (fusel alcohols), aromatic alcohols, esters, carbonyls and various sulphur-containing compounds [4]. High concentrations of fusel alcohols impart off-flavours, while low concentrations of these compounds and their esters make an essential contribution to beer flavour [5]. The main contribution of higher alcohols to beer flavour is by a general intensification of alcoholic taste and aroma and by imparting a warming character. A second very important role of fusel alcohols is in providing precursors for ester synthesis [4]. Esters are of major industrial interest as they impart the fruity aroma of beer. There are two main groups of flavour-active esters in fermented beverages: the first group contains the acetate esters (such as isoamyl acetate), the second group is the ethyl esters (such as ethyl hexanoate). The acetate esters are produced in much higher levels than the ethyl esters. The ester profile is very strain dependent and strongly effects the perception of the flavour of the beer produced by it [6].

One very important yeast trait is represented by the flocculation characteristic. Abnormal flocculation can result in either partially fermented product or an over fermented one. It is generally recognized that the first generation of yeast flocculates poorly. Serial re-pitching enhances flocculation performance. Smart *et al.* [7] remark that this is due to inadvertent selection of subpopulations during harvesting, but also the stresses imposed by serial re-pitching of the yeast affects the expression of key cell wall mannoproteins that permit flocculation to proceed. Genetic instabilities of brewer's yeast strains or heterogeneities such as in this study, however, could also contribute to this. At Heineken, flocculation of a homogeneous yeast strain is only determined after three serial fermentations.

Yeast genetic stability is one of the requirements for an "ideal" yeast as it offers the certainty of a consistent fermentation and eventually constant product quality.

The WS34/70 yeast strain is originally from Weihenstephan (Germany) and is used worldwide within the brewing industry for lager beer fermentations. Recently, our research work carried on studying this lager yeast strain has revealed a number of strain variants within its overall architecture. The intricate composition of WS34/70 along with the natural instability of the yeast population within a production environment highlights the advantage of selecting a variant capable of casting the characteristics of ideal yeast.

The current study focuses on screening different characteristics of two variants within the WS34/70 mixture. The objective is to analyze and select the best variant that could be used in production, as a single strain, capable of displaying similar or higher fermentation characteristics than the initial mixture.

Materials and Methods

Yeast variants. The two variants were isolated and selected from the WS34/70 lager yeast purchased from Weihenstephan, Germany as yeast agar slant. The initial mixtures of the WS34/70 population as well as the two variants were preserved at -80°C (Table 1).

Table 1. Yeast strains used in the study

Strain	Source	Year	Description
WS34/70	Weihenstephan	2006	Commercial lager yeast
Variant 1	WS34/70	2006	Natural variant selected from WS34/70 purchased from Weihenstephan
Variant 2	WS34/70	2006	Natural variant selected from WS34/70 purchased from Weihenstephan

The identification of the variants within the initial population was done using pulsed field gel electrophoresis. This is a technique that allows separation of large DNA molecules, typically ranging in size from 50 to 10 000 kb (10 Mb). The DNA molecules of the 16 chromosomes (haploid) of *Saccharomyces cerevisiae* range in size from approximately 200 kb to 3 Mb, making them suitable for separation by this technique [8].

Wort and growth conditions for the fermentation performance assay. The fermentation tests were performed using 17°Plato all malt wort, collected from a brewery. Zinc was added into the wort (0.5 ppm final concentration of Zn^{++}). The propagation step employed 2 hl tanks, pitching at 8ºC and propagation at 11ºC. At an extract of 8–9º Plato, the propagated yeast was transferred into 10 hl fermentation tanks. The following fermentation recipe was used: 11ºC until an extract of 7º Plato, than 14ºC. When diacetyl level dropped below 15 ppb, deep cooling at 0ºC was performed. Samples were taken every day during fermentation.

Yeast cell concentration. Yeast cell concentration and yeast viability were determined using the NucleoCounter YC-100 System.

Wort and growth conditions for the flocculation assay. The strains were grown in 30 ml bottles with 20 ml all malt wort collected from a brewery after cooling and diluted to 15°Plato. In the wort 0.5 ppm final concentration Zn^{++} was added as zinc chloride and 0.5 ml l^{-1} Dow Corning®Antifoam 1510 (10% active, food-grade silicone emulsion) was added. The yeast was grown at 20ºC for 3 days. The growth-step was repeated 3 times for a more reliable result. The flocculance buffer used was 50 mM acetate buffer, containing 0.1% (m/v) $CaCl_2$.

Sample preparation for the flocculation assay. The yeast sample was centrifuged for 5 min at 3000 rpm. The pellet was washed once with cold demi-water. The brown sediment on the top of the yeast pellet was removed with cold demi-water. Part of the yeast pellet was re-suspended in ± 5 ml of water to an OD660 of 2.30 ± 0.05. When the OD was too low or too high more yeast or more demi-water was added. Out of the yeast suspension thus prepared, 1800 µl were taken into glass tubes in duplicate and centrifuged for 5 min at 3000 rpm. The supernatant was removed using a vacuum pump. After an addition of 1800 µl of flocculance buffer to the yeast pellet the suspension was incubated for 30 min at room temperature. Afterwards, the yeast suspension was mixed and OD660 was measured in the spectrophotometer for 1 min.

Yeast flocculation. The measure of flocculance was done using the delta optical density (delta OD) per min of the yeast suspension. In this respect spectrophotometer Hitachi U-2900 was used.

Wort and growth conditions for the genetic stability assay. The strains were grown in a similar way as for the flocculation assay. The growth-step was repeated 5 times, every step

lasting 3 days at 20ºC with continuous stirring at 120 rpm. The yeast from the 5th step was diluted to 10^3 cells ml^{-1} and plated on Wallerstein Laboratorius Nutrient (WLN). After a 3 days incubation period at 30ºC, single colonies were analyzed using pulsed field gel electrophoresis.

Cell suspension preparation for the phenotype expression. The variants are plated on WLN medium, incubated overnight at 30ºC. Isolated colonies were removed from the plate using a sterile swab and transferred into a sterile capped tube containing 9 ml of phosphate buffered saline (PBS). The turbidity of the suspension must be 62%T (transmittance).

The inoculation media for the phenotype expression. In order to study the phenotype expression of the variants, phenotype microarrays (PM) with carbon sources and nitrogen sources were used. Special PM inoculating fluids were prepared for each type of plate, from stock solutions. They comprised special inoculation media for yeast, a special dye, PM additive solutions and yeast suspension.
For PM with carbon sources, the additive solution contained L-glutamic acid monosodium, disodium pyrophosphate and sodium sulphate. For PM with nitrogen sources, the additive solution only contained disodium pyrophosphate and sodium sulphate. In the latter case the inoculating fluid had also D-glucose.

Microplates with carbon sources. The microplates with carbon sources had 144 wells with a different carbon source in each well and a negative control. Yeast growth kinetics could be monitored, with recordings at every 15 min. The carbon sources comprised simple sugars but also amino acids and weak acids used as carbon source.

Microplates with nitrogen sources. The microplates with nitrogen sources had 144 wells with 143 different nitrogen sources in each well and a negative control. Yeast growth kinetics could be monitored, with recordings every 15 min. The nitrogen sources comprised amino acids (the essential amino acids as well as other amino acids), aliphatic and aromatic amines and few di-peptides.

Yeast phenotype reading. The carbon sources and nitrogen sources plates were incubated in the OmniLog™ from Biolog (Biolog, Inc., 21124 Cabot Blvd. Hayward, CA 94545 U.S.A.). This is a thermal controlled incubator/reader. The temperatures used were 25ºC and 30ºC. It had a computer-controlled interface and offered the possibility of random access plate addition.

Results and Discussion

Two variants selected from the commercial yeast lager strain WS34/70 were analyzed for their fermentation performance as well as for their particular characteristics in terms of flocculation properties, genetic stability and their cellular traits as phenotype expression.

Characterization of two variants isolated from the WS34/70 population. The two variants are referred to as variant 1 and variant 2. The initial population of the WS34/70 strain is referred to as the control strain and the analysis has identified at least 21 different electrophoretic karyotype patterns therefore there are at least 21 different component yeast strains within the WS34/70 population. Comparisons of some of these brewer's yeast strains

were followed during fermentation in all malt 17ºP wort in 10 hl cylindroconical fermentation vessels within a pilot plant. All fermentations were performed in duplicate with the initial mixture WS34/70 population used as control. The wort used for each trial presented similar quality (pitching wort characteristics are shown in table 2), therefore the fermentation behaviour of the control strain displayed similar patterns in both trials: with variant 1 and variant 2. In the graphs there are presented only the data of one control, in duplicate.

Table 2. Pitched wort characteristics

Analyzed component	Unit	Variant 1	Variant 2
Extract	%(m m^{-1})	17.13	16.99
Apparent extract after final attenuation and limit			
Apparent extract after final attenuation	%(m m^{-1})	2.78	3.13
Apparent final attenuation limit	%	83.8	81.6
Colour	EBC	14	10
pH		5.16	5.20
Nitrogen (Kjeldahl method)	mg l^{-1}	1502	1466
Free amino nitrogen (ninhydrin method)	mg l^{-1}	247	243

a) Extract reduction (Fig. 1), number of cells (Fig. 2), delta apparent extract (Fig. 3), pH evolution (Fig. 4)

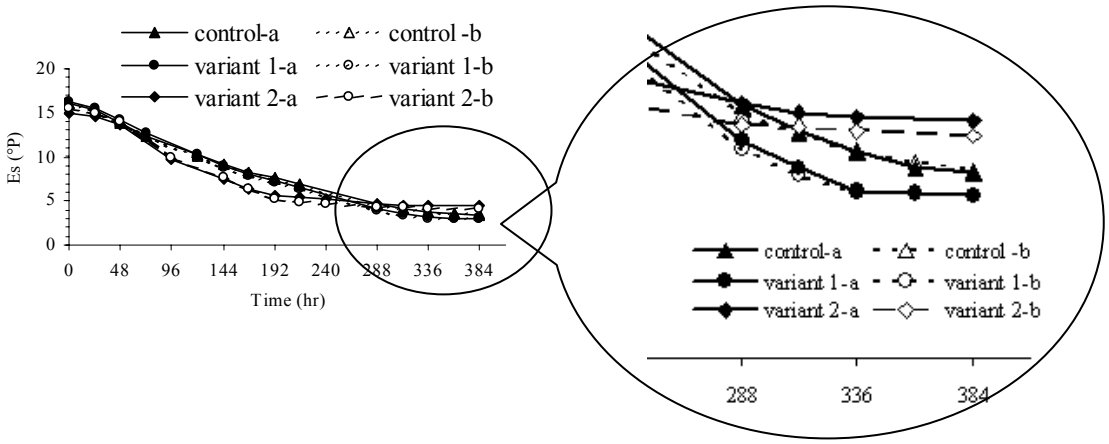


Figure 1. Extract reduction during fermentation

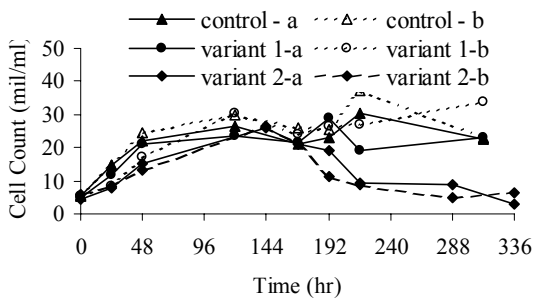


Figure 2. Total cell count during fermentation

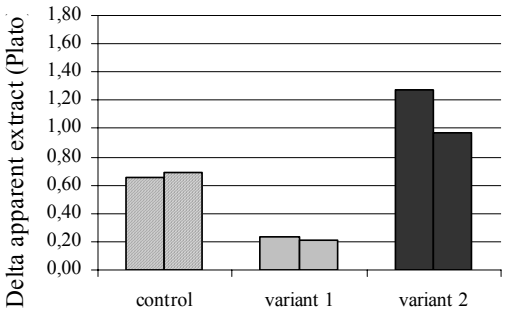


Figure 3. Delta apparent extract

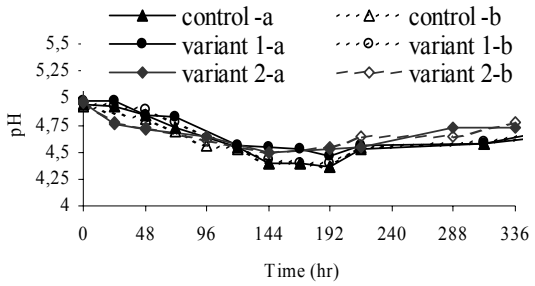


Figure 4. Value of pH during fermentation

Duplicate fermentations were performed with each yeast strain in 10 hl fermentation vessels with automatic cooling. Variant 1 showed a faster and more complete fermentation in comparison with both control strain and variant 2 (Fig. 1). This result supported by the delta apparent extract graph (Fig. 3) in which the better performance of variant 1 in terms of sugar uptake and final attenuation is obvious. Variant 2 showed a faster attenuation rate in the beginning of fermentation but slowed down afterwards, reaching in the end a poorer (higher) final attenuation. Delta apparent extract, with this variant, was very high in comparison with the control strain and variant 1.

Analysis of yeast cell number in suspension during fermentation (Fig. 2) showed that variant 2 exhibited a high flocculation characteristic compared to variant 1 or the WS34/70 control strain. This is consistent with the poor attenuation of the wort fermented with variant 2 in contrast with variant 1 and the WS34/70 control strain.

The pH during fermentation (Fig. 4) also reflects the higher flocculation potential of variant 2. During fermentation, pH characteristically drops due to uptake of wort amino-acids (the main pH buffering capacity in wort) and proton extrusion by actively growing and fermenting yeast. This can be seen for all three strains up to approximately 150 hours into the fermentation. Following this the pH in all three conditions starts to rise, this is due to cell lysis characteristically within the newly forming yeast cone where the stress on the yeast is the most severe. Variant 2 exhibits the highest pH value at the end of the fermentation reflecting the fact that more yeast has flocculated and resides in the yeast cone under the greatest stress.

b) Esters formation: iso-amyl acetate (Fig.5), ethylacetate (Fig.6), total higher alcohols (Fig.7), free amino nitrogen (FAN) evolution during fermentation (Fig.8), **taste test score (Table 3)**

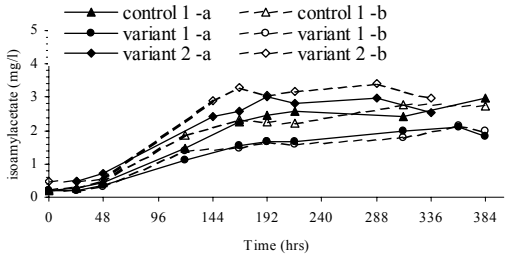


Figure 5. Isoamyl acetate produced during fermentation

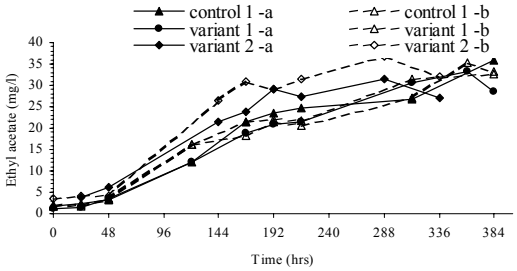


Figure 6. Ethyl-acetate produced during fermentation

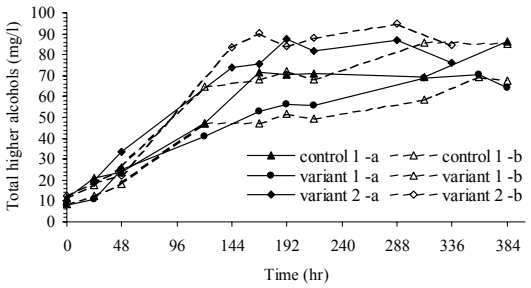


Figure 7. Total higher alcohols produced during fermentation

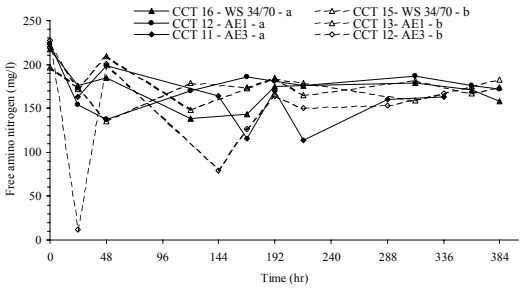


Figure 8. Free amino nitrogen evolution during fermentation

The acetate esters are the most important contributors to the volatile ester fraction of beer [9]. Out of this class two esters are of outmost importance: ethylacetate (solvent-like aroma) and isoamylacetate (fruity, banana aroma). Their taste thresholds are 33 mg/l for ethyl acetate and 1.6 mg/l for isoamyl acetate [3, 10]. In lager beers only these two esters reach the threshold level. The presence of different esters however may have a synergistic effect on the individual flavours. As most esters are present around their threshold values, minor changes in concentration can have dramatic effects on the beer flavour perception.

Other fermentation by-products are higher alcohols and aldehydes. Their formation is linked to amino-acid and carbohydrate metabolism particularly during growth. Thus, the more amino-acids and sugars are taken up, the higher alcohols and related aldehydes are produced [11].

The higher alcohol production is also important because it can reduce the aldehydes content and produces the precursor molecules for the acetate ester production.

Nykanen and Suomalainen cite in their book "Aroma of Beer, Wine and Distilled Alcoholic Beverages" (1983) a reference (Verieyen, 1971) in which the quality of a good product can be assessed by the ratio R = amount of etylacetate/amount of acetaldehyde. The higher the ratio the better the end product is. The trial conducted with both variants of the WS34/70 yeast contradicted this supposition and supports the ratio between the amount of ethylacetate / amount of isoamyl acetate to be as low as possible for a good quality product (Walsh, unpublished data).

Table 3. The acetate esters ratio and taste test score of the end product

Type of strain	Control strain	Variant 1	Variant 2
Ethylacetate/acetaldehyde ↑	**7,15**	6,05	2,9
Ethylacetate/isoamylacetate ↓	10,2	13,85	**10,12**
Taste test score	6,4	6,4	6,45

The flocculation characteristics of the two variants were analysed. Brewing yeast flocculation remains a critical requirement for adequate fermentation performance. Under brewing conditions cell-surface hydrophobicity is a major determinant of flocculence. There are strains with altered cell-surface hydrophobicity that might express a stronger or a weaker flocculation ability, corresponding to high or low cell-surface hydrophobicity, respectively.

The method used to assess the flocculation characteristics of the two variants was a quantitative one, using a spectrophotometer to measure the number of cells in suspension. Thus, in order to measure the flocculation ability of the cells the maximal decrease in optical density per minute was chosen. The OD value during an interval of 60 seconds is shown in

Fig. 9. By subtracting the OD value measured after 60 sec from the OD value measured at time zero, delta OD per min was determined (Fig. 10).
Variant 1 exhibited the characteristics of a medium flocculent yeast strain (0,1<delta OD/min<0,5), while variant 2 displayed the characteristics of a highly flocculent yeast strain (0,5<delta OD/min<1,0). These flocculation tests were supported by the fermentation behaviour of the two variant strains, as they displayed totally different flocculation characteristics during fermentation trials.
Thus, the poor final attenuation (Fig. 3) was consequence of lower yeast concentration in suspension for variant 2 compared to variant 1 and the WS34/70 population (Fig.2). The higher final pH (Fig.4) of variant 2 would be caused by increased yeast cell lysis in the cone compared to other two less flocculent yeasts.

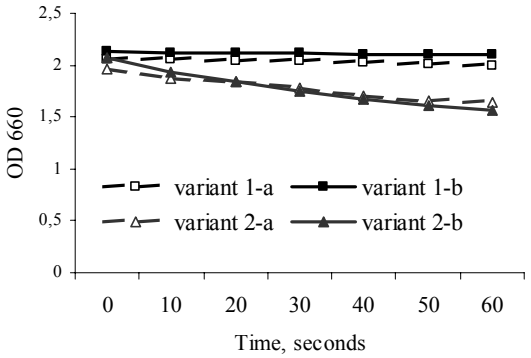


Figure 9. OD660 value during 60 seconds

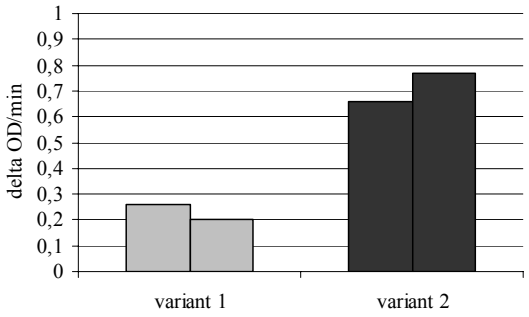


Figure 10. Delta OD per min for each variant

Genetic stability of a strain ensures that the fermentation characteristics are maintained during subsequent rounds of serial re-pitching. The genetic characterization of the two variants was performed by karyotyping, using pulsed field gel electrophoresis, through five rounds of serial fermentations.

Table 4. Genetic stability of the two variants using pulsed field gel electrophoresis

Type of strain	No. of colonies analyzed	Genetic stability	Conclusion
Variant 1	36	100% variant 1	Genetically stable
Variant 2	45	100% variant 2 + extra chromosome	Genetically not stable

By analyzing DNA isolated from 36 individual yeast colonies from variant 1 and 45 colonies from variant 2 it was discovered that one of the variants displayed genetic instability compared to the other variant. Variant 1 exhibited complete genetic stability through five rounds of serial fermentation while variant 2 was completely transformed by gaining an extra chromosome.
This observation is supported by results obtained in a study of the population dynamics of the lager yeast WS34/70 in a production environment [12]. In this case both variant 1 and variant 2 composed approximately 5% and 7% respectively, by mass, of the total yeast population in generation 0. Following four serial re-pitchings, the generation 4 yeast harvests contained less than 1% of variant 1 confirming the lower flocculence of this strain and suggesting that more flocculent strains were selected during yeast harvest and re-pitching as one would expect. Variant 2, despite its much higher flocculence, was also reduced to less than 1% in the generation 4 yeast harvest but the variant that came out the genetic stability tests i.e. variant 2

+ extra chromosome, increased from less than 1% to over 5% of the total yeast population in the generation 4 yeast harvest.
This suggests that these small-scale laboratory tests are not only useful for comparative studies within yeast populations they are also useful prediction tools for yeast behaviour in an industrial environment.

Phenotype expression (Biolog)
Genetic changes can result in phenotypic changes. The phenotype expression of the control strain and the two variants with respect to carbon and nitrogen metabolism was analyzed. The growth media was represented by C-sources and N-sources, monitoring the metabolic kinetics. A comparison between the control and the variant tested was done.
Phenotypic microarrays use the redox chemistry, employing cell respiration as a universal reporter. If the phenotype is strongly "positive" in a well, the cells respire actively, reducing a tetrazolium dye and forming a strong colour. If it is weakly positive or negative, respiration is slowed or stopped and fewer colours or no colour is formed. The OmniLog captured a digital image of the MicroArray colour change 4 times each hour and stored the quantitative values into computer files that could be displayed as kinetic graphs.
The metabolic profile of the two variants on carbon sources (Fig. 11) is quite similar. They didn't use amino-acids as carbon sources, while the uptake of sugars normally encountered in wort displayed identical patterns. The only difference was represented by the uptake of sucrose. This sugar was metabolized by variant 2 with a slightly faster rate.
Analyzing the metabolic profile of the variants on nitrogen sources (Fig. 12) the lack of affinity for amines and amides as N-sources can be easily observed on both variants (rows D and E in Fig. 12).
Line F contains the 5 nucleobases: adenine, cytosine, guanine, thyamine and uracil, as well as the nucleosides: adenosine, cytidine, guanosine, thymidine, uridine, inosine. Both variants show growth only on cytosine (variant 2 slightly faster). Variant 1 also shows a weak growth on adenine after some time.

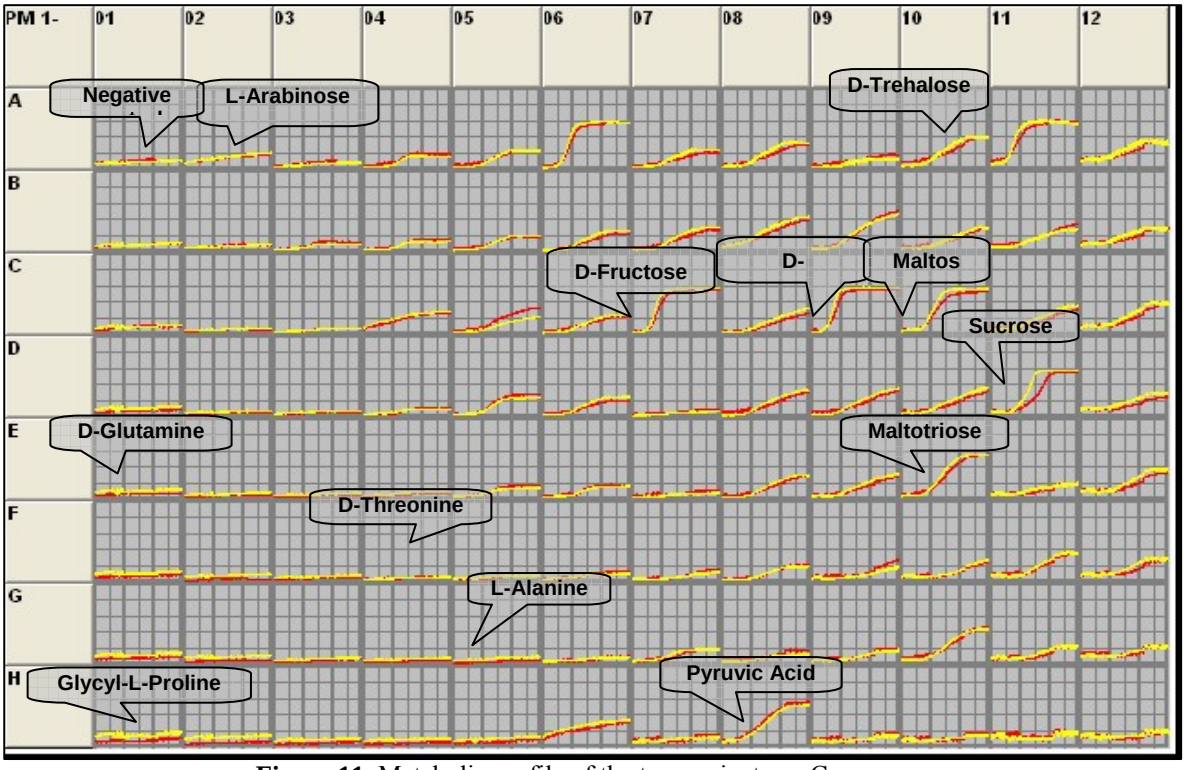


Figure 11. Metabolic profile of the two variants on C-sources
(variant 1(red) and variant 2 (yellow) at 30°C)

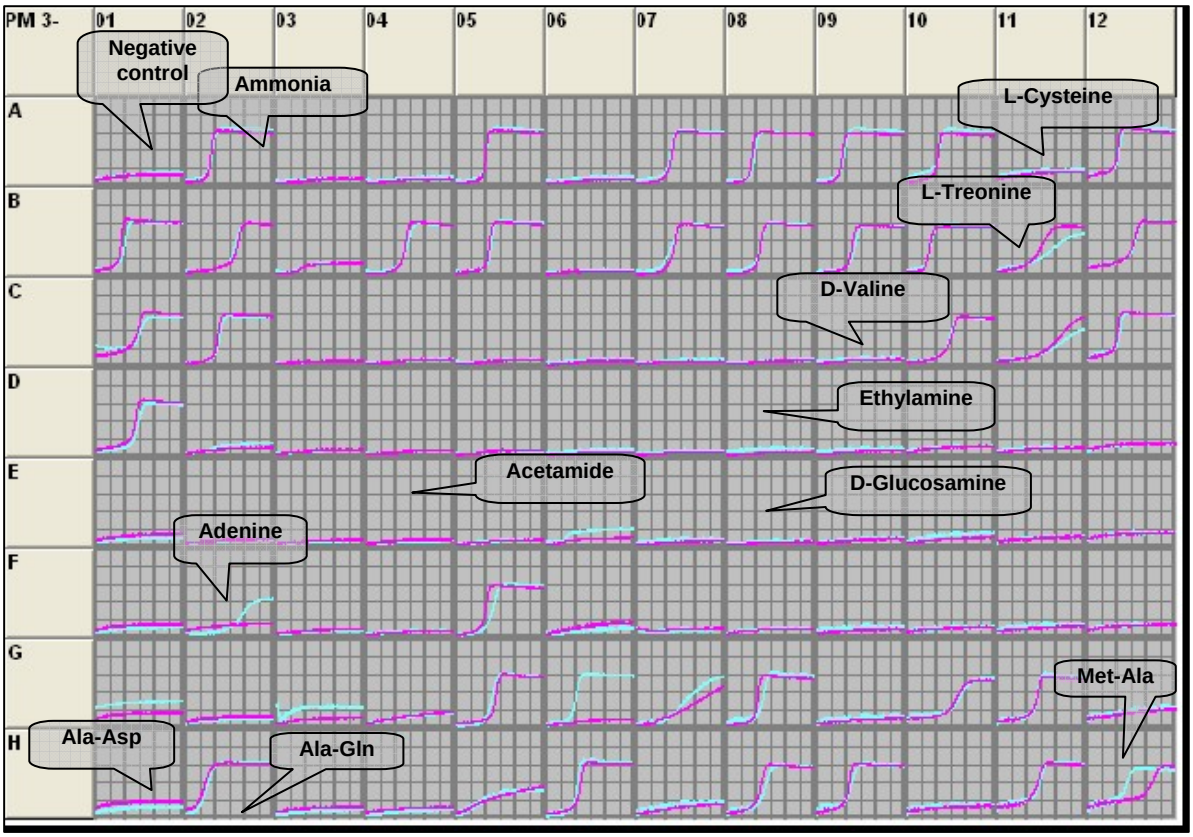


Figure 12. Metabolic profile of the two variants on N-sources
(Variant 1(blue) and variant 2 (pink) at 30°C)

Conclusions

The brewer's yeast strain WS34/70 is a mixture of very closely related strains, which nevertheless exhibit different behaviour in key fermentation characteristics like flocculation, final attenuation or flavour development. In this study two of these variants, a medium flocculent strain and a highly flocculent strain have been compared in their fermentation behaviour, their genetic stability and their phenotypic characteristics.

Variant 1 displays better fermentation performance in comparison with variant 2 or the WS34/70 population in terms of final attenuation and stress resistance but the lower flocculation could produce issues for beer filtration and extract losses if too much yeast is left in suspension.

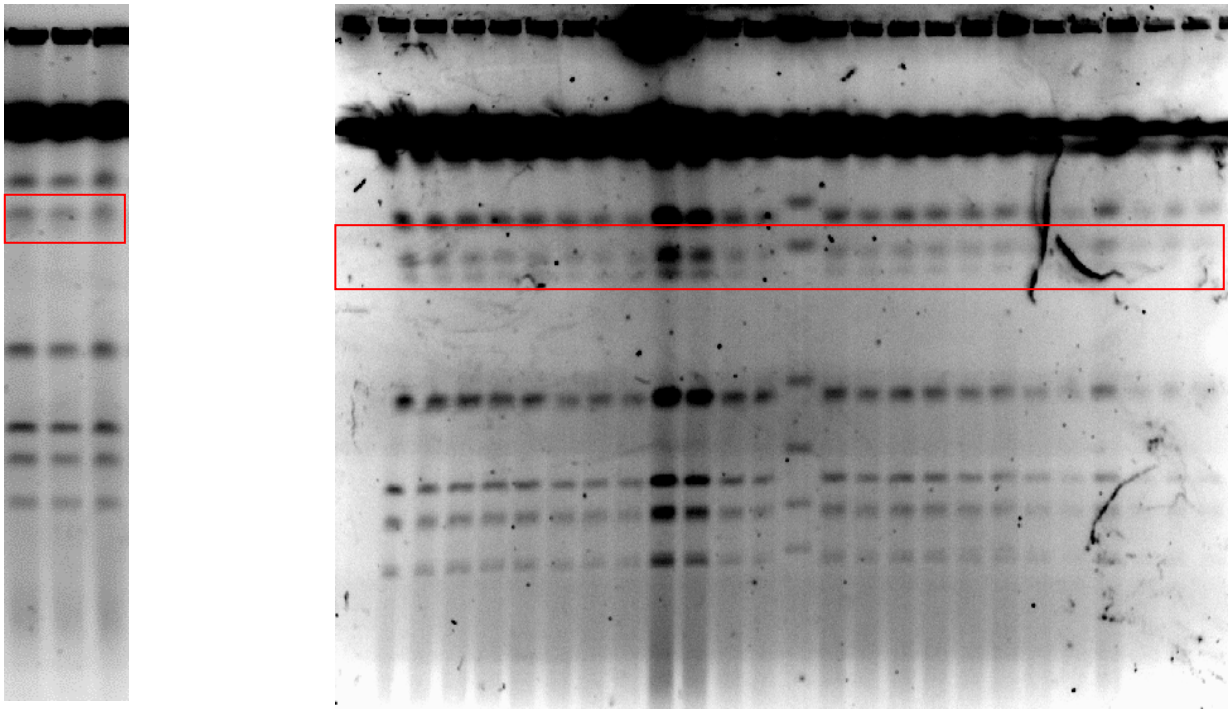
Variant 2 exhibited higher isoamyl acetate production giving a more balanced flavour profile and higher taste test scores; however, the higher flocculation produced an incomplete fermentation leading to a higher delta apparent extract and potential issues with diacetyl reduction if there is not enough yeast left in suspension at the end of fermentation.

Variant 2 also exhibited a high degree of genetic instability manifesting itself as the appearance of an extra chromosome in the karyotype during serial fermentation.

The behaviour of any heterogeneous WS34/70 population will therefore be a consequence of the distribution of different strains in the starting population, their flocculation and their genetic stability. Selecting a single (karyoptype) homogeneous strain as a production strain will depend on the process demands (tank sizes and fermentation recipes) of any particular production process as well as the flavour characteristics of the end product.

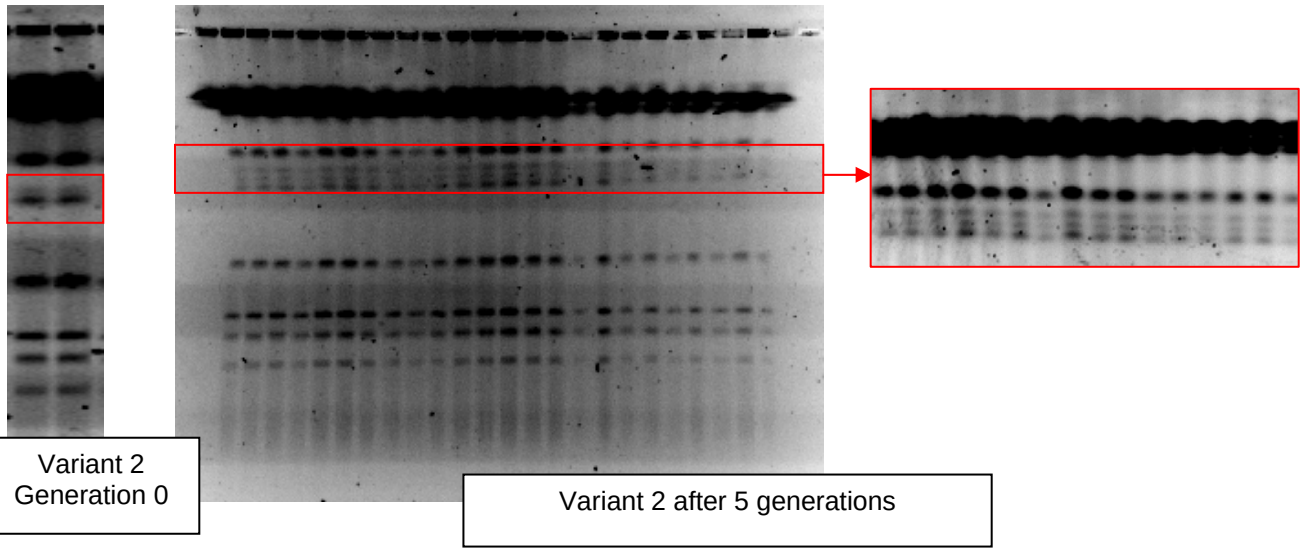
In current use of the WS34/70 yeast, brewers should pay particular attention to each propagation step and the number of serial re-pitchings in the knowledge that each of these steps will change the WS34/70 population with consequences for process control and final product quality.

Appendix 1 –Electrophoretic karyotype and genetic stability of Variant 1 and Variant 2.
Variant 1 and variant 2 differ by the ratio of the two bands indicated by the red boxes below. In variant 1 the ratio of the upper band to the lower band is greater than 1 (two bands within the red box). In variant 2 the ratio of the upper band to the lower band is less than one (within the red box), the red arrow indicates the extra chromosomal band in the genetic stability test. Each lane represents DNA isolated from a single yeast colony.



Variant 1
Generation 0

Variant 1 after 5 serial fermentations



Variant 2
Generation 0

Variant 2 after 5 generations

Acknowledgments
The authors wish to thank Heineken Supply Chain for their support of this work. Michael C. WALSH acknowledges the permission of Heineken Supply Chain to publish this work and attend the International Conference on Industrial Microbiology and Applied Biotechnology held on 9–11 October 2008 in Galati – Romania.

Abbreviations
WLN - Wallerstein Laboratorius Nutrient; PM – Phenotypic Microarrays; OD – Optical Density; T – Transmittance; PBS – Phosphate Buffered Saline; FAN – Free amino nitrogen.

REFERENCES

1. LANCASHIRE B., GOPAL C., The selection of yeast strains for brewing. Brewers' Guardian, July, 26-31, 1998.
2. STEWART G.G., Studies on the uptake and metabolism of wort sugars during brewing fermentations. MBAA TQ, **43(4)**, 265-269, 2006.
3. RENGER R.S., VAN HATEREN S.H., LUYBEN K.CH.A.M., The formation of esters and higher alcohols during brewery fermentation; the effect of carbon dioxide pressure. J. Inst. Brew, **98**, 509-513, 1992.
4. BOULTON C., QUAIN D., Brewing yeast and fermentation. Blackwell Science Ltd., 2001.
5. HAZELWOOD L.A., DARAN J-M., VAN MARIS A.J.A., PRONK J.T., DICKINSON J.R., The ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism, Appl. Environ. Microbiol., 2259-2266, 2008.
6. SAERENS S.M.G., DELVAUX F., VERSTREPEN K.J., VAN DIJCK P., DELVAUX F.R., Parameters affecting ethyl ester production by *Saccharomyces* fermentation. Appl. Environ. Microbiol., p.454-461, 2008.
7. SMART K.A., LAWRENCE S., LECLAIRE J., DAVY S., Brewing Yeast Flocculation: A model for onset and control, Proc. Inst. Brew. Dist. Asia. Pac. Conf. Hobart, AUS, CD ROM, Contribution 18, 2006.
8. JOHNSTON J.R., Molecular Genetics of Yeast - A Practical Approach, Oxford University Press, 1994.
9. NYKANEN L., SUOMALAINEN H., Aroma of beer, wine and distilled alcoholic beverages (Handbook of Aroma Research), Kluwer Academic Publishers, 1983.
10. VERSTREPEN K.J., DERDELINCKX G., DUFOUR J-P., WINDERICKX J., THEVELEIN J.M., PRETORIUS I.S., DELVAUX F.R., Flavour - Active esters: adding fruitiness to beer, J. Biosci. Bioeng., **96(2)**, 110-118, 2003.
11. MUSSCHE R.A., MUSSCHE F.R., Flavours in beer – Lecture.
12. BOLAT I.C., WALSH M.C., Population dynamics of brewers lager yeast WS 34/70. Yeasterday meeting 2008.