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Special Issue Article

Investigating flavour characteristics of British ale yeasts: techniques, resources and opportunities for innovation

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Abstract

Five British ale yeast strains were subjected to flavour profiling under brewery fermentation conditions in which all other brewing parameters were kept constant. Significant variation was observed in the timing and quantity of flavour-related chemicals produced. Genetic tests showed no evidence of hybrid origins in any of the strains, including one strain previously reported as a possible hybrid of *Saccharomyces cerevisiae* and *S. bayanus*. Variation maintained in historical *S. cerevisiae* ale yeast collections is highlighted as a potential source of novelty in innovative strain improvement for bioflavour production. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: Saccharomyces, ale yeast, brewing, flavour, strain diversity

Introduction

Flavours are highly important quality components of fermented beverages and much work has been devoted to establishing the chemical basis of taste variation in the final product. In ale brewing, research into the underlying properties of different yeast strains has tended to focus on effects of fermentation conditions and modifications of hop aromas (King and Dickinson, 2003; Saerens *et al.,* 2008; Vidgren *et al.,* 2010; Hiralal *et al.,* 2014). Such work generally recognizes that the yeast strain or strains used in the original fermentation can make a significant difference to the results obtained. However, quantifying that difference is not trivial. Complex evolution of flavour compounds during fermentation, together with a plethora of different starting materials and brewing techniques, make it difficult to attribute the precise contribution of a particular yeast strain with any degree of confidence (Jespersen *et al.,* 2000).

Here we address the need for new scientific approaches to isolate and compare yeast-derived flavours under controlled conditions. We approached this problem by performing trial fermentations in which all brewing materials and fermentation conditions were kept constant and only the yeast strain changes. By using five distinctive strains of British ale yeast, we investigated variation in their specific flavour profiles and attempted to relate this variation to strain origins, using genetic probes. Here we also discuss how best to apply genome mining of yeast strain collections to inform future strain improvement programmes, e.g. for targeted exploitation of natural genetic variation in the production of yeast flavours and fragrances.

Materials and methods

Strains

The five British ale yeast strains used in this study were all obtained as freeze-dried cultures in glass ampoules from the National Collection of Yeast

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Cultures (NCYC; Norwich, UK; http://www.ncyc.co.uk). The strains were selected from cultures deposited with the NCYC between the years 1958 and 1987. The main selection criteria were for yeast strains commonly ordered by NCYC customers and covering a period when many UK breweries closed down and much brewing information was lost. The key brewing characteristics for each strain are shown in Table 1.

Strain identification

The species identity of each brewing strain was initially determined by ribosomal DNA (rDNA) sequencing. The variable D1/D2 domain of the large subunit (LSU) ribosomal RNA gene and the ribosomal internal transcribed spacer (ITS) region were amplified directly by PCR from whole yeast cell suspensions as described previously (James *et al.,* 1996). The LSU D1/D2 domain was amplified and sequenced using primers NL1 and NL4 (O'Donnell, 1993). The complete ITS region was amplified using primers ITS4 and ITS5, and the ITS1 region was sequenced using primers ITS1 and ITS2 (White *et al.,* 1990). The amplified DNA was purified and concentrated using QIAQuick PCR purification spin columns (Qiagen) and sent to Eurofins MWG Operon (Germany) for sequencing. The LSU D1/D2 sequence for each strain was compared against the reference (type/neotype) strain of each brewing-associated *Saccharomyces* species, using the FASTA sequence similarity search programme (Pearson and Lipman, 1988). For one strain (NCYC 1006), Illumina paired-end reads from a recent whole-genome sequencing experiment [quality trimmed using Trimmomatic (Bolger *et al.,* 2014) v. 0.32] were mapped to the LSU D1/D2 and ITS1 sequences of both *S. cerevisiae* strain S288c and NCYC 1026 (the latter as determined in this study), using Stampy (Lunter and Goodson, 2011) v. 1.0.22. Samtools (Li *et al.,* 2009) v. 0.1.19 was used subsequently to identify variation across these rDNA subregions and to predict the consensus sequences for NCYC 1006. The LSU D1/D2 and ITS1 sequences determined in this study were deposited with the EMBL/GenBank database and the assigned accession numbers are shown in Table S1 (see supporting information).

PCR–RFLP analysis

A preliminary examination of the genetic background of each brewing strain was examined by PCR–RFLP analysis of the *FUN14*, *HIS3* and *RIP1* genes (Rainieri *et al.,* 2006). Each strain was also tested using *Saccharomyces* species-specific primers (Muir *et al.,* 2011; Pengelly and Wheals, 2013).

Growth temperature tests

The five ale strains were grown on yeast extract/malt extract (YM; Difco, Becton Dickenson) agar, containing 0.3% w/v yeast extract, 0.3% w/v malt extract, 0.5% w/v peptone, 1% w/v glucose and 2% agar, for 5 days at both 25 °C and 37 °C.

Trial fermentations

Freeze-dried yeast strains were resuspended in a standard YPD broth (Sunrise Biosciences). Yeast strains were propagated and harvested for use in trial fermentations in a standardized sterilized medium of malted barley (Briess Malt and Ingredients

Table 1. British ale strains used in this study

NCYC strain no.	Year deposited	Strain characteristics recorded in the NCYC database	Growth at 37°C
1006	1958	Top cropping strain; head forming; non-flocculent	Yes
1026	1958	Flocculent; non-head forming; complex ploidy (>2n)	Yes
1187	1960	Slightly flocculent (at pH 3.5 and 5.0); poor head-forming; putative hybrid strain	Yes
1228	1964	Non-flocculent	Yes
1681	1987	Bottom cropping; UK Brewing Research Foundation strain	Yes

Preliminary species identification was established as *Saccharomyces cerevisiae* by LSU rDNA D1/D2 sequencing (O'Donnell, 1993). The potential hybrid nature of NCYC 1187 was originally determined by (Pope *et al.,* 2007) using PCR–RFLP analysis, as detailed in (Rainieri *et al.,* 2006). The hybrid nature of all five strains was examined in this study by PCR, using species-specific primer pairs (Pengelly and Wheals, 2013; Muir *et al.,* 2011). No evidence of hybrid origins was obtained.

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CBW Pilsen) with an extract value of 8°Plato (36 g/l maltose, 9.5 g/l maltotriose, 10.45 g/l glucose and 14.25 g/l higher saccharides) and free amino nitrogen (FAN) content of 285 mg/l. A supplemental commercial yeast nutrient, Yeastex 82 (Kerry Biosciences), was added to the medium prior to sterilization to increase the FAN content to 310 mg/l. The FAN content was determined using the American Society of Brewing Chemists (ASBC) Wort-12 method (*Methods of Analysis*, 14th edn). Propagations were carried out in 1 litre Erlenmeyer flasks and incubated at 25 °C with shaking (150 rpm) for 48 h.

Laboratory-scale beer fermentation trials were performed in triplicate, using 1.5 litre Imhoff cones and 1 litre wort medium, consisting of 100% Light Pilsner Malt extract, bittered with Columbus variety hops to a bittering unit of 20 international bittering units (IBU) and an original extract value of 12°Plato. Beer was brewed from one standard batch and divided into five fermentation vessels. Cultured yeast was added at an inoculation rate of 6 million cells/ml of wort, and the wort was oxygenated to a rate of 8.1 mg/l dissolved oxygen. Fermentation was maintained at 69 °F/20 °C for 10 days. The fermentations were considered complete once the specific gravity remained consistent for 2 consecutive days, and the beer was removed from the fermentation vessels and collected into sterile bottles.

Flavour characterisation

Samples were obtained from fermentation vessels at 24, 48 and 72 h increments, as well as the final beer. The samples were centrifuged, degassed and analysed for specific gravity, attenuation and pH, using an Anton Paar Density Meter DMA5000 with Alcolyzer Beer and pH modules (Anton Paar, USA). All beer samples were centrifuged to remove suspended yeast cells and to eliminate the impact of yeast activity during analysis. These values were used to provide fermentation kinetic curves and validate the performance for each yeast strain.

Free (as-is) vicinal diketones (VDKs) were measured for the centrifuged samples, according to ASBC Beer-25, and analysed (Clarus 500 gas chromatograph with headspace unit and Elite 5 60 m, 1.5 DF column, Perkin-Elmer, USA), using a 2,3-hexandione internal standard.

The final concentrations of the major yeast-derived flavour-active compounds were analysed according to the ASBC Beer-29 Method for Lower Boiling Volatiles in Beer, using the Clarus 500 gas chromatograph with headspace unit and Elite BAC 1 30 m, 1.8 DF column, Perkin-Elmer) and 1-butanol as the internal standard.

Statistical analysis

Triplicate measures of the six analysed flavour compounds, taken over the specified time courses, were input to the R Statistical Computing package v. 3.1.1 (R Core Team, 2013). The *t*-test (Welch's), LM and ANOVA functions were used to assess the dependence of these values on the yeast strain under analysis and the time of measurement.

Results

Flavour profiling

Statistically significant interstrain differences were observed (Figure 1). These differences occurred in terms of both the timing of maximum production and the overall amount produced. For example, vicinal diketones (Figure 1a) peaked at day 1 in strains NCYC 1228 and NCYC 1681, and at day 2 in strain NCYC 1187, and showed peak production levels up to three-fold higher than lower-producing strains, e.g. $p = 0.023$ and $p = 0.221$ for *t*-tests of two- and three-fold differences, respectively, between day 1 measurements of NCYC 1681 and NCYC 1026. Acetaldehyde production (Figure 1b) followed a similar time course in strains with peak production at day 1, but two- to three-fold differences in the quantity produced, (e.g. $p = 0.003$ and $p = 0.216$ for *t*-tests of 1.5- and 2.5-fold differences, respectively, between day 1 measurements of NCYC 1026 and NCYC 1228). Both vicinyl diketones and acetaldehyde were fully remetabolized by the end of the experiment.

Iso-amyl acetate showed a more gradual rate of production, peaking at day 2 with up to three-fold variation (e.g. $p = 0.002$ and $p = 0.466$ for *t*-tests of two- and three-fold differences, respectively, between day 2 measurements of NCYC 1681 and NCYC 1006) and two- to three-fold differences still present in the final product (e.g. $p = 0.0001$

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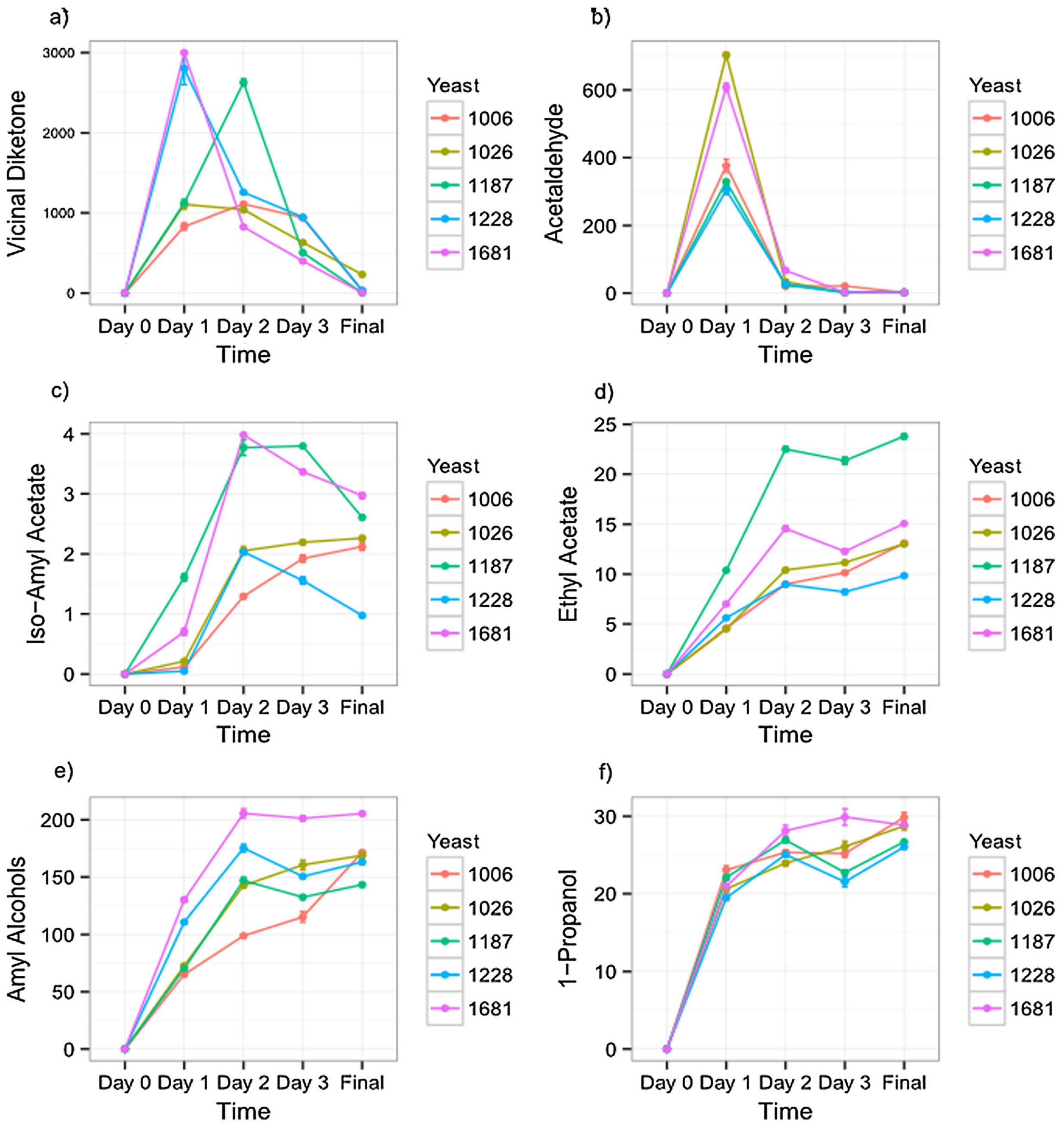

Figure 1. Time course of production of ale yeast flavour compounds: (a) vicinal diketones; (b) acetaldehyde; (c) iso-amyl acetate; (d) ethyl acetate; (e) amyl alcohols; (f) 1-propanol. Standard error bars represent results from triplicate experiments. The graphs were plotted in R, using the ggplot2 library.

and $p = 0.594$ for t-tests of two- and three-fold differences, respectively, between final measurements of NCYC 1681 and NCYC 1228). A similar pattern was observed with ethyl acetate, but NCYC 1187 was found to produce significantly more than other strains at all stages, e.g. $p = 0.00003$ and $p = 0.239$ for t-tests of one- and 1.5-fold differences, respectively, between day 2 measurements of NCYC 1187 and NCYC 1681. However, this strain was one of the poorer producers of amyl alcohol and 1-propanol levels. While amyl alcohol production across strains varied up to two-fold,

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e.g. $p = 0.0006$ and $p = 0.193$ for *t*-tests of one- and two-fold differences, respectively, between day 2 measurements of NCYC 1681 and NCYC 1006, very few interstrain differences were found in 1-propanol production, in terms of either time course of production or overall amount produced, i.e. only *t*-tests for day 3 measurements between NCYC 1681 and other strains gave *p* values <0.05, not all of which would remain statistically significant at this level following correction for multiple testing. Only acetaldehyde and vicinal diketones failed to show statistically significant differences in final day compound measurements, following correction for multiple testing.

Species identity

The five brewing strains used in this study were deposited in the National Collection of Yeast Cultures between 1958 and 1987. Their original species identities were determined by conventional chemotaxonomic methods (Kurtzman *et al.,* 2011), and all were classified as *Saccharomyces cerevisiae*. This classification was supported by the fact that each strain was found to grow readily at 37 °C (this study), a trait characteristic of *S. cerevisiae*. In contrast, the other brewing-associated species, *S. bayanus*, *S. uvarum* and *S. pastorianus*, which all have similar chemotaxonomic profiles to *S. cerevisiae*, are unable to grow at this elevated temperature. In this study, LSU D1/D2 sequencing was used to determine species identity. All five brewing strains were identified as *S. cerevisiae*, each displaying 100% sequence identity to the *S. cerevisiae* neotype strain (NRRL Y-12632^{NT}).

All five strains were also found to have variable-length poly-A/T tracts at the 5′ end of the ITS1 region (nucleotide positions 28–34, based on S288c ITS1 numbering). This is a common feature of many industrial *S. cerevisiae* strains used in brewing and baking (Kawahata *et al.,* 2007; Liti *et al.,* 2009). Visual inspection of the individual sequence traces indicated that each strain appeared to have one overall dominant (poly-A/T tract) length variant. This meant that only the most abundant sequence variant could be determined for each strain. In the case of NCYC 1006, Illumina paired-end reads were used to generate the most abundant ITS1 sequence variant. This was due to the presence of an additional polymorphic site at the 3′ end of the spacer region (nucleotide position 279, based on S288c ITS1 numbering), which prevented any sequence being directly determined. Amongst the five strains, four were found to be identical (NCYC 1006, NCYC 1026, NCYC 1187 and NCYC 1228), and these differed from NCYC 1681 simply in the length of the 5′ poly-A/T tract. In strain NCYC 1681, the most abundant length variant is 11 Ts, while in the other four strains it is 12 Ts. Figure S1 (see supporting information) shows an alignment of the ITS1 sequences for the five British ale strains and the *S. cerevisiae* reference strain S288c. A FASTA search of the EMBL/GenBank database revealed that the ITS1 sequences of NCYC 1006, NCYC 1026, NCYC 1187 and NCYC 1228 are identical to an American whisky strain (NBRC 2112), as well as two other British ale strains deposited with the NCYC (NCYC 1245 and NCYC 1333) (Kawahata *et al.,* 2007). In contrast, NCYC 1681 was found to have an ITS1 sequence identical to a Peruvian strain used to make chicha (EMBL Accession No. KC183727).

Hybrid nature

In a previous study (Pope *et al.,* 2007) it was reported that the *S. cerevisiae* ale strain NCYC 1187 contained both *S. cerevisiae* and *S. bayanus* RFLPs and should therefore be reclassified as a hybrid. In light of this finding, the same PCR–RFLP method (Rainieri *et al.,* 2006) was employed to determine the genetic make-up of all five brewing strains, including a re-examination of NCYC 1187. In parallel to this analysis, each strain was also tested with *S. cerevisiae*, *S. eubayanus* and *S. uvarum* species-specific primers (Muir *et al.,* 2011; Pengelly and Wheals, 2013).

Contrary to previous findings, only the *S. cerevisiae* homologues of the *FUN14*, *HIS3* and *RIP1* genes could be amplified from NCYC 1187. No PCR products were amplified using any of the *S. uvarum* primers. Similar results were obtained with the other four brewing strains, indicating that all five strains possessed *S. cerevisiae* genomes and providing no evidence to suggest any were of hybrid origin. Similar results were obtained with the species-specific primers. Only the *S. cerevisiae* primers, targeted to amplify the *MEX67* gene (located on chromosome XVI) (Muir *et al.,* 2011), tested positive.

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Collectively, the three DNA-based analyses identified each ale strain as *S. cerevisiae*. No evidence was obtained to indicate that any of the strains, including NCYC 1187, was hybrid in origin.

Discussion

A large number of yeast strains in the NCYC collection are recorded as having their origin in ale production by British brewers. In many cases, European Brewery Convention 'tall tube' data (Walkey and Kirsop, 1969) are available, but in most cases information on flavour characteristics is absent. By combining White Labs controlled fermentation techniques with the NCYC's extensive British ale yeast resources (a collection of several hundred strains, assembled over >60 years and representing, in effect, the brewing yeast heritage of the UK), we have attempted to fill in some of the missing flavour information. We have found that significant variation in flavour traits exists. This variation represents a valuable source of novel diversity for exploitation in future strain improvement programmes, whether for specialized brewing applications or for the commercial production of desirable compounds for the flavours and fragrances or speciality chemicals industries. Future work will assess the mechanisms involved. Associations between fermentative ability and flavour compound production will be sought. For example, excessive levels of acetaldehyde typically indicate poor condition of a yeast culture. In a typical fermentation, acetaldehyde is an intermediate compound in the metabolic pathway to ethanol production. Yeasts that are not at optimal fitness are not able to make this conversion, due to low production of alcohol dehydrogenase, which is ultimately responsible for catalysing this reaction and is affected by many environmental conditions, as well as yeast strain phylogeny (Boulton and Quain, 2006). Such traits could be pursued further through QTL mapping or analysis of whole-genome sequences (see below).

Interestingly, we find no evidence for hybridization events, such as have occurred in lager yeast genomes (Libkind *et al.,* 2011), being responsible for the observed variation. Collectively, the three DNA-based analyses identified each ale strain as *S. cerevisiae*. No evidence was obtained to indicate that any of the strains, including NCYC 1187, was an interspecies hybrid. This is in contrast to the lager yeast *S. pastorianus* (syn. *S. carlsbergensis*), whose strains have very complex genomes derived from two or more *Saccharomyces* species (Rainieri *et al.,* 2006), including *S. cerevisiae* and the cold-tolerant *S. eubayanus*, the latter recently discovered first in Patagonia (Libkind *et al.,* 2011) and then subsequently in China (Bing *et al.,* 2014). However, ale strains can exhibit multiple ploidy (Smart, 2007) and, as discovered by Liti *et al.* (2009), many brewing- and baking-related *S. cerevisiae* strains are in fact intraspecies hybrids with mosaic-like genomes. Thus, without additional genome sequence data, we cannot discount the possibility that one or more of these British ale strains may be an intraspecies hybrid.

As whole-genome sequences become available for strains such as those used in this study, we anticipate being able to investigate in more detail the genetic differences underlying yeast flavour production traits. QTL mapping studies have already been performed for oenological traits (Salinas *et al.,* 2012; Steyer *et al.,* 2012). Replication of this work in ale yeasts will require that strains be 'domesticated' and used in genetic crosses. In addition to QTL-based studies, genome sequencing outputs, such as *de novo* genome assemblies, offer further valuable insights into differences at the level of genome content and offer the potential to understand subtelomeric rearrangement, which is known to be a hotspot for generating variation with major impact on the traits described in this paper (Bergström *et al.,* 2014). Aneuploidy is also known to be widespread in British ale yeasts (Smart, 2007; Dicks *et al.* unpublished) and is another source of variation with major impacts.

Exceptional strains such as NCYC 1187 hold great promise as a source of innovation for yeast researchers and industrial users applying systems and synthetic biology approaches for a variety of applications in industrial biotechnology. We anticipate beneficial consequences, in numerous areas, from innovative bioflavour production, through pathway engineering for more environmentally friendly brewing to beers with enhanced contents of health-promoting natural products.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

Figure S1. ITS1 alignment of five British ale strains and the *S. cerevisiae* reference strain S288c

Table S1. EMBL Accession Nos for the rDNA sequences determined in this study

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