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The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation

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Abstract

Individual cells of the yeast *Saccharomyces cerevisiae* exhibit a finite replicative lifespan, which is widely believed to be a function of the number of divisions undertaken. As a consequence of ageing, yeast cells undergo constant modifications in terms of physiology, morphology and gene expression. Such characteristics play an important role in the performance of yeast during alcoholic beverage production, influencing sugar uptake, alcohol and flavour production and also the flocculation properties of the yeast strain. However, although yeast fermentation performance is strongly influenced by the condition of the yeast culture employed, until recently cell age has not been considered to be important to the process. In order to ascertain the effect of replicative cell age on fermentation performance, age synchronised populations of a lager strain were prepared using sedimentation through sucrose gradients. Each age fraction was analysed for the ability to utilise fermentable sugars and the capacity to flocculate. In addition cell wall properties associated with flocculation were determined for cells within each age fraction. Aged cells were observed to ferment more efficiently and at a higher rate than mixed aged or virgin cell cultures. Additionally, the flocculation potential and cell surface hydrophobicity of cells was observed to increase in conjunction with cell age. The mechanism of ageing and senescence in brewing yeast is a complex process, however here we demonstrate the impact of yeast cell ageing on fermentation performance.

Keywords: Yeast; Brewing; Ageing; Flocculation; Fermentation performance; Attenuation

1. Introduction

The yeast *Saccharomyces cerevisiae* has a limited replicative lifespan. Each cell within a population is only capable of a finite number of divisions prior to senescence and death. Studies of the ageing phenotype in both haploid laboratory strains and polyploid brewing strains have indicated that as a consequence of senescence yeast cells are subject to morphological, metabolic and genetic modifications [1]. Such modifications include an increase in size [2] and alterations to the shape and surface appearance of the cell [3]. In addition, generation time is altered [3], metabolism declines [4] and gene expression [5] and protein synthesis [4] become modified.

Beer quality is strongly influenced by the biochemical performance of the yeast during fermentation. The ability of yeast to separate from the beer at the required time, utilise sugars quickly and efficiently, and produce a product with a high yield of ethanol and the correct balance of flavour compounds, is of principal importance. Many intrinsic and extrinsic factors may affect the rate and quality of fermentation and the character of the final product, however replicative ageing has not previously been considered to be important to the process.

Industrial fermentations performed to produce beer are unique within the alcoholic beverage industry in that the yeast is not discarded after use but is maintained and reused a number of times in a process termed 'serial repitching'. The number of times a yeast population may be serially repitched is determined largely by a combination of product quality constraints and company policy, however it is not unusual for a yeast culture to be used between 7 and 20 times and occasionally longer.

Towards the end of fermentation, yeast begins to form large clumps of cells or 'flocs' and subsequently sediments and collects within the fermenter cone. The rate at which

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each cell sediments is believed to vary according to its replicative age [6]. Consequently, sedimentation results in the formation of zones enriched with cells of a particular age. Deans et al. [6] demonstrated that older cells accumulate at the bottom of the fermentation vessel (Fig. 1), but results within our laboratory [7] suggest that the precise location of aged cells within the cone may vary according to the characteristics of the yeast strain employed and the dimensions of the fermentation vessel.

At the end of fermentation a portion of the yeast is removed ('cropped') from the fermentation vessel for serial repitching. Typically this is the centre-top portion of the yeast crop, theoretically comprising middle aged and virgin cells [6,8]. However, increasingly yeast is removed early to decrease process time via a 'warm' or 'early' cropping regime and this facilitates removal of the lower portion of the crop, comprising a greater proportion of aged cells [9,10]. Harvesting yeast may therefore select for a population with an imbalance of young or aged individuals, depending on the cropping mechanism employed.

The immediate and long term effects of age selection on yeast fermentation performance have not been investigated. However, it has been demonstrated in both brewery and laboratory fermentations, that yeast physiology, flocculation, surface charge and viability are subject to gradual changes over the course of serial repitching [11–14]. Thus, it is suggested that cells of all ages may meet the requirements of the brewer prior to fermentation, appearing to be vital and viable, despite displaying disparity in terms of their suitability for industrial fermentation. However, genetic and phenotypic variation between yeast cultures may lead to unpredictable fermentation performance. Here we provide evidence to suggest that artificial selection for a population enriched with young or aged individuals may influence yeast fermentation performance.

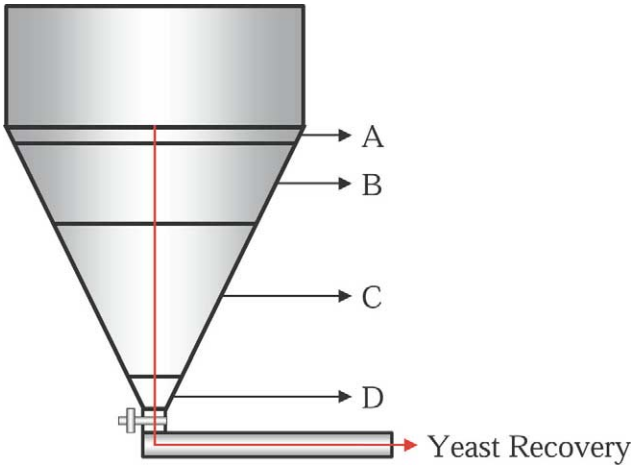

Fig. 1. Schematic illustration of the stratification of yeast during sedimentation. (A) indicates the youngest cell fraction, (D) the predominantly aged fraction. (A), (B) and (C) represent the fractions reused for successive fermentations with traditional cropping techniques. Fraction (C) represents yeast removed from the vessel during warm cropping.

2. Materials and methods

2.1. Yeast strains and growth media

Yeast strains Ltd., BB11 and BB28 were provided by Coors Brewers, Burton-on-Trent, UK. BB1 is an ale production strain, while BB11 and BB28 are lager type strains. Each strain was maintained and grown on YPD media (2% [w/v] bacteriological peptone, 1% [w/v] yeast extract, 2% [w/v] glucose). Following preparation media were sterilised by autoclaving at 121°C and 15 psi for 15 min. All media components were supplied by Oxoid. Brewery wort typically used for lager production (1060° original gravity) was obtained from Coors Brewers.

2.2. Preparation of differentially aged cell fractions

Cell fractions were prepared by sedimentation through sucrose gradients using a modified version of the protocol published by Mitchison and Vincent [15] and Egilmez et al. [16].

2.2.1. Sucrose gradient preparation

Sucrose gradients were prepared in 50-ml skirted centrifuge tubes by layering 22.5 ml of 10% (w/v) sucrose onto a base consisting of an equal quantity of 30% (w/v) sucrose. Tubes were inclined at 4°C for 48 h to produce 45 ml linear 10–30% gradients.

2.2.2. Virgin and non-virgin cell isolation

YPD was inoculated with a single colony of BB11 and incubated in an orbital shaker at 25°C for 72 h. Cells were maintained at 4°C during age synchronisation. The resulting culture was sonicated at maximum power in a Camlab sonicating waterbath followed by gentle agitation for 30 s. This was repeated three times to ensure the resulting culture contained only discrete individuals. Cells were washed twice in 0.1 M phosphate buffered saline (PBS), pH 7.3, and cell number was determined using a haemocytometer. An optimum cell suspension of 5×10^8 cells ml^{-1} in PBS (4°C) was achieved by dilution and 1-ml aliquots layered onto the surface of sucrose gradients.

Gradients were immediately centrifuged in a swing-out rotor at 1300 rpm for 5 min in a refrigerated centrifuge (IEC Centra EC4R, Int. Equipment Co., UK) set at 4°C. This resulted in two layers of cells; a less-dense upper layer, containing virgin cells and a lower compacted region comprising non-virgin individuals. The top two-thirds of the upper cell band were recovered from each gradient using a pipette, pooled and pelleted. Subsequently the lower compacted region, comprising solely non-virgin individuals, was collected and isolated. Each cell pellet was washed twice and resuspended in PBS (4°C). The resulting populations were examined for age purity using confocal microscopy.

2.3. Age determination

The age of individuals within a population was determined by enumerating bud and birth scars on the cell surface according to the method of Powell et al. [17]: 500 μl of cells (5×10^7 cells ml^{-1}) were washed twice in PBS and resuspended in 500 μl of FITC (fluorescein isothiocyanate)-labelled wheat germ agglutinin (Sigma-Aldrich, Poole, UK), at a concentration of 1 mg ml^{-1}. Cells were gently agitated at room temperature for 15 min, harvested by centrifugation (13 000 rpm for 1 min) and washed three times in 0.1 M NaCl. Cells were resuspended in 250 μl of PBS with an equal quantity of Citifluor (Agar Scientific, Cambridge, UK). Cells were examined (×100 oil immersion lens) using an LSM 410 inverted laser scanning confocal microscope (Carl Zeiss, Germany) with a 488-nm argon ion laser for fluorescence imaging and transmission detector for DIC.

2.4. Fermentations

Fermentations were performed in glass hypovials according to the method of Quain et al. [18]. 150-ml hypovials (International Bottle Company, UK) containing a magnetic flea were autoclaved at 121°C and 15 psi for 15 min prior to use. Brewery wort (1060° gravity) was sterilised (121°C and 15 psi for 15 min) and 100-ml aliquots were added to each hypovial (mini fermentation vessel). Wort was centrifuged (4000 rpm for 10 min) to remove excess lipid compounds (trub) and other particulate matter prior to sterilisation. Hypovials were sealed using suba seals and metal crimp seals using a hand-held crimper. Manipulation of the fermentation environment was performed using sterile needles (6% luer, 0.6 mm×23 gauge) attached to autoclavable silicone tubing.

Anaerobic conditions were established by passing nitrogen through the fermenter headspace at a rate of 0.1 l min^{-1}. This was performed using a cut-off plugged Pasteur pipette and sterile needle. A gas outlet port was constructed using a sterile needle and Durham tube connected via a section of silicone tubing with a narrow cut in its structure. Hypovials were stirred constantly (300 rpm) at 8°C for 12 h prior to inoculation. Nitrogen gas was distributed using a manifold and the flow rate controlled using a flowmeter (Platon).

Yeast inoculation ('pitching') was performed using a needle attached to a 2-ml disposable syringe. Aerobically grown yeast was introduced to the fermentation media to provide a final concentration of 1.5×10^7 cells ml^{-1}. Hypovials were continuously de-aerated with nitrogen during pitching and for a period of 15 min thereafter to remove air inadvertently introduced into the headspace during pitching.

Mini fermenters were transferred to a constant temperature waterbath at 15°C. Homogeneity was ensured by gentle agitation using a flat bed 15-place immersible magnetic stirrer. Fermentation progression was monitored by measuring sugar utilisation in terms of weight loss over time [19,20].

2.5. Measurement of cell flocculation – Helm's assay

Flocculation was determined using a modified Helm's assay according to the method of Bendiak et al. [21] with adjustments as suggested by D'Hautcourt and Smart [22]. Flocculation is expressed as a function of the mean absorbance of the control assay ((control A−experimental A)/control A)×100%.

2.5.1. Cell preparation

Cells were washed in sterile deionised water and resuspended in sterile deionised water to achieve a final cell concentration of 1×10^8 cells ml^{-1}. Aliquots of yeast (1 ml) were resuspended in Eppendorf tubes. 15 replicates were prepared for each assay.

2.5.2. Experimental assay

Yeast samples (1×10^8 cells ml^{-1}, 10 replicates) were washed once in 'washing buffer' consisting of calcium sulphate (0.51 g l^{-1}). Samples were recovered by centrifugation (15 000 rpm for 1 min) and resuspended in suspension buffer (calcium sulphate 0.51 g l^{-1}, sodium acetate 6.8 g l^{-1}, glacial acetic acid 4.05 g l^{-1} and ethanol 4%). Each sample was agitated thoroughly for 30 s and subsequently inverted five times in 15 s. Experimental tubes were maintained statically at 25°C for 15 min in conjunction with the control tubes. Following this sedimentation period the top 0.1 ml of the sample was removed and added to 0.9 ml deionised water in a restricted cuvette. Absorbance was measured at 600 nm using a Shimadzu UV 120-02 spectrophotometer.

2.5.3. Control assay

Yeast samples containing 1×10^8 cells ml^{-1} (five replicates) were harvested by centrifugation (15 000 rpm for 1 min) and resuspended in 1 ml of 0.5 M ethylenediamine tetraacetic acid (EDTA), pH 7.0. Each sample was agitated thoroughly for 30 s and subsequently inverted five times in 15 s. Control tubes were maintained statically at 25°C for 15 min in conjunction with the experimental tubes. Following this sedimentation period the top 0.1 ml of the sample was removed and added to 0.9 ml deionised water in a restricted cuvette. Absorbance was measured at 600 nm.

2.6. Measurement of cell surface hydrophobicity

Cell hydrophobicity was determined using two methods: the solvent partition assay [23] and a modification of the hydrophobic latex microsphere attachment assay [24]. During experimentation all yeast and solutions were

maintained at 4°C. Wide-bore pipette tips were used for all flocculent cell cultures.

2.6.1. Solvent partition

Cells were diluted to a concentration of 5×10^6 cells ml^{-1} in sterile distilled water. A 10-ml sample of yeast in a glass universal bottle was washed once and resuspended in 10 ml of phosphate, urea, magnesium (PUM) buffer. PUM buffer consisted of (g l^{-1}) K_2HPO_4 (22.2), KH_2PO_4 (7.26), urea (1.8) and $MgSO_4{\cdot}7H_2O$ (0.2). The absorbance of the cell suspension (I) was determined spectrophotometrically. Aliquots of 2.4 ml yeast suspension (three replicates) were transferred to round bottomed test tubes. Xylene (0.2 ml) was added to each tube, samples were vortex-mixed for 30 s and left to stand for 15 min. The xylene layer from each tube was removed and discarded. The aqueous layer was sampled and absorbance determined at 660 nm (F). The modified hydrophobicity index (MHI) was defined as $1-(F/I)$, where a high value indicates a hydrophobic population.

2.6.2. Hydrophobic latex microsphere attachment assay

Yeast cells were washed and resuspended in PUM buffer (pH 7.1) to achieve a final cell concentration of 2×10^7 cells ml^{-1}. Yeast cells were mixed with fluorescent hydrophobic latex microspheres (0.833 μm diameter, Sigma, UK) at a ratio of approximately 10 beads $cell^{-1}$ (2×10^8 beads ml^{-1}). Samples were vortex-mixed for 30 s and examined using a Zeiss light microscope ($\times400$ magnification). The relative hydrophobicity of each colony forming unit (CFU) was determined by enumeration of bead attachment. 100 CFUs were examined per sample in order to determine the heterogeneity of hydrophobic expression within the population. The hydrophobic coupling capacity (HCC) of each sample is expressed as the percentage of CFUs exhibiting three or more microspheres.

2.7. Measurement of cell surface charge

Cell surface charge was determined using two methods: a modified version of the alcian blue dye retention technique [25] and the latex microsphere attachment assay [26]. All yeast and solutions were maintained at 4°C throughout experimentation. Wide-bore pipette tips were used for all flocculent cell cultures.

2.7.1. Alcian blue dye retention

A yeast suspension of 5×10^6 cells ml^{-1} was achieved by dilution in 0.2 M acetate buffer (pH 4.0). Acetate buffer (pH 4.0) consisted of 4.1% acetic acid solution (previously prepared at 11.55 ml l^{-1}, stored at 4°C prior to use) and 0.9% sodium acetate solution (previously prepared at 16.4 g l^{-1}, autoclaved at 121°C and 15 psi for 15 min and stored at 4°C prior to use). 10-ml aliquots (three replicates) of yeast suspension were washed three times in acetate buffer (pH 4.0), recovered by centrifugation (4000 rpm, 10 min) and resuspended in acetate buffer containing alcian blue (0.015 g l^{-1}). The suspension was incubated for 5 min at 25°C using an orbital shaker at 120 rpm. Samples were centrifuged (4000 rpm, 10 min) and the absorbance of the supernatant determined spectrophotometrically at 607 nm. The quantity of alcian blue absorbed was determined using a standard curve. The alcian blue standard curve was produced using known concentrations prepared from the original alcian blue/acetate buffer solution. Data were expressed as μg alcian blue per mg cell dry weight. Dry weight was determined in conjunction with dye retention. 10-ml aliquots (three replicates) of yeast suspension in pre-dried glass universal bottles were washed three times in acetate buffer (pH 4.0), recovered by centrifugation (4000 rpm, 10 min) and dried using a desiccator. Dry weights of each sample were determined after 72 h.

2.7.2. Amine modified latex microsphere retention assay

Yeast cells were washed in acetate buffer containing 2% EDTA solution (from 250 mM stock solution) and resuspended to achieve a final cell concentration of 2×10^7 cells ml^{-1}. Yeast cells were mixed with fluorescent amine modified latex beads (0.833 μm diameter (Sigma, UK)) at a ratio of approximately 10 beads $cell^{-1}$ (2×10^8 beads ml^{-1}). Samples were vortex-mixed for 30 s and examined as for the charged microsphere assay. The charge coupling capacity (CCC) of each sample is expressed as the percentage of CFUs exhibiting three or more microspheres.

3. Results and discussion

3.1. Age synchronisation of yeast

The yeast strain BB11 was age synchronised to produce cell fractions comprising solely virgin or non-virgin cells. Each fraction was analysed for age purity using FITC-wheat germ agglutinin (WGA) staining of chitin in order to determine the presence of bud and birth scars [17]. Virgin cells were identifiable due to the presence of birth scars on the cell surface (Fig. 2). The virgin population was estimated to consist of approximately 94% discrete virgin cells (results not shown). Non-virgin populations were estimated to comprise approximately 88% mother cells (results not shown). Non-virgin cells were observed to exhibit a number of bud scars according to the divisional age of the cell (Fig. 3).

3.2. Cell age influences the rate of sugar utilisation during fermentation

The capacity of age synchronised fractions of BB11 to ferment efficiently was determined by monitoring small scale laboratory fermentations [18]. Populations of virgin, mixed and non-virgin cells were pitched into small scale

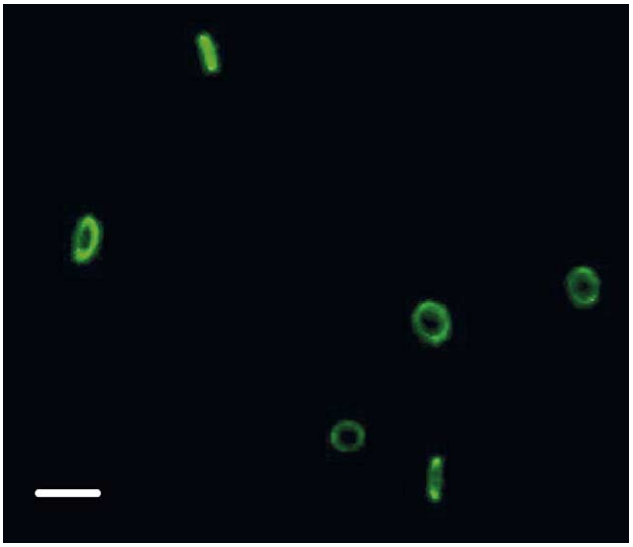

Fig. 2. Confocal micrograph illustrating a virgin cell population. Each cell exhibits a single birth scar. The size bar represents 5 μm.

laboratory fermenters containing brewery wort at 1060° gravity.

Each yeast culture was observed to produce a distinctive fermentation profile (Fig. 4), indicating that yeast cultures enriched with old or young cells perform in a different manner during fermentation. Analysis of the initial stages of fermentation indicated that virgin cells were slower to begin utilising sugars when compared to mixed aged cultures (Fig. 4). This is indicated by the extended lag phase prior to the maximum rate of sugar utilisation. In addition, yeast comprising purely non-virgin cells was observed to begin fermenting earlier than virgin or heterogeneously aged cultures (Fig. 4). It is suggested that the elongated lag phase observed during the initial stages of fermentation reflects the extended period that virgin cells spend in G_1 prior to proliferation. *S. cerevisiae* cells must meet certain requirements prior to passing START during the cell cycle, one of which is attaining a critical size [27,28]. Virgin cells do not meet these requirements initially and in order to achieve a specific size they must assimilate nutrients and convert them into biomass, which is time consuming. In addition, it has been demonstrated that the speed at which cells exit from stationary phase is influenced by the severity of the stress period previously encountered [29]. It has been established that virgin cells do not recover from cold shock as rapidly as older individuals [30] and therefore it is proposed that an energy trade-off between growth and cell repair mechanisms may occur according to the condition of the cell.

Given that a typical laboratory grown batch culture comprises approximately 50% virgin cells, 25% first-generation cells and 12.5% second-generation cells, it is suggested that the presence of virgin cells within the heterogeneously aged culture may result in a similar, albeit less conspicuous, delay at the onset fermentation. In contrast, the majority of cells within the non-virgin population satisfy requirements for START more rapidly and therefore are able to divide quickly, reducing fermentation lag time and subsequently final attenuation time (Fig. 4, Table 1). The increased fermentation time observed for virgin individuals is of particular significance to the brewing industry as an extended fermentation time has a direct impact on plant efficiency, with subsequent financial implications.

Virgin cells also fermented at a reduced rate during the middle stages of fermentation compared to non-virgin and mixed aged cultures, although there was no significant difference between the rate of fermentation for non-virgin and mixed age cell cultures. The explanation for the reduction in fermentation rate is unknown, however it has been demonstrated in haploid laboratory strains of *S. cerevisiae* that gene expression alters with age [4]. It has also been shown that the expression of genes varies during the first 24 h following pitching [31] and is anticipated to alter throughout fermentation. It has been suggested that variable sugar uptake rate may arise as a consequence of differential expression of genes required to produce sugar transport enzymes, for example the production of sugar permeases involved in maltose transport [32]. Alternatively virgin cell cultures may simply be less efficient at utilising sugars due to their small biomass. In contrast, there was no significant difference between the rate of fermentation in heterogeneous and non-virgin cultures. This may be explained by the age distribution of each population at the end of fermentation. During a typical brewery fermentation, a yeast culture will divide approximately two to three times. Theoretically, differences between the age distribution of mixed age and non-virgin populations should therefore remain at the end of fermentation. However, although this would be correct on the assumption that each individual within the population will divide at the same time and rate, in reality growth is not synchronous.

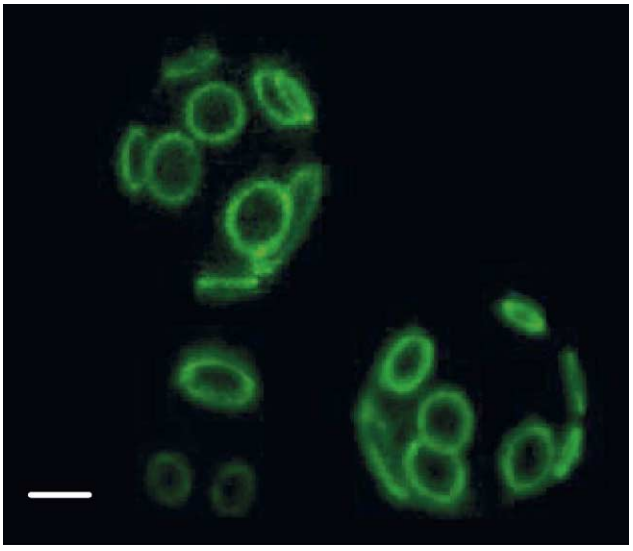

Fig. 3. Confocal micrograph illustrating aged individuals. Each individual exhibits a number of bud scars according to its divisional age. The size bar represents 3 μm.

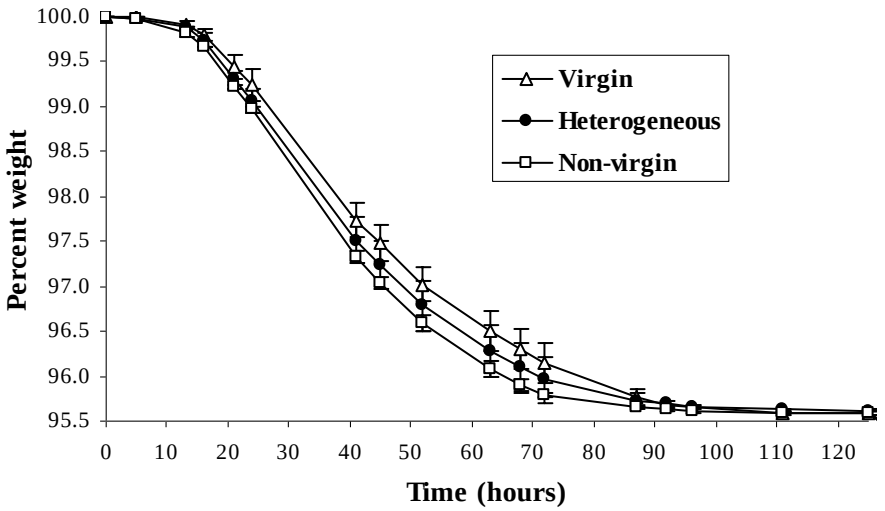

Fig. 4. The impact of yeast cell age on attenuation. Fermentations were performed in wort (1060°). Fermentation results represent the mean of four replicates. Fermentation vessel weight is expressed as a percentage where 100% indicates the weight of the original wort sample after pitching. The standard deviation is represented.

It is suggested that asynchronous division will cause the age dynamics of the population to develop into a more conventional profile and resemble a heterogeneously aged population to a greater extent than theoretically predicted. This would not be possible for a virgin population, with the maximum attainable divisional age being approximately three divisions, due primarily to the absence of aged individuals in the original population.

Despite variation in sugar uptake rate, the final gravity of beer produced with cells of different age was not observed to be significantly different. This indicates that each population of BB11 cells was equally capable of utilising fermentable wort sugars, suggesting that variation in uptake rate is purely an artefact of cell age rather than a vitality induced defect.

3.3. The impact of age on cell flocculation

Yeast flocculation provides a natural mechanism for the removal of yeast from beer at the end of fermentation [33]. The flocculation potential of a cell is governed by the properties of the cell wall, which in turn is genetically determined [33–36] and influenced by environmental factors [37–39]. Given that cell wall composition is a major determinant in flocculation [14,40] and that aged individuals exhibit variation in cell wall composition [41], it has been suggested that the flocculation potential of a cell may alter throughout the lifespan. In addition, Barker and Smart [42] have proposed that increasing cell size may lead to older cells acting as nucleation points for floc formation, although no study has been performed to substantiate these hypotheses.

Analysis of the flocculation characteristics of each yeast fraction indicated that aged individuals were more efficient at flocculating than younger counterparts (Table 2), irrespective of their brewing classification (ale/lager). BB11 virgin cells were observed to be 46% flocculent, which correlates to a 39% reduction in flocculation, compared to the control (heterogeneously aged) group. Populations of non-virgin BB11 cells were observed to exhibit an increased flocculation potential of 87% which equates to a 34% increase in flocculation. A similar pattern was detected for the FLO1 ale strain, BB1, and the NEWFLO lager strain, BB28 (Table 2), indicating that this is a phenomenon which is common amongst brewing yeast, irrespective of the genetic determinants of flocculation. It is suggested that virgin cells may be less flocculent due to their cell surface physiology. Virgin cells possess extremely smooth cell surfaces with very few protruding structural components [42]. It is well known that a rough cell surface topography favours cell to cell adhesion during the onset of flocculation [26], and it would be expected that discrete older wrinkled cells would be more flocculent than discrete younger cells.

Table 1
Summary of the effect of yeast cell age on sugar utilisation

Yeast fraction	Time to attenuation (h)	Final gravity
Virgin	111	1008.0
Mixed aged	92	1008.1
Non-virgin	87	1007.8

Table 2
The impact of yeast replicative cell age on flocculation potential

Strain	Virgins	Heterogeneous	Non-virgins
BB11	46.3 ± 8.5	64.7 ± 7.2	86.7 ± 3.7
BB28	24.5 ± 10.5	35.0 ± 11.0	51.3 ± 11.6
BB1	45.5 ± 8.6	59.3 ± 8.3	68.3 ± 4.2

Results indicate percent flocculation, where 100% denotes complete flocculation ± standard deviation. Flocculation was estimated using a minimum of five control samples and 10 replicates for experimental samples.

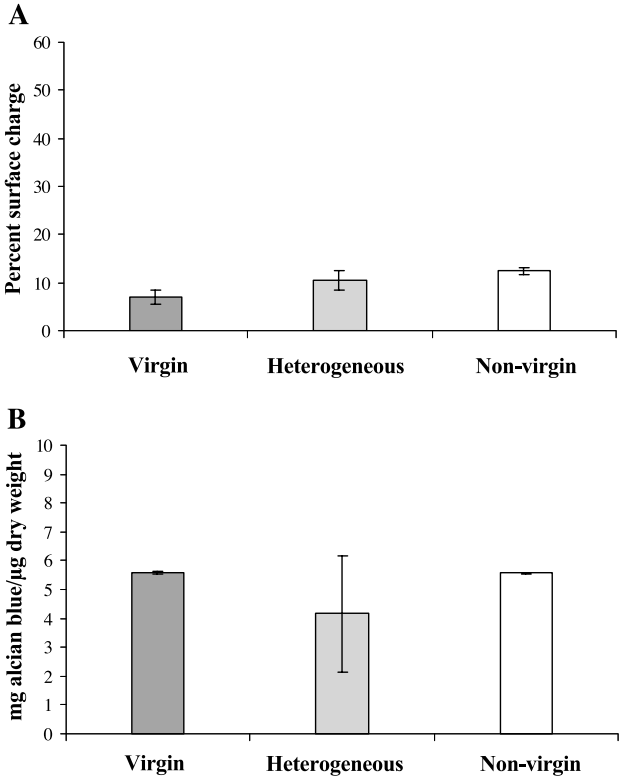

Fig. 5. A, B: The impact of yeast replicative age on cell surface charge. Surface charge was determined using charged microsphere attachment (A) and alcian blue dye retention (B). Charged bead assays were quantified by analysis of 100 individuals selected at random from each sample. Charge coupling capacity is expressed as the percentage of colony forming units exhibiting three or more microspheres.

Bony et al. [43] have demonstrated that for haploid yeast strains exhibiting FLO1 flocculation, the flocculation protein was localised at the site of bud formation. This would suggest that older cells, with a greater number of budding sites, would express a greater number of flocculins, leading to increased flocculation. Although this hypothesis would explain the results observed, analysis of the distribution of flocculins in polyploid NEWFLO brewing strains, has revealed that the flocculin is not restricted to the site of bud formation, but is present over the entire cell wall (Rhymes and Smart, unpublished data). It is suggested that a variation between young and old cells in expression of the genes responsible for the production of flocculation lectin may be the source of the difference in flocculation observed. It is proposed that the extent to which the flocculin is expressed, coupled with the cell surface morphology of aged individuals may lead to older cells acting as nucleation points for floc formation.

3.4. *The relationship between age and cell hydrophobicity and cell surface charge*

Yeast flocculation is affected by many intrinsic and extrinsic factors. The cell wall in particular is known to play a significant role in determining the flocculation capacity of a cell. Cell surface charge (SC) [44] and hydrophobicity (SH) [32] are known to influence the flocculation capacity of a strain. Age synchronised populations of BB11 cells were prepared using sucrose gradients and analysed for surface charge and cell hydrophobicity.

Cell surface charge was observed to remain constant irrespective of the divisional ages of each population. The charged microsphere attachment assay indicated a charge coupling capacity of 7% in virgin cells, 11% in mixed age cells and 13% in non-virgin cells (Fig. 5A). The alcian blue dye retention test confirmed these results, with each population absorbing a similar amount of alcian blue (Fig. 5B).

Although surface charge remained constant irrespective of cell age, cell surface hydrophobicity was observed to vary between each fraction. The hydrophobic microsphere attachment assay indicated a population of virgin cells to have a hydrophobic coupling capacity of 9% (Fig. 6A). Heterogeneous populations were 32% and non-virgins were 34% hydrophobic (Fig. 6A). In addition, analysis of cell surface hydrophobicity using the solvent partition assay indicated a hydrophobic index of 0.16, 0.51 and 0.52 for virgin, heterogeneous and non-virgin cells, respectively (Fig. 6B), demonstrating that virgin cells are significantly

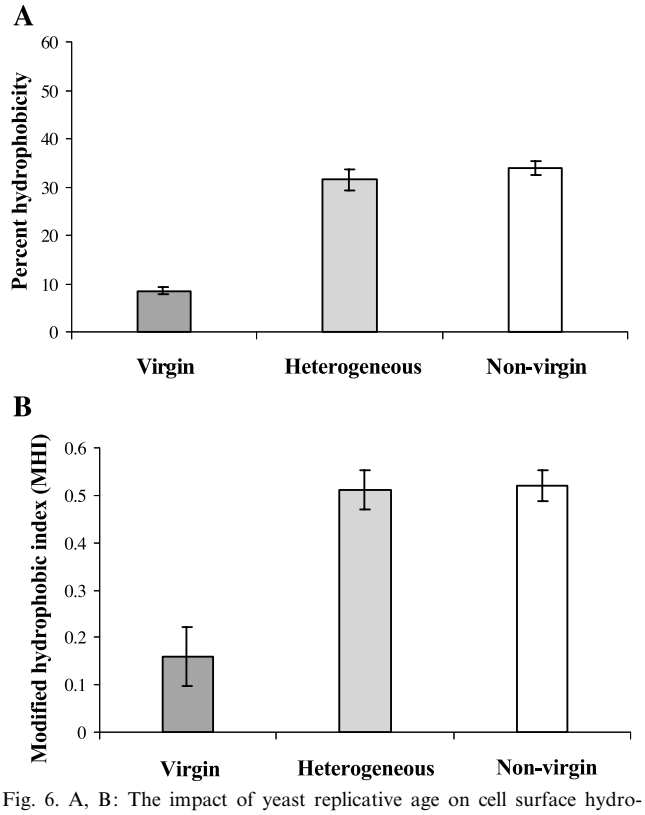

Fig. 6. A, B: The impact of yeast replicative age on cell surface hydrophobicity. Hydrophobicity was determined using latex microsphere attachment (A) and the solvent partition assay (B). Hydrophobic bead assays were quantified by analysis of 100 individuals selected at random from each sample. Hydrophobic coupling capacity is expressed as the percentage of colony forming units exhibiting three or more microspheres.

less hydrophobic than older counterparts. In consequence, it is suggested that the hydrophobic properties of the cell wall are an important factor in determining flocculation potential in BB11, supporting observations in other brewing strains [26,32,45,46]. The rationale for this observation is unknown, however it has been demonstrated that cell surface hydrophobicity is influenced by the ratio of nitrogen-rich to phosphorus-rich cell wall peptides [47]. In addition, the N terminus of the FLO1 lectin has been identified to contain highly hydrophobic regions and as thus may promote localised van-der-Waals interactions between adjacent call walls as a prelude to cell aggregation.

Given that the composition of the cell wall changes with age [41], it is suggested that newly produced virgin cells may not exhibit conformationally mature cell walls and that the presence of hydrophobic surface structures, such as flocculins and hydrophobic peptides, may increase with age. However, despite the observed relationship between hydrophobicity and flocculation, it is acknowledged that the observed difference in flocculation between heterogeneous and non-virgin cells may also be accounted for by alternative factors, such as cell wall surface topography, the relative exposure of mannoprotein fibrils, the presence of bud scars and cell wrinkling. It is also suggested that variable expression of genes involved in producing the NEWFLO phenotype, such as Lg-FLO1 [48], between young and old cells may be the cause of the difference in flocculation observed. Moreover, it is postulated that the period required for lectin maturation [49] may be longer in virgin cells due to their juvenile state. However, the effect of age on the sedimentation time required for maximum flocculation was not determined. Therefore it is not known whether aged cells flocculate more quickly than younger cells or if they flocculate at the same time albeit to a greater extent. It is suggested that within a full scale fermentation vessel aged individuals displaying an enhanced flocculation potential may sediment early, causing cell numbers in suspension to decrease and reducing the efficiency during the final stages of fermentation in terms of speed and attenuation. Alternatively, selection for a population comprising a large proportion of younger cells during serial repitching may result in slow beer clarification, yeasty off-flavours and filtration issues as a result of weak flocculation.

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References

[1] Jazwinski, S.M. (1990) An experimental system for the molecular analysis of the aging process: The budding yeast *Saccharomyces cerevisiae*. J. Gerontol. 45, B68–B74.
[2] Bartholomew, J.W. and Mittwer, T. (1953) Demonstration of yeast bud scars with the electron microscope. J. Bacteriol. 65, 272–275.
[3] Mortimer, R.K. and Johnston, J.R. (1959) Life span of individual yeast cells. Nature 183, 1751–1752.
[4] Motizuki, M. and Tsurugi, K. (1992) The effect of aging on protein synthesis in the yeast *Saccharomyces cerevisiae*. Mech. Age Dev. 64, 235–245.
[5] Egilmez, N.K., Chen, J.B. and Jazwinski, S.M. (1989) Specific alterations in transcript prevalence during the yeast life-span. J. Biol. Chem. 264, 14312–14317.
[6] Deans, K., Pinder, A., Catley, B.J. and Hodgson, J.A. (1997) Effects of cone cropping and serial repitch on the distribution of cell ages in brewery yeast. Proc. Eur. Brew. Congr. 26, 469–476.
[7] Powell, C.D., Quain, D.E. and Smart, K.A. The impact of yeast sedimentation on the distribution of cells within the cone of a cylindroconical vessel. Proc. IGB Asia Pac. Sect., in press.
[8] Powell, C.D., Van Zandycke, S.M., Quain, D.E. and Smart, K.A. (2000) Replicative ageing and senescence in *Saccharomyces cerevisiae* and the impact on brewing fermentations. Microbiology 146, 1023–1034.
[9] Loveridge, D., Ruddlesden, J.D., Noble, C.S. and Quain, D.E. (1999) Improvements in brewery fermentation performance by 'early' yeast cropping and reduced yeast storage time. In: Proc. 7th Inst. Brew. Congr. Africa Sect., pp. 95–99.
[10] Quain, D.E., Powell, C.D., Hamilton, A., Ruddlesden, J.D. and Box, W.G. (2001) Why warm cropping of yeast is best! Proc. Eur. Brew. Congr. 28, 388–396.
[11] Jenkins, C., Kennedy, A., Thurston, P., Hodgson, J. and Smart, K. Yeast handling and brewing cell wall integrity. In: Proc. IGB Asia Pac. Sect., in press.
[12] Jenkins, C., Kennedy, A., Thurston, P., Hodgson, J. and Smart, K. (2001) Impact of wort composition and serial repitching on lager brewing yeast fermentation performance and organelle integrity. Proc. Eur. Brew. Congr. 28, 378–387.
[13] Smart, K.A. and Whisker, S.W. (1996) Effect of serial repitching on the fermentation properties and condition of brewing yeast. J. Am. Soc. Brew. Chem. 54, 41–44.
[14] Teixeira, J.M., Teixeira, J.A., Mota, M., Manuela, M., Guerra, B., Machado Cruz, J.M. and Almeida, A.M. (1991) The influence of the cell wall composition of a brewers flocculent lager yeast on sedimentation during successive industrial fermentations. Proc. Eur. Brew. Congr. 23, 241–248.
[15] Mitchison, J.M. and Vincent, W.S. (1965) Separation of synchronous cell cultures by sedimentation. Nature 205, 987–989.
[16] Egilmez, N.K., Chen, J.B. and Jazwinski, S.M. (1990) Preparation and partial characterisation of old yeast cells. J. Gerontol. 45, B9–B17.
[17] Powell, C.D., Quain, D.E. and Smart, K.A. Chitin scar breaks in aged *Saccharomyces cerevisiae*. submitted.
[18] Quain, D.E., Box, W.G. and Walton, E.F. (1985) An inexpensive and simple small-scale laboratory fermenter. Lab. Pract. 34, 84.
[19] Äyräpää, T. (1973) Monitoring of fermentation by weighing of the vessel. J. Inst. Brew. 79, 274.
[20] Jacobsen, T., Hage, T. and Lie, S. (1982) A fermentation assay for wort element availability. J. Inst. Brew. 88, 387–389.
[21] Bendiak, D.S. (1995) Quantification of the Helm's flocculation test. J. Am. Soc. Brew. Chem. 52, 120–1222.
[22] D'Hautcourt, O. and Smart, K.A. (1999) The measurement of brewing yeast flocculation. J. Am. Soc. Brew. Chem. 57, 123–128.
[23] Hinchcliffe, E., Box, W.G., Walton, E.F. and Appleby, M. (1985) The influences of cell wall hydrophobicity on the top fermenting

properties of brewing yeast. Proc. Eur. Brew. Congr. 20, 323–330.
[24] Hazen, K.C. and Hazen, B.W. (1987) A polystyrene microsphere assay for detecting surface hydrophobicity variations within *Candida albicans* populations. J. Microbiol. Methods 6, 289–299.
[25] Rapoport, A. and Becker, M. (1985) Changes in the surface charge of yeast cells during their dehydration and rehydration. Mikrobiologiya 54, 450–453.
[26] Rhymes, M.R. and Smart, K.A. (2000) The relationship between flocculation and cell surface physical properties in a FLO1 ale yeast. In: Brewing Yeast Fermentation Performance (Smart, K.A., Ed.), pp. 152–159. Blackwell Science, Oxford.
[27] Lorincz, A. and Carter, B.L.A. (1979) Control of cell size at bud initiation in *Saccharomyces cerevisiae*. J. Gen. Microbiol. 113, 287–295.
[28] Pringle, J.R. and Hartwell, L.H. (1981) The Saccharomyces cell cycle. In: The Molecular Biology of the Yeast Saccharomyces (Strathern, J., Jones, E.W. and Broach, J.R., Eds.), pp. 97–142. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[29] Werner-Washbourne, M., Braun, E., Johnston, G.C. and Singer, R.A. (1993) Stationary phase in the yeast *Saccharomyces cerevisiae*. Microbiol. Rev. 57, 383–401.
[30] Smart, K.A. (2001) Biomarkers of yeast condition – predicting fitness to ferment. In: Proc. 8th Inst. Brew. Africa Sect. Congr., pp. 138–149.
[31] Higgins, V.J., Oliver, A.D., Day, R.E., Dawes, I.W. and Rogers, P.J. (2001) Application of genome-wide transcriptional analysis to identify genetic markers used in industrial fermentations. Proc. Eur. Brew. Congr. 28, 495–504.
[32] Cohen, J.D., Goldenthal, M.J., Chow, T., Buchferer, B. and Marmer, J. (1985) Organisation of the MAL loci of Saccharomyces. Physical identification and functional characterisation of three genes at the MAL6 locus. Mol. Gen. Genet. 200 (1), 1–8.
[33] Gilliland, R. (1951) The flocculation characteristics of brewing yeast during fermentation. Proc. Eur. Brew. Congr. 3, 35–57.
[34] Kobayashi, O., Hayashi, N. and Sone, H. (1995) The Flo 1 genes determine two flocculation phenotypes distinguished by sugar inhibition. Proc. Eur. Brew. Congr. 25, 361–368.
[35] Miki, B.L.A., Hung Poon, N., James, A. and Seligy, V.L. (1982) Possible mechanisms for flocculation interactions governed by gene FLO1 in *Saccharomyces cerevisiae*. J. Bacteriol. 150, 878–889.
[36] Stewart, G.G. and Russell, I. (1977) The identification, characterization and mapping of a gene for flocculation in *Saccharomyces* sp. Can. J. Microbiol. 23, 441–447.
[37] Eddy, A.A. (1955) Flocculation characteristics of yeasts II. Sugars as dispersing agents. J. Inst. Brew. 61, 313–317.
[38] Stratford, M. (1989) Yeast flocculation: Calcium specificity. Yeast 5, 487–496.
[39] Stratford, M. (1996) Induction of flocculation in brewing yeasts by change in pH value. FEMS Microbiol. Lett. 136, 13–18.
[40] Day, A.W., Poon, N.H. and Stewart, G.G. (1975) Fungal fimbriae III. The effect on flocculation in *Saccharomyces cerevisiae*. Can. J. Microbiol. 12, 558–564.
[41] Lyons, T.P. and Hough, J.S. (1970) The role of yeast cell walls in brewing. In: Brew. Digest. Aug., pp. 52–60.
[42] Barker, M.G. and Smart, K.A. (1996) Morphological changes associated with the cellular ageing of a brewing yeast strain. J. Am. Soc. Brew. Chem. 54, 121–126.
[43] Bony, M., Barre, P. and Blondin, B. (1998) Distribution of the flocculation protein, Flop, at the cell surface during yeast growth: the availability of Flop determines the flocculation level. Yeast 14, 25–35.
[44] Mestdagh, M., Rouxhet, P. and Dufour, J. (1990) Surface chemistry and flocculation of brewing yeast. Fermentation 3, 31–37.
[45] Jin, Y.-L. and Speers, R.A. (2001) Effect of cell surface hydrophobicity, charge and zymolectin density on the flocculation of *Saccharomyces cerevisiae*. J. Am. Soc. Brew. Chem. 59, 1–9.
[46] Smit, G., Straver, M., Lugtenberg, B. and Kijne, J. (1992) Flocculence of *S. cerevisiae* cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. Appl. Environ. Microbiol. 58, 3709–3714.
[47] Hazen, K.C. and Hazen, B.W. (1993) Surface hydrophobic and hydrophilic protein alterations in *Candida albicans*. FEMS Microbiol. Lett. 107, 83–88.
[48] Kobayashi, O., Hayashi, N., Kuroki, R. and Sone, H. (1998) Region of Flo1 proteins responsible for sugar recognition. J. Bacteriol. 180, 6503–6510.
[49] Stratford, M. and Assinder, S. (1991) Yeast flocculation: FLO1 and NewFlo phenotypes and receptor structure. Yeast 7, 559–574.