

1 **Physiological and transcriptomic response of *Saccharomyces pastorianus* to cold storage**

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9 **Abstract**

10 Removal of yeast biomass at the end of fermentation, followed by a period of storage before re-inoculation into
11 a subsequent fermentation, is common in the brewing industry. Storage is typically conducted at cold
12 temperatures to preserve yeast quality, a practice which has unfavourable cost and environmental implications.
13 To determine the potential for alleviating these effects, the transcriptomic and physiological response of
14 *Saccharomyces pastorianus* strain W34/70 to standard (4°C) and elevated (10°C) storage temperatures was
15 explored. Higher temperatures resulted in increased expression of genes associated with the production and
16 mobilisation of intracellular glycogen, trehalose, glycerol and fatty acids, although these observations were
17 limited to early stages of storage. Intracellular trehalose and glycerol concentrations were higher at 4°C than at
18 10°C, as a consequence of the cellular response to cold stress. However, significant changes in glycogen
19 degradation or cellular fatty acid composition did not occur between the two sets of populations, ensuring that
20 cell viability remained consistent. It is anticipated that this data may lead to changes in standard practice for
21 handling yeast cultures, without compromising yeast quality. This work has significance not only for the
22 brewing industry, but also for food and biofuel sectors requiring short term storage of liquid yeast.

23

24 **Keywords** – Brewing, cold shock, lager yeast, membrane fluidity, repitching, yeast stress

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26

27 **Introduction**

28 The practice of serial repitching, whereby yeast biomass is recovered from a fermentation (cropped) and used to
29 inoculate a subsequent fermentation (repitched) is common practise in the brewing industry. This cycle is
30 typically repeated 5-10 times, although in some instances extended repitching can be performed with some
31 cultures used indefinitely. The extent to which a yeast culture is repitched depends largely on company policy as
32 well as the health and fermentation potential of the yeast culture (McCaig and Bendiak 1985b). However, in
33 recent years the trend has been to reduce the number of repitchings, predominantly due to a drive for quality in
34 conjunction with intensification of the fermentation process.

35 Cropped yeast is often required to be stored between successive fermentations, providing operational flexibility.
36 Brewing yeast biomass is typically stored cold, either in the form of a slurry comprising a suspension of yeast in
37 beer taken directly from the fermenter, or as a 'pressed cake' (Quain and Tubb 1982; Boulton and Quain 2001).
38 Storage in cake form is usually associated with breweries employing traditional top-cropped fermentations and,
39 despite economic benefits, has been suggested to exert an adverse effect upon yeast quality as a consequence of
40 increased contamination risks due primarily to exposure to atmospheric air. Difficulty in controlling temperature
41 and variation in population heterogeneity due to desiccation or localised metabolic activity within the cake are
42 additional risks (Boulton and Quain, 2001). As a result the most accepted practice is to store cropped yeast in
43 the form of a slurry in an enclosed vessel for a defined period of time (normally for less than 48 hr). Yeast slurry
44 is typically held at between 0-4°C in anaerobic conditions under pressure. Periodic agitation is also applied to
45 homogenise the culture and assure an even temperature across the population prior to repitching (Briggs *et al.*
46 2004).

47 One of the primary aims of storage is to maintain strain integrity while preventing contamination or
48 deterioration of the physiological state of the cropped yeast (McCaig and Bendiak 1985a; Lodolo *et al.* 2008).
49 This is particularly important since changes to yeast physiology during serial repitching constitute a major
50 source of variation to fermentation performance (Sall *et al.* 1988). Brewing yeast quality during storage is
51 influenced by a number of factors including duration, rate of agitation, presence of air, nutrient availability, and
52 temperature (McCaig and Bendiak 1985a; McCaig and Bendiak 1985b). Of these, temperature arguably plays
53 the most important role since it has a direct influence on the metabolic activity of the yeast (Murray 1984;

54 McCaig and Bendiak 1985a). Current standard practice is to store yeast within the recommended range of 0-4°C
55 to reduce metabolic activity and preserve viability and vitality. Higher storage temperatures are believed to be
56 detrimental as, under such conditions, intracellular carbohydrate reserves (primarily glycogen) are broken down,
57 forcing the yeast population to degrade vital cellular components potentially resulting in autolysis and cell death
58 (Quain and Tubb 1982). Conversely, application of near-freezing storage temperatures can lead to considerable
59 cold stress thereby compromising transcriptional efficiency, impairing the microorganism's translational
60 capacity and perhaps more importantly reducing the plasma membrane's fluidity (Al-Fageeh and Smales 2006;
61 Aguilera *et al.* 2007; Gibson *et al.* 2007). The latter can adversely affect the functioning of membrane bound
62 enzymes, reduce diffusion rates and hinder the cross-membrane transport of proteins and metabolites (Ruis and
63 Schuller 1995; Al-Fageeh and Smales 2006). Indeed, thermal downshift has been shown to elicit considerable
64 regulation in *S. cerevisiae*'s global gene expression along with crucial adaptations at the level of cellular
65 physiology which mainly involves accumulation of trehalose, glycerol and changes in fatty acid distribution
66 (Lashkari *et al.* 1997; Sahara *et al.* 2002; Schade *et al.* 2004; Al-Fageeh and Smales 2006; Murata *et al.* 2006;
67 Aguilera *et al.* 2007).

68 Although it has previously been reported that storage of propagated brewing yeast slurry at 4 and 10°C does not
69 appear to elicit notable differences in cellular physiology (Garcia-Rios *et al.* 2017), the impact of storage at
70 these temperatures on subsequent fermentation performance is not well understood (Gibson *et al.* 2009).
71 Brewing yeast transcriptional changes occurring as a consequence of storage temperature have also not
72 previously been investigated. Transcriptomic analyses demonstrated by haploid model laboratory and industrial
73 yeast strains to different degrees of thermal downshift suggested time-dependent upregulation of genes
74 associated with biosynthesis of reserve cellular carbohydrates (glycogen and trehalose), rRNA and ribosomal
75 machinery, glycerol, intracellular fatty acids and cold shock proteins (Lashkari *et al.* 1997; Sahara *et al.* 2002;
76 Schade *et al.* 2004; Murata *et al.* 2006; Panadero *et al.* 2006; Garcia-Rios *et al.* 2017). Whilst these studies have
77 undoubtedly provided considerable insight into yeast behaviour at low temperatures, their direct relevance to
78 cold storage of lager brewing yeast *Saccharomyces pastorianus* is limited. This is due partly to the allopolyploid
79 lager genome, which comprises significant contribution from the cryotolerant *Saccharomyces eubayanus*
80 (Libkind *et al.* 2011; Peris *et al.* 2014). In addition, selective pressures imposed by low temperature

81 fermentations over many years (Dunn and Sherlock 2008; Gallone *et al.* 2016) and the concomitant presence of
82 additional environmental stresses such as nutrient limitation, anaerobiosis and plausible ethanol stress are also
83 likely to have resulted in physiological differences to previously studied strains (Gibson *et al.* 2007). In this
84 study we investigate transcriptomic changes within yeast populations in association with key physiological
85 characteristics during storage of serially repitched lager yeast slurries.

86 **Materials and methods**

87 **Yeast strain and wort composition**

88 *Saccharomyces pastorianus* (W34/70) received from Hefebank Weihenstephan GmbH (Germany) was utilized
89 for all experiments. Wort (hopped, sterile) was obtained from a commercial brewery by sampling from the
90 paraflow (plate heat exchanger) into a sterile transfer vessel immediately following boiling. Wort exhibited a
91 total extract content of 14.5°P (1056 g/cm³), comprising fermentable sugars [fructose – 2.7 g/L, glucose – 19
92 g/L, maltose - 99 g/L, maltotriose - 23 g/L] and free amino nitrogen (FAN) (217 mg/L).

93 **Yeast propagation & pre-storage fermentation**

94 Representative W34/70 colonies from YPD agar (1% yeast extract, 2% neutralized bacteriological peptone, 2%
95 dextrose and 2% agar; all in w/v) slopes were cultivated under aerobic conditions using incremental volumes of
96 sterile wort (20 mL, 100 mL, 250 mL, and 1 L) at 25°C for 24, 24, 24 and 48 hr respectively. Harvested cells
97 were inoculated into sterile, air-saturated wort (14°Plato) in triplicate bioreactors (Techfors-S, Infors HT,
98 Switzerland) at a rate of 1.8×10^7 cells / mL to represent pre-storage fermentations. Fermentations were
99 conducted at 10°C in the initial phases followed by a controlled rise to 13°C until attenuation was achieved. No
100 significant difference between cellular characteristics, such as cell density (1.36×10^8 cells/ml; CV < 5%), cell
101 viability (98%; CV < 1%) and cellular budding index, or wort characteristics, including final wort gravity
102 (2°Plato; CV < 1%), ethanol (6% alcohol by volume; CV < 5%) and pH (4.1; CV < 5%), could be observed
103 between triplicate fermentations indicating homogeneity between cultures used for subsequent storage. Pre-
104 storage fermentation profiles have been shown within supplementary data (Fig. S1).

105 **Brewing yeast ‘cropping’ and storage**

106 As round-bottomed Infors bioreactors were used to conduct fermentations, cone cropping was not permissible
107 (since no cone was present in the vessels). As a result, an in-house method was developed to promote yeast

108 sedimentation and slurry collection. Spent wort (including yeast) was collected by opening the valve at the
109 bottom of the fermenter into sterile externally-graduated reaction vessels (capacity 2.5 L). Following transfer,
110 vessels were stored in cooled incubators (Sanyo, Osaka, Japan) and maintained at 10°C for a further 24hr,
111 without stirring, to facilitate yeast sedimentation. The bulk of the supernatant was then removed under nitrogen
112 pressure leaving behind approximately 30% (w/v) of ‘cropped’ brewing yeast slurry. Cropped yeast slurries
113 from 3 bioreactors were aseptically pooled to maintain homogeneity, and equal aliquots (200 mL) were
114 transferred into Duran vessels using nitrogen top-pressure.

115 Cropped yeast was stored in Duran vessels (500 mL; Infors, HT, Switzerland) sealed with 2-port stainless steel
116 plates (Infors, HT, Switzerland) to maintain anaerobiosis. One of the ports was fitted with a sterile gas filter and
117 served as a gas inlet whilst the other served as a gas outlet and sampling port. Both ports were sealed during
118 storage to prevent air (oxygen) ingress into the vessel. Two sets of triplicate storage vessels were maintained in
119 static incubators at either 4°C or 10°C. Sampling of stored yeast slurry was performed aseptically whilst
120 flushing the headspace with nitrogen via the gas inlet port, using sterile, stainless steel syringe needles (10 inch,
121 18 gauge) (Sigma Aldrich, UK). Samples were taken at key time points as indicated in the relevant results
122 sections, selected to represent the different stages of yeast storage and considering 48 hr to be the typical
123 maximum storage time. Yeast slurry was washed once with sterile saline (0.8 % NaCl), flash frozen (using
124 liquid nitrogen) and stored at -20°C until required for analysis.

125 **Cell viability, concentration and budding index**

126 Cell concentration was determined using a haemocytometer. Methylene violet (0.01% in 2% w/v sodium citrate)
127 (3RAX, Sigma-Aldrich, UK) was utilized to determine cell viability according to the method of Smart *et al.*
128 (1999). A minimum of 400 cells were scored for each sample. Viability and budding index (representing the
129 fraction of budding cells in the population) were determined using a haemocytometer and expressed as a
130 percentage of the total population based on triplicate analyses.

131 **Microarray data acquisition and analysis**

132 RNA was extracted from yeast pellets using a RiboPure-Yeast Kit (Ambion, Applied biosystems, UK). Sample
133 preparation, hybridization to a yeast genome 2.0 array and scanning was performed according to the
134 manufacturer’s instructions (GeneChip Expression Analysis, Affymetrix). Following scanning, the GeneChip

135 command console (AGCC, Affymetrix Inc., USA) was used to generate non-scaled RNA (.cel) files which were
136 analysed using GeneSpring GX 11 (Agilent Technologies, USA) software and pre-normalized as a single
137 experimental group using the Robust Multichip Average (RMA) algorithm, as described previously (Gibson *et al.*
138 *et al.* 2008; Gibson *et al.* 2009). Probe selection was performed using the Xspecies (cross species) hybridization
139 approach through probe masking files generated using a parser script written in Perl (Gibson *et al.* 2008; Gibson
140 *et al.* 2009). Differential expression was deemed to have occurred when, compared to the start of cold storage,
141 the transcript levels changed by more than 1.3 fold at significance of $p < 0.05$ (assessed by one way ANOVA
142 with Benjamini-Hochberg (BH) false discovery rate (FDR) correction).

143 **Intracellular glycogen, trehalose & glycerol**

144 Intracellular glycogen and trehalose in recovered yeast cells were determined using a method based on that
145 described by Parrou and Francois (1997), as detailed previously (Garcia-Rios *et al.* 2017). Yeast slurry was
146 diluted to 1×10^9 cells/mL and glycogen and trehalose were quantified separately based on specific enzymatic
147 digestion to glucose using a D-glucose assay kit (GOPOD, Megazyme). Analysis was conducted in triplicate
148 and results expressed as mg glucose / g slurry dry weight (SDW).

149 Intracellular glycerol determination was conducted as reported previously (Wojda *et al.* 2003). Final glycerol
150 content was estimated using a commercial kit (Megazyme, Ireland) following the manufacturer's instructions.
151 Results were presented as μg glycerol / mg SDW over triplicate analyses.

152 **Intracellular fatty acids**

153 Intracellular fatty acid (FA) distribution was assessed in cell pellets containing 1×10^9 cells using GC-MS as
154 described previously (Garcia-Rios *et al.* 2017). Besides the major long-chain FAs (C14:0, C16:0, C18:0, C16:1
155 and C18:1), the profile of relatively less prevalent medium-chain FAs (C10:0 and C12:0) were also determined.

156 The membrane unsaturation index (UI) and percentage Medium Chain Fatty Acid (MCFA) distribution were
157 calculated as follows - $UI = \frac{\sum(C14:0 + C16:0 + C18:0)}{\sum(C16:1 + C18:1)}$

158 $MCFA = \frac{\sum(C10:0 + C12:0)}{\sum(C10:0 + C12:0)}$

159 **Statistical analysis**

160 Probability (p) values were generated following pairwise comparisons between means using unpaired Student's
161 T test (SPSS 16.0.0). $p < 0.05$ was deemed to be significant in each instance. Normal distribution for the

162 variables was established using the One-Sample Kolmogorov-Smirnov Test whilst group variance on the
163 dependent variable was tested using Levene's Test of Homogeneity of Variance.

164 **Results**

165 In order to investigate the relationship between storage temperature, yeast physiology and the cellular
166 transcriptome, yeast slurries were stored at standard (4°C) and elevated (10°C) temperatures for 72 hours. The
167 10°C upper limit was applied since this matched the lowest temperature encountered in fermentations conducted
168 prior to storage. Analysis over 72h was performed since this exceeds the 48h maximum storage time widely
169 regarded as exceeding that recommended for good brewing practise (Boulton and Quain 2001; Briggs *et al.*
170 2004). Besides the cellular transcriptome, expression of key genes were monitored through storage to provide an
171 indication of the relative response of yeast to temperature at the molecular level. In addition, yeast populations
172 were analysed for viability, budding index, carbohydrate reserves, glycerol and aspects of membrane fluidity.

173 **Cell viability and budding index**

174 During storage, cropped brewing yeast cell viability remained consistently above 95% irrespective of
175 temperature (Fig. 1). Budding index was also consistent through storage and remained lower than 3% for yeast
176 stored at 4°C (1.7 ± 0.13 to 2.3 ± 0.64 percent) and at 10°C (1.9 ± 0.33 to 2.3 ± 0.63 percent). This data
177 indicates a lack of active cell division during slurry storage, most likely due to a combination of thermal
178 downshift combined with oxygen and nutrient limitation. However, it also provides reassurance that yeast
179 populations were representative of brewery-stored cultures, with typically high viability accompanied by a
180 reduced divisional rate.

181 **Impact of higher storage temperature on cropped yeast global transcriptome**

182 For transcriptome comparison as a function of temperature, fermented slurry samples harvested after 0, 6, 24
183 and 48 hr of incubation at 4°C and 10°C were compared. The 6 hr time point was chosen to encompass any
184 immediate and rapid alterations in global gene expression following stressful environmental exposure, as
185 proposed by Gasch and co-workers (Gasch *et al.* 2000), whilst the latter sampling points represent the typical
186 storage durations adopted within the brewing industry. Transcript abundance in slurry stored at 10°C was
187 compared with that at 4°C treating them as distinct experiments.

188 None of the ORFs were differentially expressed at the 0 hr sampling point between the two populations
189 suggesting similar transcriptional status at the beginning of differential storage. 506 genes (8.8 % of the total
190 genome) were found to be differentially expressed after 6 hr, with increased transcript abundance in 174 entities
191 (3% of total) and reduced transcript numbers in 332 entities (5.8% of total). Only 3 entities were differentially
192 expressed between W34/70 populations after 24 hr, whilst no difference in global transcription was evident after
193 48 hr.

194 Genes demonstrating significant changes in transcript abundance after 6 hr of cold storage were annotated using
195 the MIPS FunCat scheme (Ruepp *et al.* 2004). Amongst the genes with higher transcript abundance, functions
196 associated with cellular metabolism, particularly vitamin biosynthesis and secondary metabolism, were
197 significantly over-represented (Table 1) suggesting higher energy requirement for yeast survival in the initial
198 stages of storage at 10°C. Almost 12% of ORFs with increased transcript abundance after 6 hr of incubation at
199 10°C were involved in stress responses, particularly cellular detoxification (see Table 1). Further MIPS analysis
200 of genes within the “cell rescue, defence and virulence” category revealed the presence of genes associated with
201 oxidative stress (*GRX1*, *PST2*, *FMP46*) and osmotic/salt stress (*MYO3*, *GCY1*, *RRD2*). Storage at the higher
202 temperature also seemed to cause induction of genes associated with protein modification, auto-proteolysis and
203 reorganisation of the mitochondrion (Table 1). Together these observations indicate the prevalence of increased
204 stress during the initial phases of slurry storage at 10°C.

205 ORFs which were repressed when the storage temperature was increased from 4 to 10°C comprised 41.5% of
206 the total, and included genes associated with cellular transcription, particularly the rRNA machineries
207 (processing, modification and synthesis) (Table 1). A large fraction of ORFs (15.9% of total) encoding proteins
208 known to bind to nucleic acids were also present in this group. This suggests reduced cellular efforts to overhaul
209 its transcriptional apparatus when stored at the higher temperature, an observation complemented by lower
210 transcript abundance of genes involved in nucleotide/nucleoside/nucleobase metabolism along with nucleo-
211 cytoplasmic transport of proteins and RNA (Table 1). Genes involved in the biogenesis of various cellular
212 compartments, including cytoskeletal proteins and nuclear membrane were also repressed following an increase
213 in storage temperature (Table 1).

214 Amongst the 3 differentially expressed genes after 24 hr of cold storage, *STE14* was induced following an
215 increment in the storage temperature whilst *YJL144W* and *HSP10* were repressed. Both *YJL144W* and *HSP10*
216 are stress-responsive genes; *YJL144W* is induced during osmotic stress and starvation (Gasch *et al.* 2000) whilst
217 *HSP10* encodes for a mitochondrial chaperonin involved in protein folding and sorting (Laroche *et al.* 2001).
218 None of the microarray entities demonstrated significant change in expression after 48 hr. As a result, no
219 conclusions could be drawn except that the transcriptional status of *S. pastorianus* W34/70 is seemingly
220 unaffected by an increase in storage temperature after 48 hr of anaerobic maintenance, at least under the
221 laboratory-scale conditions used in current study.

222 **Monitoring gene expression during cold storage of cropped lager brewing yeast slurry**

223 Genes known to be involved in the metabolism of glycogen, trehalose, glycerol and intracellular fatty acids (GO
224 classification obtained from *Saccharomyces* Genome Database, (Gallone *et al.* 2016)) were investigated after 0,
225 6, 24 and 48 hr of incubation at 4°C and 10°C.

226 **Expression of genes associated with reserve carbohydrate metabolism during storage at 4°C**

227 Of the 18 genes involved in the biosynthesis (GO:0005978) and catabolism (GO:0005980) of glycogen in *S.*
228 *cerevisiae*, 10 demonstrated a significant difference in transcript levels during 4°C-storage (Fig. 2a and 2b).
229 *GSY1* and *GSY2*, which encode glycogen synthases responsible for glycogen chain elongation, along with
230 *GLC3*, whose protein product catalyzes glycogen branching, were induced after 24 hr (Fig. 2a). Progressive
231 repression of *GLG2*, whose protein product (along with GLG1p) initiates glycogen formation, was observed
232 throughout 48 hr of cropped slurry storage at 4°C (Fig. 2a). *GLC8* encodes the regulatory subunit of type 1
233 serine/threonine protein phosphatase (PP1) whilst PIG1p tethers PP1 to the glycogen synthase GSY2p thereby
234 aiding the latter's activation for glycogen accumulation. Transcript levels for both *GLC8* and *PIG1* were lower
235 during the initial stages of 4°C-storage (Fig. 2b). Products of *PGM2* and *UGP1* catalyze the sequential
236 conversion of glucose-6-phosphate to glucose-1-phosphate and glucose-1-phosphate to UDP-glucose
237 respectively, thereby providing the necessary substrates for glycogen (and trehalose) synthesis (Daran *et al.*
238 1997). After initial down-regulation, *PGM2* was induced in a sustained manner until 48 hr whilst transcription
239 of *UGP1* remained largely unchanged after 24hr (Fig. 2b). Amongst genes associated with glycogen catabolism,

240 glycogen phosphorylase encoding *GPH1* demonstrated initial repression followed by sustained induction to
241 yield peak levels after 48 hr of 4°C-storage (Fig. 2b).
242 Amongst the 11 genes involved in trehalose biosynthesis (GO:0005992), catabolism (GO:0005993) and
243 transport (GO:0015771), expression of 8 genes (including *PGMI* and *UGPI* mentioned earlier) were
244 significantly altered during storage at 4°C. Similar to glycogen metabolism, most of the genes involved in
245 trehalose metabolism were suppressed during the first 6 hr of storage (Fig. 2c). Of the genes associated with
246 trehalose biosynthesis, *TPSI*, which encodes the synthase subunit of trehalose-6-phosphate/synthase complex,
247 was induced after 24 hr (Fig. 2c). Final *TPSI* transcript levels were 1.9 fold higher than those preceding storage.
248 Following initial down regulation, transcription of genes encoding the phosphatase (*TPS2*) and one of the two
249 structural subunits (*TSL1*) of the trehalose-6-phosphate synthase complex returned to levels comparable to the
250 start of cold storage (Fig. 2c). Cytosolic neutral trehalase encoding *NTH1* and *NTH2* were significantly induced
251 following 24 hr of W34/70 incubation at 4°C whilst transcript abundance of the vacuolar acid trehalase-encoded
252 by *ATH1* remained largely stable until 48 hr (Fig. 2c).

253 **Expression of genes associated with glycerol metabolism during storage at 4°C**

254 Transcript abundance of 8 (out of 12) genes involved in glycerol biosynthesis (GO:0006114), catabolism
255 (GO:0019563) and transport (GO:0015793) were significantly regulated during 4°C-storage. Amongst the genes
256 associated with glycerol biosynthesis, glycerol-3-phosphatase encoding *GPP1* and *GPP2* demonstrated marked
257 induction after 48 hr (Fig. 2d), whilst *YIG1*, whose protein product facilitates glycerol metabolism under
258 anaerobic conditions, was gradually repressed (Fig. 2d). Amongst the glycerol transporter genes, *STL1* was
259 heavily repressed whilst *FPS1*, associated with glycerol efflux, was marginally induced (Fig. 2d). All of the
260 differentially expressed genes associated with glycerol catabolism, namely *DAK1*, *GUT1* and *GUT2*, were
261 repressed during cropped W34/70 storage at 4°C (Fig. 2e).

262 **Expression of genes associated with yeast fatty acid biosynthesis and transport during storage at 4°C**

263 Five of the 8 genes associated with yeast MCFA biosynthesis (GO:0051792), UFA biosynthesis (GO:0006636),
264 UFA elongation (GO:0019368) and FA transport (GO:0015908 and GO:1902001) were significantly expressed.
265 The two genes, *EEB1* and *YMR210W*, part of a cohort of three genes involved in MCFA biosynthesis,
266 demonstrated sustained induction until 24 hr followed by a stabilization in expression (Fig. 2f). *ELO1*, which is

267 involved with the elongation of synthesized unsaturated fatty acid precursors, demonstrated peak expression in
268 the initial storage stages followed by repression (Fig. 2f). Amongst the FA transport genes, *PXA1*, which
269 encodes a subunit of the ABC transport complex for importing FAs into the peroxisomes for beta-oxidation,
270 showed maximal transcript abundance after 24 hr of 4°C-storage (Fig. 2f). *RSB1*, whose product is a putative
271 membrane transporter transferring long chain bases from the cytoplasm into the extracytoplasmic space showed
272 initial repression followed by increased transcript levels after 24 hr (Fig. 2f). Notably the $\Delta 9$ -desaturase
273 encoding *OLE1*, responsible for UFA biosynthesis, did not show any significant change in expression during
274 storage.

275 **Changes in gene expression when cropped lager yeast storage temperature was increased from 4°C to** 276 **10°C**

277 In addition to monitoring gene expression at the traditionally practiced temperature of 4°C, evaluation of the
278 activity of genes involved in yeast glycogen, trehalose, glycerol and fatty acid machinery was also conducted
279 during storage at 10°C. Specifically, transcript abundance at 10°C was compared to that at 4°C at each of the
280 four sampling points (0, 6, 24 and 48 hr). This comparison revealed that changes in transcript abundance were
281 confined to the initial stages of storage (6 hr), after which time no changes were observed (Table 2). Amongst
282 glycogen biosynthesis genes, *GLG2* and *GAC1*, which functions to regulate protein phosphatase I, were induced
283 during the first 6 hr (Table 2). None of the glycogen catabolism genes were influenced by the storage
284 temperature applied. This suggests partial remodelling of the W34/70 transcriptome to facilitate de-
285 phosphorylation of glycogen synthase (GSY2p) and hence glycogen accumulation (Francois and Parrou 2001;
286 Wu *et al.* 2001). Furthermore, increased storage temperature did not influence trehalose biosynthesis, catabolism
287 or transport genes. However, considerable repression of genes responsible for glycerol biosynthesis (*GPP1* and
288 *GPP2*) was observed, along with concomitant induction of ORFs associated with glycerol dissimilation (*GCY1*
289 and *GUT1*) (Table 2). Interestingly, the use of a higher storage temperature also resulted in lower transcript
290 abundance of the glycerol transporter-encoding *FPS1*. Increasing the storage temperature did not affect the
291 expression of the majority of the genes involved in FA metabolism with only *ELO1* demonstrating differential
292 expression (Table 2).

293 **Storage temperature-dependent changes in lager yeast physiology**

294 **Intracellular carbohydrate reserves**

295 To support the transcriptome analysis, brewing yeast slurries were analysed for cellular glycogen content over
296 72 hr of anaerobic storage at 10 and 4°C (Fig. 3a). Glycogen levels in yeast slurry stored at 4°C remained
297 statistically constant and was relatively higher when compared to yeast stored at 10°C. Irrespective of the
298 temperature deployed, glycogen breakdown occurred as a function of the duration of storage.

299 Intracellular levels of the disaccharide trehalose were largely independent of both time and temperature of
300 storage over the first 24 hr of cold incubation (Fig. 3b). After 36 hr, increased trehalose levels ($p < 0.05$) were
301 observed in brewing yeast stored at 4°C but not for that stored at 10°C. Following 72 hr, yeast obtained from
302 4°C storage had significantly ($p < 0.05$) higher trehalose compared to 10°C; final levels indicating a 14%
303 increase in trehalose content under traditional slurry storage regime as opposed to a 9% drop at the higher
304 storage temperature.

305 **Intracellular glycerol**

306 The glycerol content within W34/70 cell populations stored at 4°C remained constant until 48 hr of storage (Fig.
307 3c). After 72 hr these were higher when compared to samples taken at 0 hr (1.5 ± 0.1 compared to 2.1 ± 0.2 $\mu\text{g} /$
308 mg SDW), corresponding to the transcriptome data reported above. An increase in storage temperature to 10°C
309 resulted in higher intracellular glycerol after 12 hours of incubation followed by gradual dissimilation and return
310 to basal levels after 36 hr with no change thereafter. Intracellular glycerol content was slightly elevated after 72
311 hours of slurry storage at 4°C in comparison to W34/70 maintenance at 10°C.

312 **Storage temperature-dependent changes in membrane fluidity**

313 To obtain a holistic view of variations in individual long-chain (LCFA) and medium-chain (MCFA) fatty acids
314 and corresponding impact over plasma membrane fluidity, the yeast membrane unsaturation index (UI) and
315 percent MCFA content were calculated (Fig. 4). UI was largely unaffected by the storage temperature and a
316 slight decrease (albeit not significant) was observed throughout storage (Fig. 4a). Although the MCFA content
317 in 10°C stored slurry was lower than 4°C-stored populations after 24 hr of storage (Fig. 4b), no significant
318 difference could be observed between the two sets of yeast populations.

319 **Discussion**

320 Maintenance of yeast physiological condition between successive fermentations is important in dictating both
321 the consistency of subsequent fermentations and final beer quality. In this study, the impact of storage
322 temperature on gene expression and key performance indicators for yeast condition were assessed.

323 Brewing yeast cell viability, the most widely utilized indicator of yeast quality in the brewing industry, was
324 observed to be consistent independent of storage temperature. These observations partially support those of
325 McCaig and Bendiak (1985b) who reported cell viability maintenance following 2 days of yeast storage at 1, 4
326 and 10°C. However, these authors suggested that prolonged storage at 10°C yielded poor cell viability, a
327 hypothesis not supported in the current study where longer term yeast slurry storage (3 days) at 10°C did not
328 result in any decline in cell viability. The low budding index (< 2%) during cold incubation of cropped yeast
329 was comparable to earlier values reported by Miller *et al.* (2012).

330 More than 99% of the temperature-dependent changes in cropped yeast gene expression occurred in the early
331 phase of storage suggesting that major transcriptome events are limited to this stage. Induction of genes
332 associated with cellular energy was observed in cells stored at 10°C compared to those at 4°C. This is probably
333 due to the ability of lager brewing yeast to conserve metabolic function between 8 -12°C on account of its
334 hybrid nature, and likely a result of the *S. eubayanus* contribution to the genome (Sato *et al.* 2002; Dunn and
335 Sherlock 2008; Nakao *et al.* 2009; Libkind *et al.* 2011; Gallone *et al.* 2016). Analysis of ORFs related to
336 oxidative and osmotic stress indicated that increasing the storage temperature from 4 to 10°C resulted in an
337 enhanced yeast stress response during early storage. This is in contrast to previous reports linking an induction
338 of antioxidant and oxidative stress gene activity following temperature downshift to low temperatures (Huang *et*
339 *al.* 1998), and in near-freezing conditions (Aguilera *et al.* 2007). The reason for this phenomenon is not yet
340 known and warrants further investigation, but may also be inherent to the strains employed and the robust nature
341 of brewing strains at relatively cold temperatures. Alternatively, it is worth considering that the capacity of lager
342 strain W34/70 to survive at 10°C without adverse effects may be dependent on gene activity associated with
343 alleviating stress factors. It should be noted that extensive remodelling of yeast transcriptional and translational
344 machinery at near-freezing temperatures has been widely reported (Sahara *et al.* 2002; Schade *et al.* 2004;
345 Murata *et al.* 2006; Isasa *et al.* 2016; Garcia-Rios *et al.* 2017) and is predominantly driven by widespread
346 inactivation of cellular ribosomes, protein synthesis and increased stabilization of mRNA secondary structures

347 (Al-Fageeh and Smales 2006; Aguilera *et al.* 2007). Storage of yeast slurry at higher temperature could perhaps
348 reduce such requirements as evident by a reduction in cellular efforts towards refurbishing
349 transcriptional/translational apparatus at the higher temperature applied here.

350 A high glycogen content in cropped yeast is vital for maintaining cell viability during cold storage since it fuels
351 yeast basal metabolism in the absence of assimilable nutrients (Quain and Tubb 1982). Elevated storage
352 temperatures have been linked to an accelerating metabolism rate and cellular energy demand, resulting in
353 increased degradation of yeast glycogen (McCaig and Bendiak 1985a). However, under the conditions
354 employed in the current study, slurries stored at 10°C did not significantly exhibit an altered glycogen
355 mobilization rate. Furthermore, higher storage temperatures prompted an initial transcriptional response towards
356 glycogen accumulation, although this was not sustained during the later stages of storage. The expression of
357 glycogen-associated genes was largely supported by the physiological data obtained. Simultaneous induction of
358 genes associated with both glycogen accumulation and mobilization observed during cropped W34/70 slurry
359 storage at 4°C mimics the response of *S. cerevisiae* to cold shock (Sahara *et al.* 2002; Schade *et al.* 2004;
360 Murata *et al.* 2006), heat shock and oxidative stress (Parrou *et al.* 1997). In contrast to the proposed recycling of
361 glycogen in response to heat and oxidative stresses (Parrou *et al.* 1997), Schade *et al.* (2004) reported
362 accumulation of this carbohydrate following thermal downshift. This was not observed in the current study,
363 albeit conducted using a different yeast species, gaseous environment and nutritional availability. It is therefore
364 proposed that under brewery storage conditions, glycogen accumulation may not be an absolute prerequisite for
365 cold shock adaptation. In support of this hypothesis, an absence of glycogen accumulation in *S. cerevisiae*
366 cultures maintained in a chemostat at low temperatures and under nutrient limiting conditions has been
367 previously observed. (Tai *et al.* 2007).

368 Trehalose is a well-recognised cellular protectant which is known to accumulate in response to numerous yeast
369 stress factors (Odumeru *et al.* 1993; Majara *et al.* 1996; Parrou and Francois 1997; Hounsa *et al.* 1998).
370 Significantly higher levels of this disaccharide were observed in W34/70 populations stored at near-freezing
371 temperatures, presumably due its requirement for maintaining and stabilising cellular membrane structures
372 (Kandror *et al.* 2002; Kandror *et al.* 2004). In contrast, the presence of this sugar did not appear to be imperative
373 for yeast survival at 10°C. In the absence of an exogenous carbon source and extracellular uptake (due to non-

374 significant regulation of transporter *MAL11* in current study) (Plourde-Owobi *et al.* 2000), trehalose
375 accumulation could have occurred at the expense of internal cellular carbon reserves (Jorgensen *et al.* 2002),
376 including glycogen. A difference in yeast trehalose synthesis as a function of temperature was not observed at
377 the level of transcriptome regulation. Such disparity between gene expression and cell physiology could partly
378 be explained by the sensitive post-translational control exerted upon most enzymes involved in trehalose (and
379 glycogen) metabolism (Hwang *et al.* 1989; Huang *et al.* 1998; Parrou *et al.* 1999; Gasch *et al.* 2000). Similar to
380 glycogen, simultaneous activation of both trehalose accumulation and mobilization genes appeared to occur
381 under both storage conditions presumably for rapid and sensitive regulation of its internal reserves (Gasch *et al.*
382 2000).

383 The observation that low levels of intracellular glycerol were accumulated during the later stages of storage at
384 4°C was supported by the genetic response; simultaneous activation and repression of key glycerol biosynthesis
385 and catabolism genes was noted. Since *S. cerevisiae* has a general propensity to curtail glycerol production
386 under nutrient limiting conditions (O'Connor and Wyrick 2007), glycerol accumulation in brewing yeast cells
387 may be a protective mechanism against freeze injury and, like trehalose, has been suggested to be a vital factor
388 in maintaining cellular viability (Panadero *et al.* 2006; Zhao *et al.* 2015). During the early stages of slurry
389 storage at 10°C, a transient increase in intracellular glycerol concentration was observed. This was surprising
390 given that previous reports have indicated that higher glycerol accumulation occurs in *S. cerevisiae* following a
391 temperature drop to 4°C than to 12°C (Panadero *et al.* 2006). One reason for such a discrepancy could lie in the
392 pivotal role played by the glycerol export channel FPS1p (encoded by *FPS1*) in glycerol accumulation. Cold-
393 induced accumulation of glycerol has been attributed to lower activity levels of FPS1p (Tamas *et al.* 1999;
394 Panadero *et al.* 2006) whereas *FPS1* deletion in *S. cerevisiae* was shown to enhance intracellular glycerol and
395 impart freeze tolerance (Tumu *et al.* 2012). However in the current study, significantly lower transcription of
396 *FPS1* was observed in the early stages of lager yeast storage at 10°C when compared to the traditional storage
397 regime. Decreased *FPS1* activation could thus lead to reduced glycerol efflux eventually resulting in a net
398 intracellular accumulation of this solute.

399 The 'homeoviscous' adaptive response (Sinensky 1974) in yeast aims to compensate for cold stress-induced
400 reduction in membrane fluidity primarily through an increase in the membrane's unsaturation levels or the

401 shortening of its mean fatty acid chain length via increments in MCFA proportions (Redon *et al.* 2011). The
402 absence of any obvious difference in UI between W34/70 populations at 10 and 4°C suggests no obvious change
403 in membrane unsaturation. Despite induction of genes associated with MCFA biosynthesis, the MCFA content
404 in 4°C-stored yeast populations was observed to be relatively constant over time. Other possible mechanisms for
405 membrane fluidity maintenance include alterations to the membrane's triacylglycerol and phospholipid
406 distribution (Redon *et al.* 2011) or increased disorder in the distribution of membrane fatty acids by the action of
407 glycerol (Boggs and Rangaraj 1985; Beranova *et al.* 2008). Nevertheless, changing the storage temperature from
408 4 to 10°C did not elicit any obvious physiological adaptation in FA distribution.

409 Maintenance of pitching yeast quality following cropping is vital for the performance and consistency of
410 subsequent fermentations. Although this study was completed using a single lager brewing yeast strain and did
411 not investigate the impact of increased storage temperature on subsequent fermentation performance, the data
412 presented here suggests that when yeast is cropped and stored, cell physiology is not significantly affected by a
413 shift in storage temperature conditions (4°C to 10°C for a maximum of 72 hr). Lack of significant changes in
414 cell viability, intracellular glycogen or cellular fatty acid and confinement of temperature dependent changes in
415 yeast transcriptome to early stages of storage, followed by attainment of a steady transcriptional state, indicates
416 that marked differences in subsequent fermentation characteristics are unlikely. However, further assessment is
417 required to discern the impact of increased yeast storage temperature over multiple pitching cycles. Due to the
418 expense associated with maintaining yeast at low temperatures, this study offers the brewer the opportunity to
419 explore alterations to yeast storage regime and enhancing cost efficiency without impacting on the quality of the
420 yeast culture.

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426

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545

Legends

Table 1. MIPS Functional classification of the differentially transcribed genes after 6 hrs of increasing the cropped yeast storage temperature from 4°C to 10°C. A total 506 genes were divided into groups of induced (174) and repressed (332) genes before functional annotation. Differential expression was defined as a fold change of >1.3 with $p < 0.05$. The cut off for the enrichment magnitude for the functional categories was $p < 0.05$. “Grp occ” - number of genes in the cluster belonging to the specified functional category. “Genome occ” – number of genes in the genome belonging to the specified functional category.

Table 2. Fold-change in expression level of genes associated with glycogen, trehalose, glycerol and fatty acid production after 6h, 24h and 48 hrs of storage at 10°C and 4°C. Data points represent the mean of three per-gene normalized transcription values. All genes presented show a statistically significant change in expression (corrected $p < 0.05$; Benjamini-Hochberg FDR) and a fold change cut off ≥ 1.3 . Descriptions for gene functions were obtained following those of the *Saccharomyces* Genome Database (SGD) (Cherry et al., 2012)

Fig 1. Cell viability during anaerobic W34/70 slurry storage at 10°C and 4°C for 72 hours in the absence of agitation. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean

Fig 2. Transcription profiles of genes associated with the regulation of yeast glycogen (a and b), trehalose (c), glycerol (d and e) and fatty acids (f) during yeast storage at 4°C. All presented genes show a statistically significant change in expression of $p < 0.05$ (one-way ANOVA with BH-FDR correction) and fold change of > 1.3 . Data points represent the mean of three per-gene normalized transcription values.

Fig 3. Intracellular glycogen (a), trehalose (b), and glycerol (c) during yeast storage at 10°C and 4°C for 72 hr. For glycogen and trehalose final values are expressed as mg glucose released per gram of slurry dry weight (SDW). Values represent the mean of three independent replicates and the error bars represent the standard deviation around the mean.

Fig 4. Alterations in unsaturation index (UI; a) and medium chain fatty acids (MCFA; b) in yeast populations maintained at 10°C and 4°C for 72 hours. Values represent the mean of three independent replicates. Error bars represent the standard deviation around mean. $UI = \Sigma(C16:1 + C18:1) / (C14:0 + C16:0 + C18:0)$; $MCFA = \Sigma(C10:0 + C12:0)$.

Table 1.

MIPS Functional Category - 10°C vs 4°C - 6 Hr (continued later)	Up-regulated			Down-regulated		
	Grp Occ	Genome Occ	P - Value	Grp Occ	Genome Occ	P - Value
01 METABOLISM						
01.01.09.07.01 biosynthesis of histidine				0.91	0.24	4.24E-02
01.03 nucleotide/nucleoside/nucleobase metabolism				6.42	3.65	7.99E-03
01.03.01 purin nucleotide/nucleoside/nucleobase metabolism				2.14	0.99	4.24E-02
01.06.06.11 tetracyclic and pentacyclic triterpenes metabolism				1.52	0.58	4.06E-02
01.07 metabolism of vitamins, cofactors, and prosthetic groups	7.59	2.67	1.01E-03			
01.07.01 biosynthesis of vitamins, cofactors, and prosthetic groups	6.96	1.79	1.11E-04			
01.20 secondary metabolism	3.79	1.27	1.49E-02			
01.20.27 metabolism of secondary products derived from aspartic acid and threonine	0.63	0.01	2.58E-02			
02 ENERGY	10.1	5.98	2.64E-02			
02.08 pyruvate dehydrogenase complex	1.89	0.08	1.62E-04			
02.13.03 aerobic respiration	3.79	1.25	1.40E-02			
02.16.09 mixed acid and butanediol fermentation	0.63	0.01	2.58E-02			
10 CELL CYCLE AND DNA PROCESSING						
10.01.03.03 ori recognition and priming complex formation				1.22	0.4	4.14E-02
10.03.03 cytokinesis (cell division) /septum formation and hydrolysis				2.44	1.15	3.42E-02
11 TRANSCRIPTION				41.5	17.5	7.29E-26
11.02.01 rRNA synthesis				3.97	1.35	3.97E-04
11.04 RNA processing				28.1	7.11	1.70E-33
11.04.01 rRNA processing				23.2	3.34	2.77E-46
11.06 RNA modification				7.03	1.04	4.92E-14
11.06.01 rRNA modification				2.75	0.29	9.95E-08
11.06.02 tRNA modification				3.97	0.7	1.91E-07
12 PROTEIN SYNTHESIS				18.3	7.83	1.57E-10
12.01 ribosome biogenesis				13.4	5.05	1.18E-09
12.10 aminoacyl-tRNA-synthetases				2.44	0.63	8.65E-04
14 PROTEIN FATE (folding, modification, destination)						
14.04 protein targeting, sorting and translocation	8.22	4.56	2.80E-02			
14.07.03 modification by phosphorylation, dephosphorylation, autophosphorylation				4.89	3.03	3.91E-02
14.07.11 protein processing (proteolytic)	4.43	1.45	7.77E-03			
14.07.11.01 autoprolytic processing	1.89	0.42	2.83E-02			
16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT				32.4	17.1	2.66E-12
16.03 nucleic acid binding				15.9	5.56	1.72E-12
16.03.03 RNA binding				12.5	3.09	3.51E-15
16.07 structural protein binding				2.1	0.91	2.82E-02
16.13 C-compound binding	1.3	0.14	2.11E-02			
16.13.03 fatty acid binding (e.g. acyl-carrier protein)	0.6	0.01	2.58E-02			
16.19 nucleotide/nucleoside/nucleobase binding				6.7	3.67	4.07E-03
16.19.03 ATP binding				6.1	3.11	2.77E-03
20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND ROUTES						
20.01.10 protein transport				4.3	2.28	1.67E-02
20.01.21 RNA transport				4.3	1.4	1.57E-04
20.09.01 nuclear transport				5.5	1.38	3.51E-07
32 CELL RESCUE, DEFENSE AND VIRULENCE	15.8	9.03	3.77E-03			
32.01 stress response	12.0	7.34	2.18E-02			
32.07 detoxification	4.4	1.9	3.09E-02			
32.07.07.03 glutathione conjugation reaction	1.3	0.08	6.27E-03			
34 INTERACTION WITH THE ENVIRONMENT						
34.07 cell adhesion	1.3	0.21	4.27E-02			
34.07.01 cell-cell adhesion	1.3	0.16	2.59E-02			
40 CELL FATE				7.0	4.45	1.90E-02
40.01 cell growth / morphogenesis				6.7	3.88	7.89E-03
42 BIOGENESIS OF CELLULAR COMPONENTS						
42.04 cytoskeleton/structural proteins				6.4	4.11	2.71E-02
42.10 nucleus				4.6	2.43	1.28E-02
42.10.05 nuclear membrane				1.8	0.48	4.40E-03
42.16 mitochondrion	7.6	2.78	1.45E-03			
43 CELL TYPE DIFFERENTIATION	12.0	7.37	2.27E-02			
43.01 fungal/microorganismic cell type differentiation	12.0	7.37	2.27E-02			
43.01.03 fungal and other eukaryotic cell type differentiation	12.0	7.37	2.27E-02			
43.01.03.09 development of asco- basidio- or zygospor	7.0	2.72	3.73E-03			
11 TRANSCRIPTION				41.5	17.5	7.29E-26
11.02.01 rRNA synthesis				3.97	1.35	3.97E-04
11.04 RNA processing				28.1	7.11	1.70E-33
11.04.01 rRNA processing				23.2	3.34	2.77E-46
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14.07.11 protein processing (proteolytic)	4.43	1.45	7.77E-03			
14.07.11.01 autoprolytic processing	1.89	0.42	2.83E-02			

Table 2.

Genes	10°C Vs 4°C					
	6h		24h		48h	
	Reg	FC	Reg	FC	Reg	FC
Glycogen						
<i>GIP2</i>	up	1.7	-	-	-	-
<i>GLG2</i>	up	1.7	-	-	-	-
<i>GAC1</i>	up	1.3	-	-	-	-
Trehalose						
None						
Glycerol						
<i>GPP1</i>	down	2.5	-	-	-	-
<i>GPP2</i>	down	2.1	-	-	-	-
<i>GCY1</i>	up	2.2	-	-	-	-
<i>GUT1</i>	up	1.3	-	-	-	-
<i>FPS1</i>	down	1.5	-	-	-	-
Fatty Acid						
<i>ELO1</i>	down	1.5	-	-	-	-

Reg – Regulation; FC – Fold Change

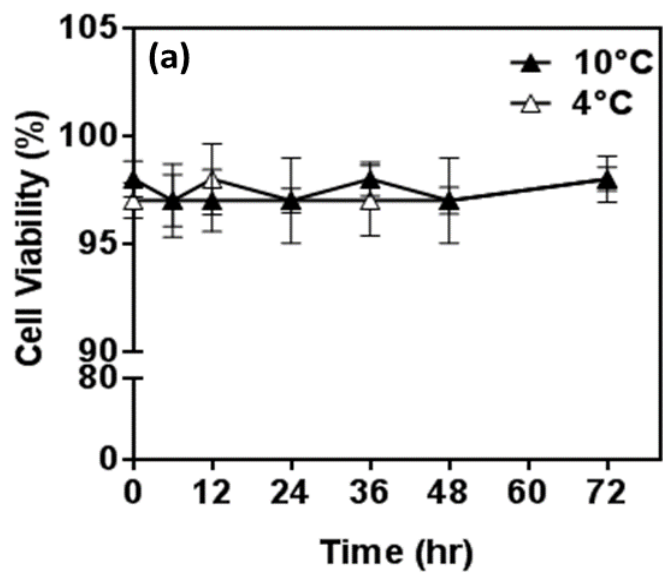


Fig. 1

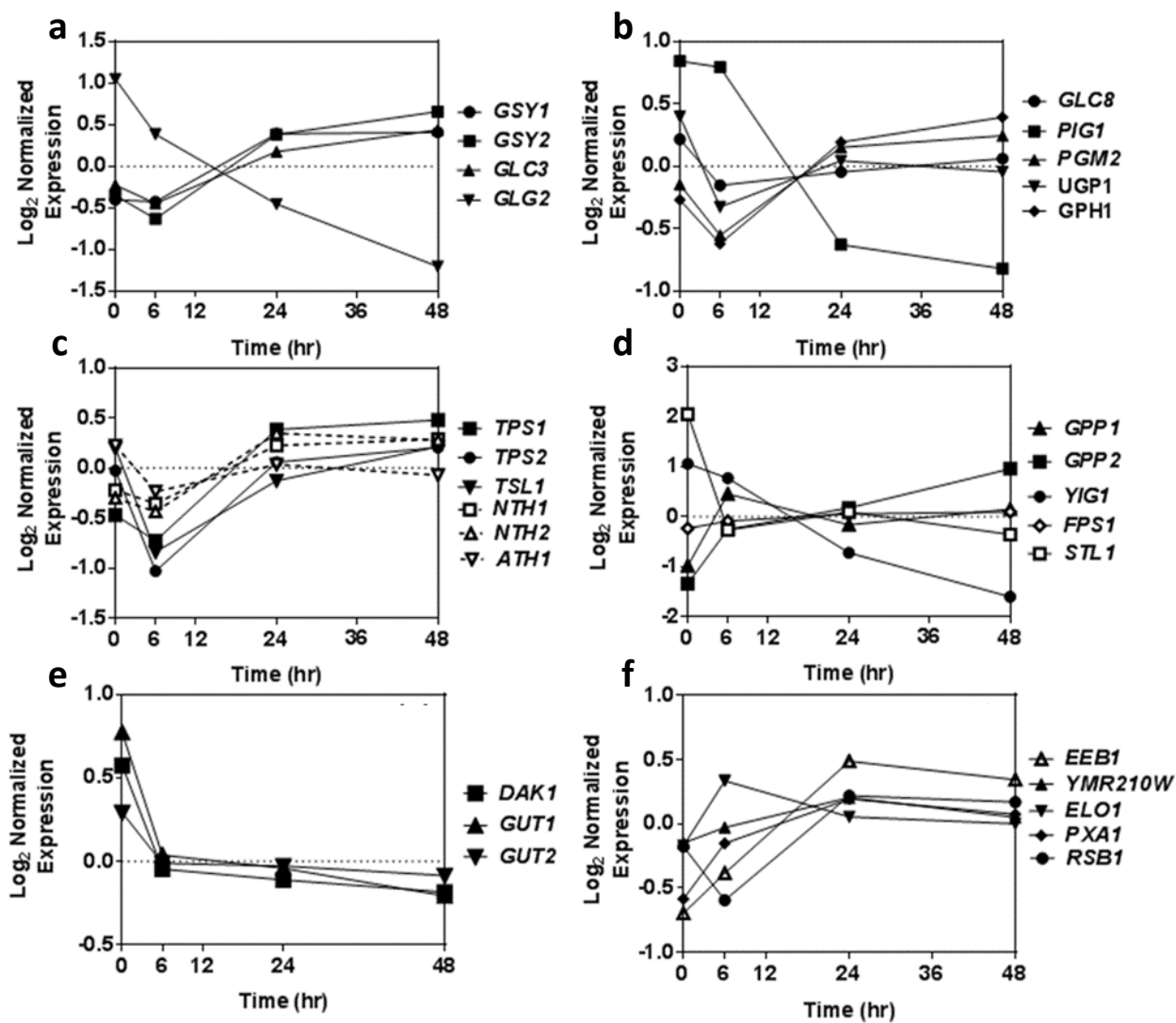


Fig. 2

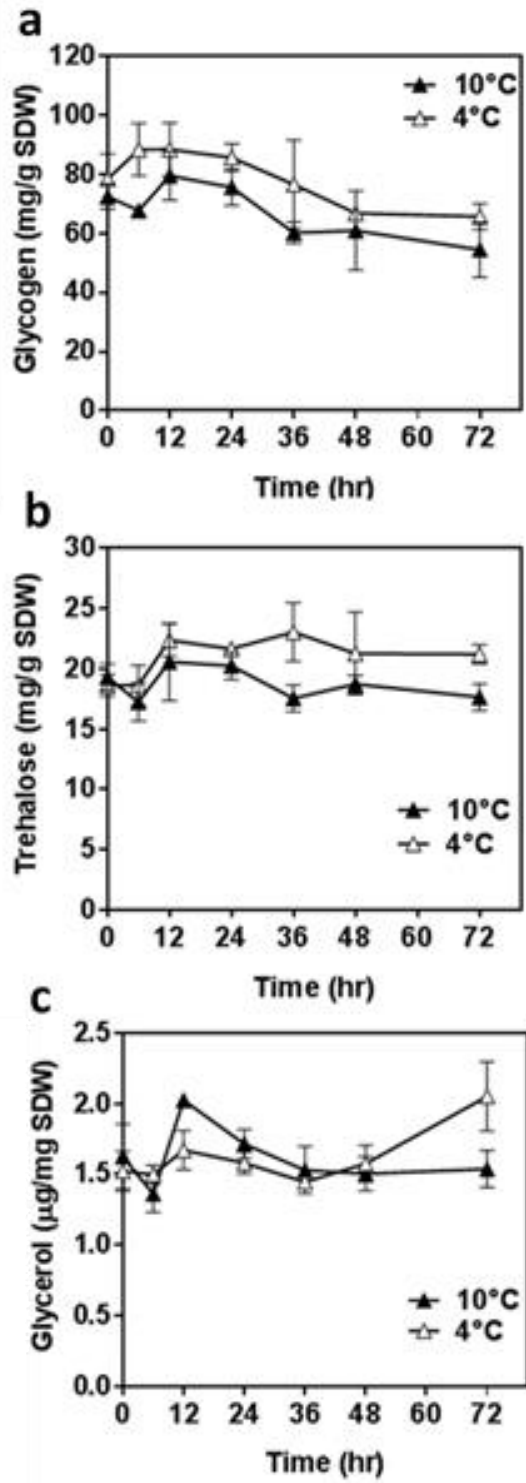


Fig. 3

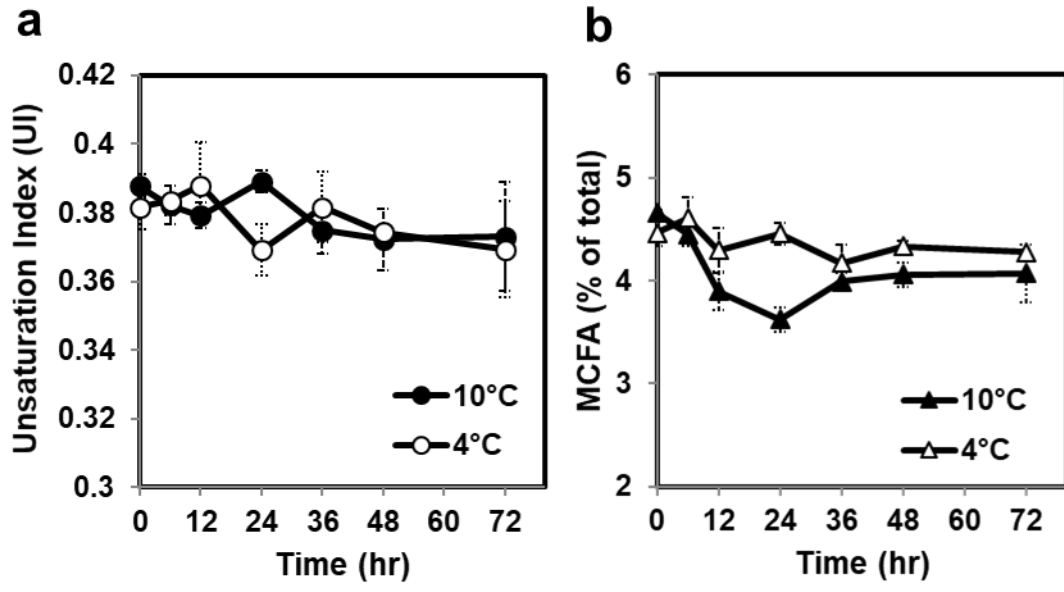
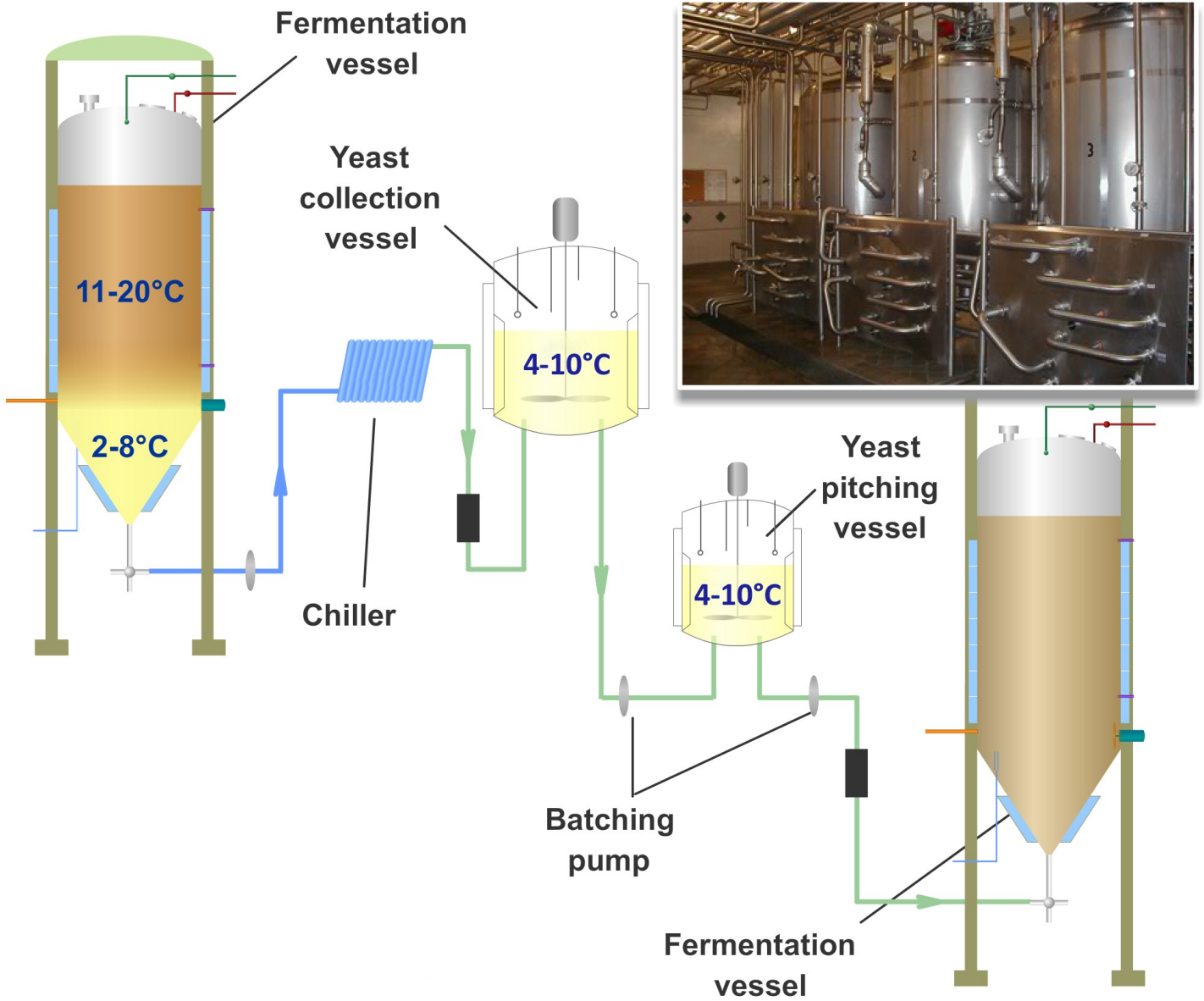


Fig. 4



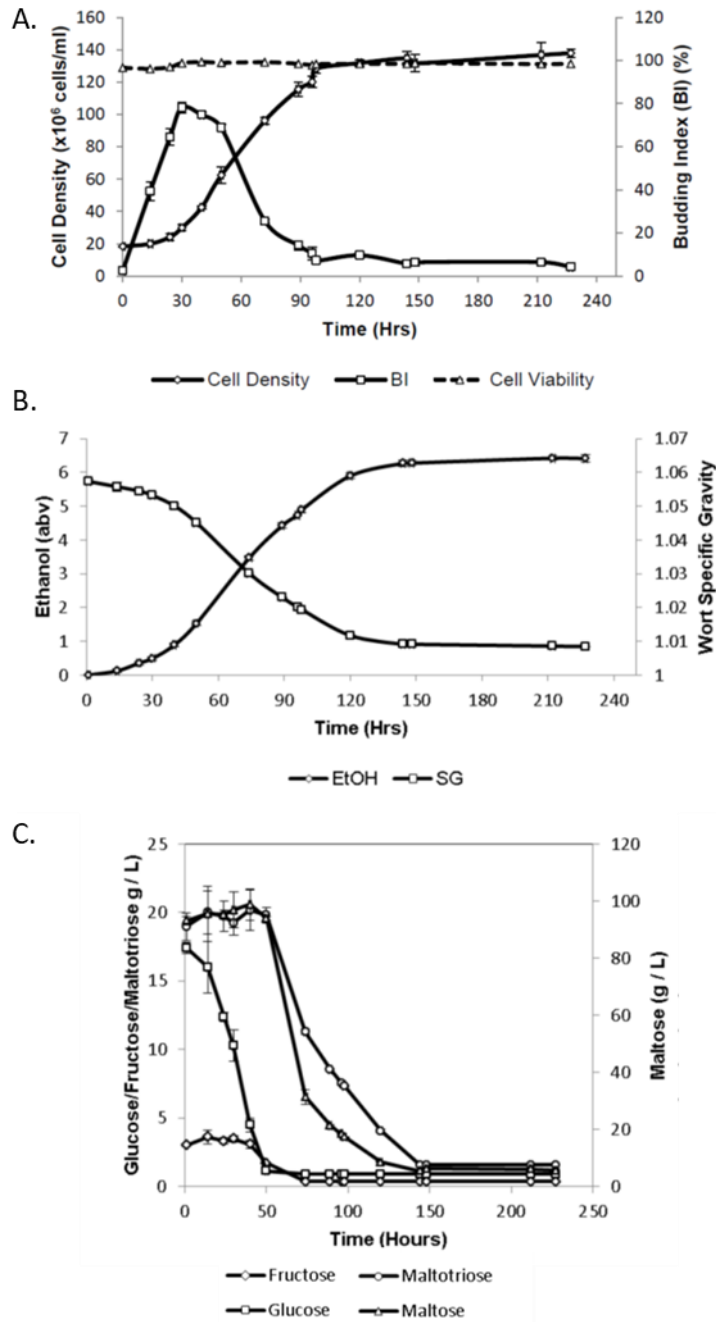


Figure S1 – Fermentation profile of *Saccharomyces pastorianus* W34/70 prior to storage. (A) Yeast cell viability, density and budding index along with wort characteristics including (B) specific gravity, ethanol and (C) major fermentable sugars were assessed. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.

