The Relationship Between Wort Sugar Concentration and Yeast Carbon Partitioning during Brewing Fermentations

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Abstract

High gravity (HG) and very high gravity (VHG) fermentations are increasingly attractive within the brewing industry as a means of optimising process efficiency and energy-saving. However, the use of highly concentrated worts is concomitant with a number of biological stress factors which can impact on yeast quality and fermentation performance. In order to eliminate or reduce potentially detrimental effects, brewing yeast respond to their environment by shunting carbon into different metabolic end products which assist in the protection of cells, but also impact on final ethanol yield. The purpose of this research was to investigate the impact of substrate sugar concentration on carbon partitioning in brewing fermentations. This was conducted using a series of lab-scale fermentations with worts of 13ºP, 18ºP and 24ºP, pitched using lager and ale yeast strains. Fermentation performance was assessed with respect to the uptake of wort sugars and the production of key carbon-based metabolites, leading to a calculation of yeast central carbon flux. Analysis of carbon assimilation and dissimilation revealed that changes in intracellular trehalose, glycogen, higher alcohols and esters were observed, however the production of yeast biomass acted as the major trade-off with ethanol

production. The data presented here shows for the first time the requirements of yeast populations during HG and VHG conditions and the factors which have a major impact on key performance indicators. This data has major significance for fermentation-based industries globally and is especially important for those sectors seeking to maximise yield from existing resources through high gravity fermentations.

Keywords: Ethanol yield, High gravity, *Saccharomyces*, Sugar utilisation, Yeast stress

Introduction

High Gravity (HG) brewing is a well-established technique where worts of high sugar concentration (15-20ºP) are employed to produce high alcohol beer, which is then adjusted to sales-gravity by dilution (Stewart, 2010, Stewart 2016). This method is increasingly attractive as an effective strategy towards enhanced process productivity, reduced investments and overall energy cost savings throughout the brewing industry (Stewart, 2009, Stewart, 2010, Puligundla *et al.*, 2011). Due to the success of this approach, efforts to explore the possibility of using Very High Gravity (VHG) worts of 20-25ºP have increased (Vidgren *et al.*, 2009, Gibson, 2011). However, in order to achieve this, problems related to the use of concentrated worts must be overcome, including decreased foam stability (Cooper, 1998, Brey, 2004), poor hop utilization (Stewart, 2010), longer fermentation times (Boulton & Quain, 2001), and inconsistencies in final product flavour matching (Stewart, 2009, Stewart, 2010, Puligundla *et al.*, 2011). Furthermore, the use of high gravity worts can result in a number of biological stress factors which are known to influence yeast quality during fermentation leading to a reduction in efficiency and potentially impacting on serial repitching (Stewart, 2009, Stewart, 2010, Puligundla *et al.*, 2011, Dekoninck *et al.*, 2012).

Brewing yeast acts as the workhorse during fermentation and, from the brewing perspective, are essential in converting wort components to alcohol and flavour compounds. However, sugars are also required for a number of other biological processes including growth and division as well as for cellular homeostasis, which incorporates the maintenance of redox balance, generation of energy, production of storage carbohydrates and activation of anti-stress pathways. As a result, yeast is capable of producing a range of carbon metabolites including ethanol, carbon dioxide, glycerol, trehalose, glycogen, higher alcohols and esters, as well as polysaccharides (including glucan and mannan) and lipid structures used for yeast biomass

production (Figure 1). The ratio of compounds produced can be dictated by the fermentation conditions, including parameters which impact growth such as temperature and oxygen, the raw materials provided, and the nutritional requirements of the strain. Furthermore, in order to counteract or limit the impact of environmental stress factors, yeast cells respond by shunting carbon into different metabolic end products. Although such metabolites assist in the protection of cells, inevitably this diversion of carbon will impact final ethanol yield. Consequently, the manner in which brewing yeast adapt their central carbon flux in response to the wort environment is critical in determining both fermentation efficiency as well as the health of the yeast culture.

Typical approaches to quantifying carbon distribution are based on mass balance analysis. Antoine Lavoisier first described this in 1790 based on the realization that sugars are transformed into carbonic acid, alcohol, and yeast biomass (Lavoisier, 1790). Subsequently Karl Napoleon Balling published a fermentation mass balance formula based on the concept that fermentable wort solids contribute to yeast mass increase. This formula has been applied in brewing practice for over 100 years (De Clerck, 1958, Nielsen, 2004) and is accepted as standard by the American Society of Brewing Chemists (Beer-6A, 2014). However, Balling's formula was derived based on assumptions that 0.11 g of carbohydrate is converted to yeast mass for each gram of ethanol produced in fermentation, and that all fermentable dissolved wort solids are monosaccharides. These assumptions are not wholly justified based on current knowledge of yeast metabolism and wort composition during brewing fermentations. Cutaia (2007) compared stoichiometric values to Balling's classic formula during a brewing fermentation, taking into consideration the major wort carbohydrates (glucose, fructose, maltose and maltotriose) and factors associated with yeast growth including sterols and dissolved oxygen. The author concluded that a significant departure from the traditional wort profile, such as the application of high gravity brewing, could result in inaccurate estimates

using Balling's original formula (Cutaia, 2007). Despite these observations it should be noted that in the study conducted by Cutaia (2007), both fermentable mono- and disaccharides were considered to be completely fermented, which may not necessarily correspond to reality in production scale HG or VHG brewing fermentations. Consequently, while significant insights into the subject in general have been made, the apportioning of carbon contribution to yeast metabolites under HG and VHG conditions has not been fully explored.

This study aims to evaluate the carbon partitioning of brewing lager and ale yeast under various wort gravities, and to highlight potential approaches for managing fermentation efficiency and understanding yeast health at HG and VHG fermentations. It is anticipated that the data presented here will provide a greater understanding of the response of yeast to high gravity conditions based on carbon flux, which could potentially lead to strategies for directing carbon utilisation in the future.

Materials and methods

Yeast strains and growth media

Lager strain (*Saccharomyces pastorianus*) designated Lager1 was obtained from Molson Coors Brewing Company (UK) Limited, and lager strain W34/70 was obtained from Hefebank Weihenstephan (Germany). Ale strains (*Saccharomyces cerevisiae*) NCYC1332 and M2 were collected from the National Collection of Yeast Culture (NCYC, Norwich, UK). All yeast strains were maintained on YPD agar plates containing 1 % (w/v) yeast extract, 2 % (w/v) neutralized bacteriological peptone, 2 % (w/v) D-glucose and 1.2 % (w/v) agar at 4°C. All chemicals were purchased from Fisher Scientific (UK) and all media were autoclaved at 121°C and 15 psi for 15 min immediately after preparation and prior to use.

For yeast propagation, single yeast colonies were taken from stock plates and inoculated into 10 mL YPD media. After incubation for 48 hours at 25°C on an orbital shaker at 120 rpm, each cell suspension was transferred to a pre-sterilized 250 mL conical flask containing 100 mL YPD media and the yeast was grown at 25°C and 120 rpm for 48 hours. Finally, the suspension was transferred to a pre-sterilized 2 L conical flask containing 800 mL YPD media. The yeast culture was again incubated aerobically at 25ºC for 48 hours with constant shaking at 120 rpm. Cells were recovered by centrifugation at 4,000 rpm for 5 min at 4ºC and a viable cell count was determined using a haemocytometer in conjunction with methylene blue staining, in order to calculate pitching rates as described below.

Wort preparation

Industrially produced 25ºP wort, obtained from Molson Coors Brewing Company (UK) Limited, was diluted with sterile reverse-osmosis water to obtain 18ºP (HG) and 24ºP (VHG) worts. A separate 13ºP wort, representing a 'standard' gravity medium was also obtained from the same brewery. All worts were supplemented with 0.2 mg/L Zn^{2+} by addition of $ZnSO_4 \cdot 7H_2O$ (Fisher Scientific, UK). In order to mimic the gaseous environment associated with industrial fermentation conditions, 13 ºP, 18ºP and 24ºP worts were provided with oxygen to achieve approximately 13, 18 and 24 ppm final concentration (1 ppm per degree Plato), respectively.

Fermentations

Fermentations were carried out using glass hypo-vials according to the method described previously (Quain *et al.*, 1985, Powell *et al.*, 2003). Well-mixed yeast slurry was pitched into 100 mL wort to create five different experimental conditions based on starting gravity and pitching rate (Table 1). For high gravity fermentations (18ºP and 24ºP), an 'adjusted' pitching rate based on brewery practice (1.0×10^6 viable cells/mL per degree Plato) was employed in

addition to a standardised pitching rate (1.5×10^7 viable cells/mL). These different pitching rates were applied to reveal any effects of initial cell numbers on carbon dissimilation. All fermentations were performed within a closed (anaerobic) system with constant stirring (350 rpm) for up to 120 hours. It is acknowledged that within the brewing industry lager fermentations are typically conducted at 12-18°C, and ale fermentations at 18-25°C. However, in this study all fermentations were conducted at 15°C, regardless of yeast type. This was primarily performed to remove temperature as a variable (since it is widely recognised that this will impact growth rate and cellular metabolism), while also providing data directly relevant to the commercially significant lager yeasts analysed. Correspondingly, it is recognized that the data presented here related to ale yeasts could be impacted by the lower fermentation temperatures, which may have a bearing when translating the results to industrial settings.

For each set of fermentations, a series of vessels were prepared as described above to allow for destructive sampling. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours post-pitching and, at each time point, three vessels were removed and immediately stored on ice. The number of yeast cells in suspension was determined immediately using a haemocytometer; viability was simultaneously measured by methylene blue staining (Pierce, 1970). Cell pellets and aliquots of 50 mL wort/beer were separated by centrifugation at 4,000 rpm for 5 min at 4ºC and stored at -80ºC prior to further analysis.

Analytical methods

The specific gravity of the fermenting wort was measured using a handheld density meter (DMA 4500, Anton Paar, UK) and sugar composition was determined by HPLC using the method described by Gibson et al (Gibson *et al.*, 2008). Separation of ethanol and glycerol was performed using 1 mL of sample via an HPLC column (300 × 7.8 mm, Phenomenex ROA column, USA) with 2.5 mM H_2SO_4 as eluent into a refractive index detector (RI 2031 plus,

146 JASCO, Japan). Flavour compounds were determined by headspace gas chromatograph-mass
147 spectrometer (GC-MS) based on an established method (Ashraf *et al.*, 2010). All the
148 HPLC/GC-MS samples above were placed in an automatic sampler set to follow a random
149 running order. CO_2 evolution was determined to indicate fermentation progression based on
150 weight loss of the entire fermentation vessel over time.

151 Intracellular trehalose and glycogen were assessed according to a method described by Parrou
152 and Francois (Parrou & Francois, 1997). Briefly, glucose was released from each carbohydrate
153 by enzyme digestion with trehalase and amyloglucosidase, respectively (Sigma, UK), and
154 subsequently assessed using a commercial glucose assay (Megazyme, Ireland). The
155 concentration of trehalose or glycogen was expressed in µg glucose per 1×10^8 cells. Yeast
156 biomass was expressed in dry cell weight by drying at 55ºC until a constant weight was reached.

157 *Estimation of yeast carbon partitioning*

158 In order to estimate yeast carbon partitioning under different fermentation conditions, certain
159 assumptions were made based on the observations of Cutaia (2007): (I) carbon conversion by
160 assimilation of non-carbohydrate materials from wort, such as free amino nitrogen, was
161 considered negligible; (II) carbon conversion to fermentation products other than ethanol,
162 carbon dioxide, glycerol, yeast biomass, glycogen, trehalose, higher alcohols and esters, was
163 considered to be negligible.

164 *Derivation of equations*

165 Equation 1 was used to determine carbon contents in wort and beer samples based on carbon
166 mass conservation within each compound (Table 2). Briefly, carbon content was quantified
167 from the sugar contents of the initial and residual worts, and the concentrations of ethanol,
168 carbon dioxide and glycerol, as well as higher alcohols and esters in the final beer. Equation 2

was used to quantify the carbon concentrations in trehalose and glycogen based on the derived glucose units.

Equation 1 Calculation of carbon content in wort/beer

$$C1\ (g/L) = \mathrm{Cg} \times \mathrm{Rc}$$

Where Cg is the concentration for a given compound (g/L), Rc is the ratio of carbon in the compound (Table 2)

Equation 2 Calculation of carbon content in trehalose and glycogen

$$C2\ (g/L) = \frac{Nt}{10^8} \times Ci \times 10^{-5} \times 40.0\ \%$$

Where Nt is the total cell numbers, Ci is the concentration of trehalose or glycogen in the form of glucose ($\mu g/10^8$cells), 10^{-5} is a unit conversion factor and 40.0 % represents the carbon percentage in glucose (Table 2).

Total carbon input and carbon partitioning

The total carbon input was determined to be the sum of the carbon content of wort carbohydrates consumed during each set of fermentations and was calculated from the original and residual wort sugars using Equation 1. These values were then used to quantify carbon investment in each metabolite, including ethanol, carbon dioxide, glycerol, higher alcohols and esters, as well as trehalose and glycogen. Carbon partitioning data was expressed as a percent of the total carbon input.

Apart from the allocated carbon proportion of the total carbon input, the 'un-allocated' percentage of carbon was attributed to yeast biomass (as an artefact of cell maintenance, growth and division), according to the observations of Cutaia (2007). Additionally, as an alternative metric, the carbon concentration in the yeast biomass was calculated based on a predicted

carbon content of 48 % in dry baker's yeast (Van Hoek *et al.*, 1998) and expressed as a percent of the total carbon consumption.

Statistical analysis

Three independent biological samples (for both yeast and wort analyses) were taken at each time point during fermentation as described above. Each sample was analysed in triplicate and statistical analysis was performed using SPSS version 20.0 for windows (Chicago, USA). Data were subjected to one-way analysis of variance (ANOVA) with a least significant difference test (LSD) or paired samples T-test to determine the significant differences between the samples. Differences were considered significant at $P < 0.05$.

Results and discussion

Identification of optimum strategies for directing carbon towards the desired end products is a challenging task owing to the complexity of metabolic networks. Studies of carbon partitioning in microorganisms have led to scientific and industrial breakthroughs in the disciplines of metabolomics (Van Gulik *et al.*, 2000, Rui *et al.*, 2010), genetic engineering (Nevoigt *et al.*, 2002, Underwood *et al.*, 2002, Cadiere *et al.*, 2011), and targeted enzyme production (Sauer & Eikmanns, 2005, Grose *et al.*, 2007). An example specifically related to beer is the shift of carbon flux towards glycerol at the expense of ethanol formation using a brewing yeast overexpressing gene *GPD1*, leading to 5.6-fold increase of glycerol production and 18 % reduction of ethanol yield (Nevoigt *et al.*, 2002). However, the navigation of carbon flow has largely remained an interesting and under-explored topic in brewing fermentations, especially when related to wort sugar concentration. To address this, we provide a comparative estimation of carbon partitioning during HG and VHG fermentations using brewer's wort. During each set of fermentations, utilisation of wort carbohydrates, as well as generation of main carbolic products were determined (Supplementary data), and the data at start (0 hour) and end (120

hour) points were used to determine both the carbon concentration of wort carbohydrates consumed and the carbon metabolites produced.

Effect of wort gravity on carbohydrate utilisation and total carbon input

In each instance, fermentation progression was characterised by a typical decrease in wort gravity (Figure 2); increasing the starting wort concentration resulted in higher attenuation regardless of yeast strain or pitching rate applied. 13P15M (see Table 1 for explanation of terminology) and 24P15M conditions yielded the lowest and highest attenuation gravity, respectively. Although similar attenuation gravities were obtained with 18P15M and 18P18M fermentations, the 24P15M conditions resulted in an elevated final gravity when compared to 24P24M, most pronounced in fermentations conducted using the ale strain NCYC1332. Additionally, at 24ºP, 24P15M conditions were found to display the slowest fermentation rate for each of the four yeast strains. However, a faster attenuation was achieved by increasing the initial cell density to 2.4×10^7 cells/mL (24P24M condition). In contrast, at 18ºP, an accelerated fermentation rate at 18P18M compared to 18P15M fermentations was only seen with strain Lager1.

For each set of fermentations the utilisation of carbohydrates was calculated, including consumption of monosaccharides (fructose and glucose), disaccharides (sucrose and maltose) and trisaccharides (maltotriose). Subsequently the total carbon input (defined by carbon assimilation by yeast) was determined and apportioned based on carbohydrate group (Table 3). Sugar consumption was observed to increase with increasing wort density, and no significant difference was found between the four strains for the consumption of monosaccharides, disaccharides or trisaccharides.

Corresponding to the data reported above, the total carbon input increased with elevated original wort gravity, with approximately 39 g/L, 51 g/L and 72 g/L for 13ºP, 18ºP and 24ºP

fermentations, respectively (Table 2). At each condition, no significant difference in total carbon input ($P < 0.05$) was found for the yeast strains investigated, except for ale yeast NCYC1332 at 24P15M, which consumed a lower amount of carbon overall than the other strains investigated, due to the relative lower consumption of maltotriose. It should be noted that the measurement of total carbon consumption was based on the net utilisation of all wort sugars, and hence the notable amounts of residual maltotriose present in the final beers was taken into consideration. This quantification therefore offers some improvement over the method of Cutaia (2007), where all the monosaccharide and disaccharide were assumed to be fully fermented.

Effect of wort gravity on yeast carbon dissimilation

The total carbon input data obtained (Table 3) was used to quantify the 'carbon investment' in each metabolite. Data are expressed as a percentage of the total carbon input and are summarized in Table 4 and 5 for each brewing yeast strain.

Ethanol is one of the major products derived from central carbon metabolism of brewing yeast and is an important key performance indicator for HG and VHG brewing. Here, ethanol occupied the most abundant form of carbon output in all fermentations, representing greater than 50 % of the total carbon input. At 24ºP fermentations, a higher amount of carbon ($P < 0.05$) in the form of ethanol was observed at higher pitching rates (24P24M compared to 24P15M) for each of the strains examined, except for lager strain W34/70, which exhibited similar carbon investment in ethanol under both conditions. At 18ºP, only strain NCYC1332 showed a higher carbon to ethanol conversion at the higher pitching rate (18P18M compared to 18P15M) ($P < 0.05$), whilst no significant difference ($P < 0.05$) was observed for the other strains. Calculation of the proportion of carbon attributed to ethanol and carbon dioxide

provided expected results; ethanol and carbon dioxide were present in ca. 2/1 ratio as anticipated based on a standard fermentation equation.

Glycerol is produced during fermentations in a redox-neutral process in order to maintain cellular redox balance and to act as an essential compatible solute during osmoregulation in yeast (Wang *et al.*, 2001). Analysis of carbon to glycerol indicated that only 2-4 % of carbon was directed towards the production of this molecule, and the percent output in response to different conditions varied between the strains independent of wort gravity and pitching rate. The exception to this was strain Lager1, which directed higher ($P < 0.05$) amounts of carbon into glycerol at higher pitching rates (18P18M and 24P24M) than when pitching lower numbers of cells (18P15M and 24P15M).

Trehalose is widely accepted as an important stress protectant in yeast cells, conferring stability to the plasma membrane (Neves *et al.*, 1991, Mansure *et al.*, 1994, Petit & Francois, 1994, Plourde-Owobi *et al.*, 2000, Jules *et al.*, 2004), and glycogen is regarded as a major storage carbohydrate in yeast, serving as an energy source for maintaining cellular functions. In this study, a comparatively minor proportion of carbon was directed to trehalose and glycogen synthesis, representing approximately 0.1-0.2 % and 0.2-0.3 % of the total carbon input for higher and lower pitching rates respectively. With regard to trehalose, it was interesting to note that both lager strains directed a higher amount of carbon to trehalose at 24P24M conditions than at 24P15M, whereas the ale strains did not. In contrast, all strains (lagers and ales) directed similar amount of carbon to trehalose at lower gravity (13P15M, 18P15M and 18P18M). With respect to glycogen, when comparing 13P15M, 18P15M and 24P15M conditions, only the ale strain M2 showed a decreased allocation of carbon with increasing wort gravity; similar proportions were seen in all other strains irrespective of conditions. In addition, higher amounts of carbon in the form of glycogen were observed at 18P15M than at 18P18M conditions,

285 irrespective of yeast strain. The trend for carbon investment in glycogen was also seen for
286 Lager1 at higher wort gravities (24P15M vs 24P24M), however this was not seen for the other
287 strains analysed.

288 Approximately 0.2-0.3 % of the total carbon input was diverted to the production of higher
289 alcohols and esters in each of the four yeast strains examined. When comparing 18P15M and
290 24P15M, strain W34/70 directed slightly higher amounts of carbon into these flavour
291 compounds at 24ºP than 18ºP fermentations whereas the carbon investment of the other strains
292 was not affected by wort density (18 or 24ºP). Additionally, analysis of carbon to flavour
293 compounds indicated that there was no significant difference either between 18P18M and
294 18P15M, or between 24P24M and 24P15M, regardless of yeast strain.

295 Carbon proportion attributed to yeast biomass was estimated in two ways. Initially, carbon-
296 based products other than ethanol, carbon dioxide, glycerol, trehalose, glycogen, higher
297 alcohols and esters were included in the 'un-allocated' portion of the total carbon input. This
298 carbon was attributed to yeast biomass production (yeast biomass 1, Tables 5), produced as an
299 artefact of cell maintenance, growth and division based on the observations of Cutaia (2007).
300 As a means of comparison, the carbon portion associated with generation of yeast biomass was
301 also calculated based on a previous study indicating that approximately 48 % of carbon is used
302 for cellular growth (Van Hoek *et al.*, 1998). Consequently, this was expressed as a percentage
303 of the total carbon input (yeast biomass 2; Tables 5). Although there are variations between
304 these two estimations, they yielded broadly comparable data. At 24ºP fermentations, the
305 proportion of carbon in the form of biomass was observed to be lower ($P < 0.05$) when pitching
306 rate was increased (24P24M compared to 24P15M) for all yeast strains except for W34/70,
307 which showed a similar carbon flow into yeast biomass. In contrast, at 18ºP fermentations, the
308 values were similar ($P < 0.05$) irrespective of pitching rate for both lager strains and one of the

ale strains. The exception being the ale strain NCYC1332 which displayed a lower carbon percentage in the form of biomass at 18P18M when compared to 18P15M. Consequently, the effect of wort gravity and pitching rate on biomass production appeared to be strain-specific, however an overall trend was observed indicating a direct carbon trade-off between ethanol yield and biomass production. This is supported by data indicating that the majority of strains investing in a low carbon to biomass ratio also directed a high proportion of carbon to the formation of ethanol.

These results indicate that the percent carbohydrate conversion to final metabolites (including trehalose, glycogen, higher alcohols and esters) is not majorly affected by initial wort gravity *per se*. At standard gravity the findings reported here are consistent with the assumption of Cutaia (2007) in previous measurements. However, under HG and VHG conditions the absence of a change in carbon utilisation was perhaps surprising, since it was anticipated that increased carbon proportion to cellular protectants such as trehalose and glycerol might be observed. The rationale for this was that at high gravities yeast would require greater concentrations of compounds required to protect or stabilize cell structures. Importantly, the data presented here indicates that even though overall levels of trehalose and glycerol are elevated in HG and VHG brewing fermentations, the percentage of carbon directed to these molecules remained consistent. This observation suggests that (I) the concentration of anti-stress agents may not actually be a measure of the yeast stress response under the conditions applied, as the carbon content in the form of these metabolites was basically conserved under both standard and higher gravity conditions; (II) carbon directed towards these products has little impact on ethanol yield. However it should be noted that there are other important considerations which define the success of a fermentation, including fermentation efficiency and yeast 'fitness' at high gravities (Mansure *et al.*, 1994). If metabolites other than ethanol, carbon dioxide and glycerol do not impact significantly on ethanol yield then it may be pertinent to focus on

elevating such compounds due to their important functional properties in the yeast. It is possible that only a small increase, negligible in terms of ethanol proportion, would result in significant savings in fermentation time and yeast quality.

The data presented here also reveals a strong trade-off between biomass production and ethanol yield, indicating that ethanol production is not only limited by available wort carbohydrates, but is also affected by the growth of the yeast culture. This indicates that increasing pitching rate may be an effective strategy to shift the carbon flux towards ethanol formation during VHG brewing fermentations. While this is not a novel proposal, it certainly suggests that more emphasis should be placed on understanding the precise relationship between cell number and performance at VHG; investigations should be conducted in-house using individual yeast strains to fully appreciate the link between cell number and key performance indicators. Related to this, wort oxygenation is almost certainly of similar significance, since oxygen is required for synthesis of sterols and unsaturated fatty acids (UFAs)), without which cell division cannot occur (Rosenfeld *et al.*, 2003). Sterols are significant not simply to ensure 'healthy' cell membranes, but also due to their 'sparking function' (Rodriguez and Parks, 1983, Gaber *et al.*, 1989), important in allowing the cell to progress from G1 to S in the cell cycle (Rodriguez and Parks, 1983, Gaber *et al.*, 1989). Consequently, since sterol synthesis essentially dictates the extent of yeast population growth, oxygen (as an essential biosynthetic compound) acts to influence the proportion of wort sugars used for the generation of yeast biomass at the expense of ethanol. The fact that cellular oxygen requirements are strain-specific (Jakobsen & Thorne, 1980) indicates that a holistic approach to optimizing process parameters at VHG (incorporating pitching rate, oxygenation and sugar concentration) should be implemented. Furthermore, it should be emphasised that cellular growth has a direct impact on the generation of flavour and sensory compounds, either as by-products of metabolism and synthesis of building blocks (for example through amino acid synthesis), or as a means of redox

balance. As such, from a brewing perspective, the importance of these parameters in terms of matching final product specifications should not be underestimated.

Despite the likely relationship between pitching rate, oxygenation, biomass production and ethanol yield, it should be noted that in this study the derived equations employed for determination of yeast mass balance were based on brewer's wort carbohydrate utilisation and measurable outputs. Consequently the 'remaining' carbon proportion was assumed to comprise yeast biomass, which, although a reasonable assumption, was not able to be evaluated accurately. As such, certain carbon biochemical networks may be under-represented, including the pentose phosphate pathway and routes through nitrogen metabolism. Although a direct calculation of carbon content in biomass yielded broadly comparable data, it should be noted that it was established based on the increase of yeast dry mass and a carbon content of 48 % in a baker's yeast (Van Hoek *et al.*, 1998). In this instance, the occurrence of cell lysis and the carbon composition in individual brewing yeast could also be underestimated, especially under HG and VHG conditions. A further potential source of discrepancy could be related to the measure of carbon dioxide production; although measurement of weight loss is a simple and economic way to quantify carbon dioxide evolution when analysing multiple small scale fermenters, it is accepted that it may not be as accurate as methods that can be applied at scale. Despite this, the observed ethanol to carbon dioxide ratio was remarkably consistent and certainly within the range representing a theoretical ratio of these compounds. However, with respect to the precise carbon quantification, it should be acknowledged that it may represent a source of either over- or under-estimation, and further investigation may be required to achieve a more accurate framework when looking at a narrower range of defined conditions. Moreover, although the start and end points of carbon metabolism are of commercial significance, analysis of intermediate compounds such as pyruvate may also provide some useful information regarding carbon flux distribution (Rui *et al.*, 2010, Quiros *et al.*, 2013, Soons *et al.*, 2013)

particularly with regard to flavour generation. Despite these comments, the data presented here demonstrate significant differences in carbon flux between yeast strains and between environmental conditions. This systematic investigative approach to industrial brewing yeast central carbon metabolism in response to high density wort is novel and may prove to be extremely useful for optimising industrial VHG fermentations.

Conclusions

In this study, an approach was described to evaluate the carbon partitioning of brewing lager and ale strains under a series of lab-scale fermentations at 15ºC using 13ºP, 18ºP and 24ºP brewer's wort. It should be noted that although the fermentation temperature employed was not reflective of industrial ale fermentations, it was applied throughout to allow direct comparison with the commercially significant lager strains analysed, and to remove the relationship between temperature and growth rate as a variable. If temperature had been adjusted for brewing type, this may have masked trends related to the intrinsic capacity of strains to partition carbon. Irrespective, an estimation of total carbon input was calculated based on overall sugar utilisation, related to wort and beer carbohydrate content after eliminating carbon associated with residual sugars present in the final beer. Analysis of carbon partitioning revealed that carbon-based metabolites including trehalose, glycogen, higher alcohols and esters had only a minor effect on overall carbon distribution, whereas yeast biomass acted as a major trade-off with ethanol production. It is proposed that improved fermentation efficiency and yeast health could be achieved by navigation of carbon towards yeast functional compounds such as trehalose without negatively impacting ethanol yield. It is also suggested that the control of cell growth is arguably the most important strategy affecting the conversion of carbon to ethanol. Consequently, we suggest a holistic approach should be taken to harmonize wort gravity, pitching rate and oxygenation for a particular yeast strain. It is anticipated that this data will be immediately useful in highlighting the yeast functional

response to high gravity conditions, as well as in demonstrating the varying requirements of yeast strains. These results may also be applied to provide important insight into the suitability of current production strains for VHG fermentations, or for the selection of novel yeasts with desirable properties more suited to high sugar conditions. While this data has direct implications within brewing, it also impacts on related sectors such as those associated with biofuels, oenology and distilling worldwide.

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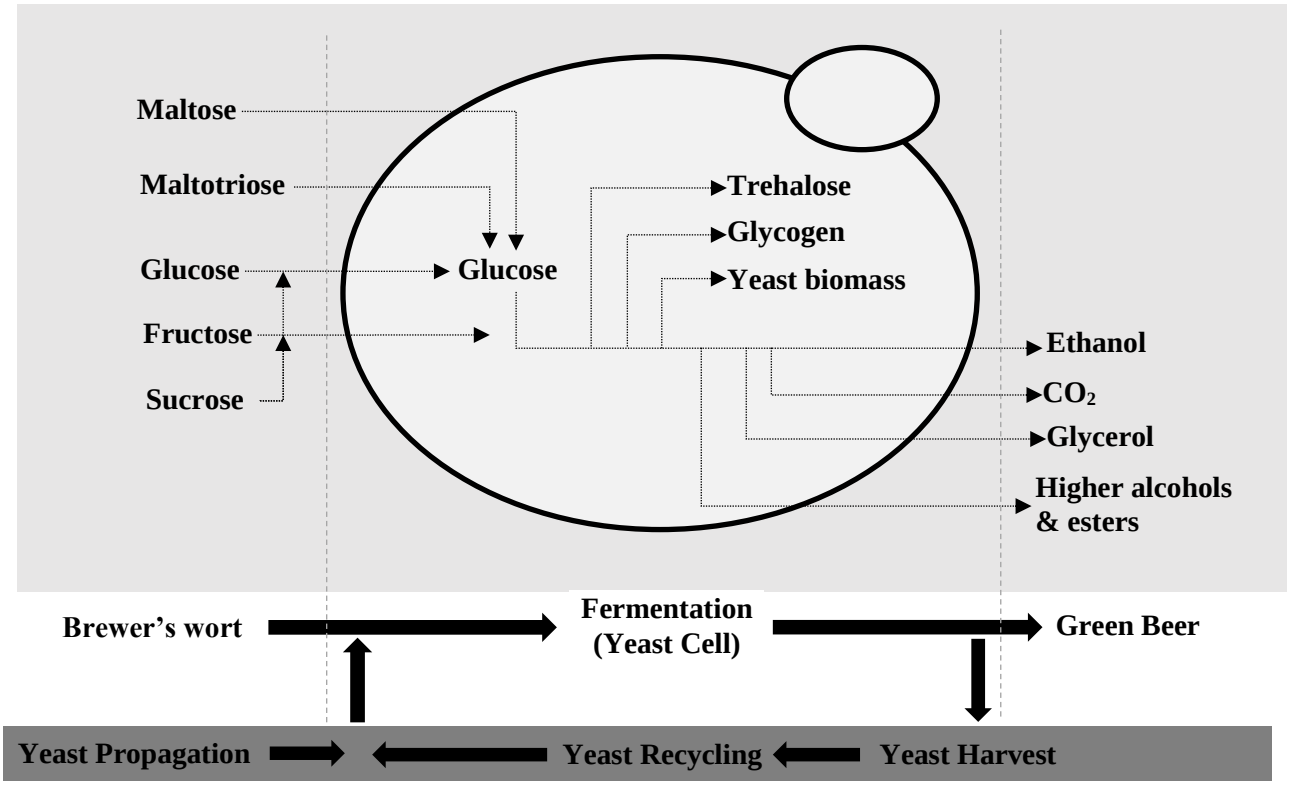


Figure 1 Central metabolism of wort carbohydrates, indicating the major carbon-based metabolites produced by brewing yeast

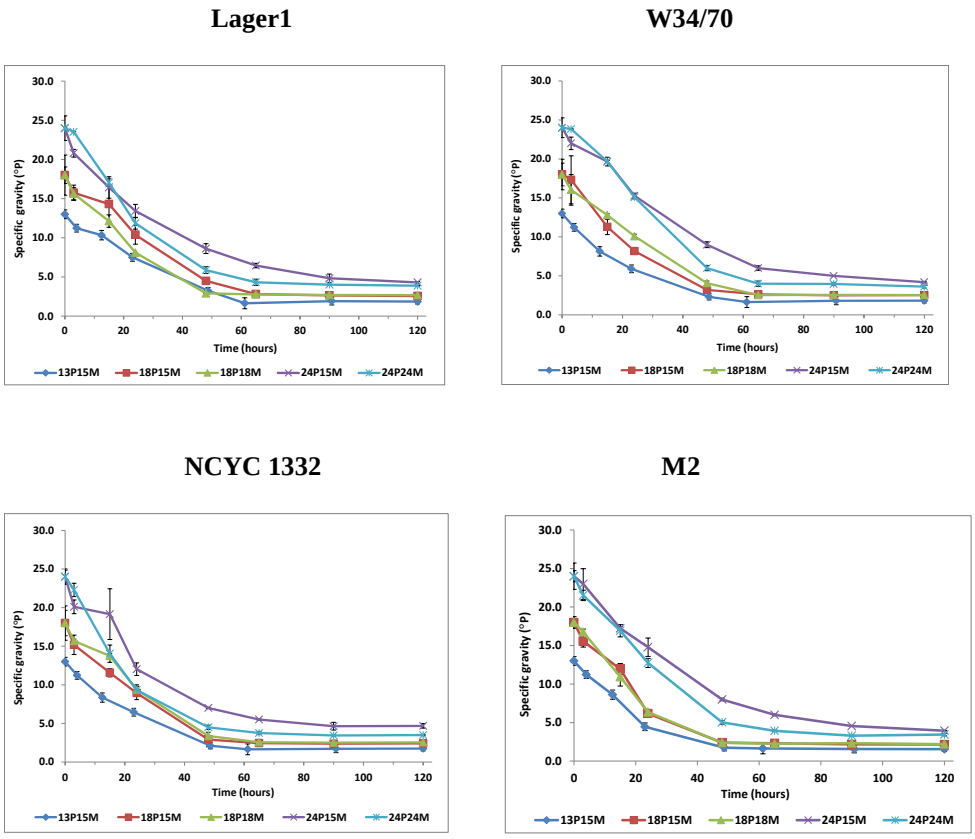


Figure 2 Decrease of specific gravity during fermentations. Data points represent the mean of triplicate samples ± standard deviation from independent experiments. Fermentation conditions were described in Table 1.

Table 1 Experimental parameters applied. The abbreviations listed under test conditions are used throughout the text and refer to the corresponding set of experiments.

Test conditions	***Wort gravity (°P)***	***Pitching rate (Viable cells per mL)***
13P15M	13	1.5×10^7
18P15M	18	1.5×10^7
18P18M	18	1.8×10^7
24P15M	24	1.5×10^7
24P24M	24	2.4×10^7

Table 2 Molecular weight and carbon percentage of key carbon-based compounds analysed (*ratio of carbon in corresponding compound)

Compounds	***Total molecular weight***	***Molecular weight of carbon component***	***Carbon (%)* (Rc)***
Fructose	180.1	72.0	40.0
Glucose	180.1	72.0	40.0
Sucrose	342.0	144.0	42.1
Maltose	342.0	144.0	42.1
Maltotriose	504.4	216.0	42.8
Ethanol	46.1	24.0	52.1
Carbon dioxide	44.0	12.0	27.3
Glycerol	92.1	36.0	39.1
Ethyl acetate	88.1	48.0	54.5
Ethyl propionate	102.1	60.0	58.7
Ethyl butyrate	116.2	72.0	62.0
Isobutanol	74.1	48	64.8
Isoamyl acetate	130.2	84.0	64.5
2-methyl-1-butanol	88.2	60.0	68.1
Ethyl hexanoate	144.2	96.0	66.6

Table 3 Total carbon consumption and corresponding attribution from each carbohydrate group. Data represents the mean ± standard deviation of three independent experiments.

Strain	*Carbon utilization (g/L)* [a]			*Total carbon input (g/L)* [b]
	Monosaccharides	*Disaccharides*	*Trisaccharide*	
			13P15M	
Lager1	5.1 ± 0.4	25.2 ± 1.0	8.3 ± 0.9	38.6 ± 0.9
W34/70	5.2 ± 0.6	25.1 ± 0.6	8.3 ± 0.3	38.7 ± 1.8
NCYC1332	5.2 ± 0.5	25.2 ± 1.0	8.3 ± 0.5	38.7 ± 1.5
M2	5.1 ± 0.8	25.2 ± 0.5	8.3 ± 0.6	38.6 ± 2.2
			18P15M	
Lager1	7.1 ± 1.2	34.1 ± 1.5	10.1 ± 1.3	51.1 ± 1.3
W34/70	6.9 ± 0.9	34.3 ± 0.7	10.1 ± 1.0	50.1 ± 0.9
NCYC1332	6.8 ± 0.6	34.2 ± 1.8	10.1 ± 0.9	50.1 ± 1.6
M2	6.9 ± 1.6	34.0 ± 0.9	10.1 ± 0.5	50.9 ± 0.9
			18P18M	
Lager1	7.0 ± 1.0	34.4 ± 2.1	10.6 ± 0.9	52.0 ± 1.5
W34/70	7.0 ± 0.7	34.5 ± 1.0	10.4 ± 0.5	51.8 ± 1.5
NCYC1332	6.9 ± 0.9	34.4 ± 1.6	10.5 ± 0.6	51.8 ± 1.4
M2	6.9 ± 1.0	34.6 ± 1.1	10.6 ± 0.9	52.1 ± 1.5
			24P15M	
Lager1	10.0 ± 3.1	46.3 ± 0.5	13.1 ± 0.6	68.5 ± 1.0
W34/70	10.2 ± 2.1	46.3 ± 0.9	13.5 ± 1.8	69.8 ± 1.4
NCYC1332	10.0 ± 1.1	45.1 ± 0.6	12.2 ± 0.9	66.3 ± 0.8
M2	10.1 ± 3.0	46.0 ± 0.5	13.0 ± 0.5	69.0 ± 1.8
			24P24M	
Lager1	10.1 ± 1.2	47.0 ± 1.0	14.4 ± 1.4	71.5 ± 1.2
W34/70	10.2 ± 0.9	47.1 ± 0.9	14.4 ± 0.9	71.6 ± 0.9
NCYC1332	10.3 ± 1.1	46.9 ± 0.5	14.1 ± 1.8	71.1 ± 1.1
M2	10.0 ± 0.8	46.9 ± 1.1	14.1 ± 0.8	71.1 ± 0.8

[a] Carbon consumption was quantified based on carbon conservation of the utilized carbohydrate (difference between the original wort and the final beer carbon content) for each carbohydrate group: monosaccharides (fructose and glucose); disaccharides (sucrose and maltose); and trisaccharides (maltotriose). Carbohydrate concentration was determined by HPLC.

[b] Total carbon input (carbon consumption by the yeast) was the sum of the carbon utilization from each carbohydrate group.

Table 4 Carbon partitioning to major metabolites under different fermentation conditions. Data is presented as a percentage of the total carbon consumption and represents the mean ± standard deviation of three independent experiments.

Yeast strain	*Carbon output* [a]	*Fermentation conditions*				
		13P15M	***18P15M***	***18P18M***	***24P15M***	***24P24M***
Lager1	Ethanol	59.3 ± 1.0	59.7 ± 0.5	59.8 ± 0.3	58.9 ± 0.5	60.8 ± 0.3
	Carbon dioxide	30.8 ± 0.7	30.6 ± 0.2	29.9 ± 0.5	30.5 ± 0.3	29.8 ± 0.5
	Glycerol	3.0 ± 0.2	2.8 ± 0.1	3.5 ± 0.2	2.5 ± 0.1	3.5 ± 0.2
	Trehalose	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.09 ± 0.01	0.17 ± 0.01
	Glycogen	0.23 ± 0.05	0.32 ± 0.01	0.22 ± 0.01	0.30 ± 0.02	0.23 ± 0.03
	Higher alcohols and esters	0.20 ± 0.02	0.22 ± 0.04	0.22 ± 0.02	0.21 ± 0.01	0.20 ± 0.03
W34/70	Ethanol	59.8 ± 0.6	60.1 ± 0.7	60.4 ± 0.7	60.2 ± 0.5	60.7 ± 0.4
	Carbon dioxide	29.7 ± 0.3	30.2 ± 0.3	30.2 ± 0.2	30.1 ± 0.2	29.6 ± 0.2
	Glycerol	2.9 ± 0.3	2.8 ± 0.1	2.5 ± 0.1	2.2 ± 0.3	2.3 ± 0.2
	Trehalose	0.08 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.20 ± 0.01
	Glycogen	0.25 ± 0.04	0.27 ± 0.03	0.23 ± 0.01	0.21 ± 0.03	0.29 ± 0.01
	Higher alcohols and esters	0.17 ± 0.03	0.15 ± 0.02	0.20 ± 0.03	0.23 ± 0.03	0.21 ± 0.02
NCYC 1332	Ethanol	57.9 ± 0.7	57.1 ± 0.7	59.0 ± 0.4	57.3 ± 0.5	59.0 ± 0.2
	Carbon dioxide	28.2 ± 0.6	28.4 ± 0.4	29.2 ± 0.5	28.4 ± 0.3	29.1 ± 0.2
	Glycerol	3.2 ± 0.2	3.3 ± 0.3	3.0 ± 0.3	2.7 ± 0.2	2.8 ± 0.2
	Trehalose	0.10 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
	Glycogen	0.30 ± 0.05	0.32 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.28 ± 0.01
	Higher alcohols and ester	0.30 ± 0.02	0.29 ± 0.03	0.32 ± 0.01	0.29 ± 0.03	0.26 ± 0.1
M2	Ethanol	58.6 ± 0.2	58.9 ± 0.5	59.1 ± 0.5	59.2 ± 0.4	60.2 ± 0.3
	Carbon dioxide	29.2 ± 0.3	29.1 ± 0.5	30.0 ± 0.3	29.5 ± 0.1	30.1 ± 0.7
	Glycerol	3.1 ± 0.1	3.0 ± 0.5	2.8 ± 0.2	2.7 ± 0.2	2.8 ± 0.1
	Trehalose	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
	Glycogen	0.31 ± 0.02	0.33 ± 0.01	0.26 ± 0.02	0.21 ± 0.01	0.26 ± 0.01
	Higher alcohol and ester	0.16 ± 0.01	0.24 ± 0.01	0.30 ± 0.01	0.28 ± 0.02	0.32 ± 0.0

[a] Calculations were based on the values obtained from analysis of the compounds at start and end point of each set of fermentations. Carbon proportion attributed to biomass is presented in Table 5.

Table 5 Carbon proportion attributed to biomass using different formulae indicated by [a] and [b]. Data is presented as a percentage of the total carbon consumption and represents the mean ± standard deviation of three independent experiments.

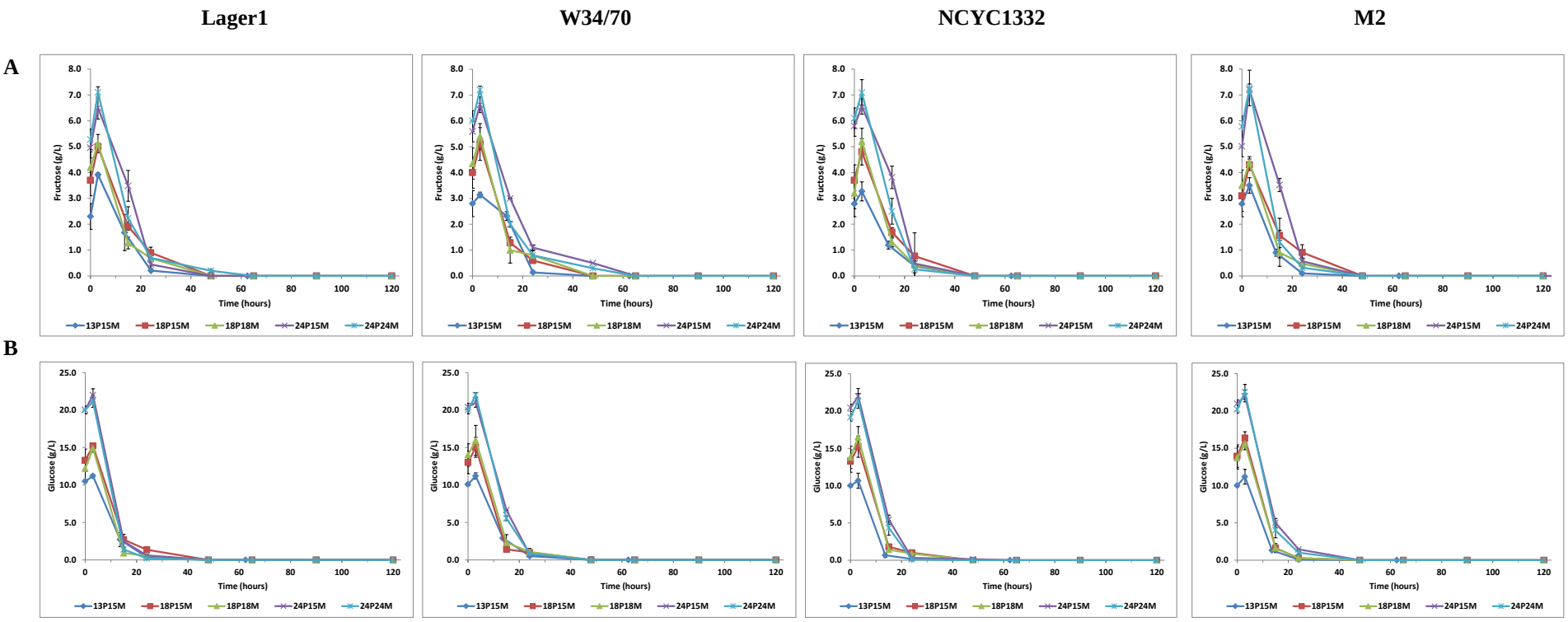
Yeast strain	*Carbon output*	*Fermentation conditions*				
		13P15M	***18P15M***	***18P18M***	***24P15M***	***24P24M***
Lager1	Yeast biomass 1 [a]	6.4 ± 0.9	6.3 ± 0.3	6.3 ± 0.9	7.5 ± 0.5	5.3 ± 0.4
	Yeast biomass 2 [b]	6.6 ± 0.4	6.1 ± 0.5	5.9 ± 0.2	6.9 ± 0.3	5.7 ± 0.1
W34/70	Yeast biomass 1 [a]	7.1 ± 0.8	6.4 ± 0.7	6.4 ± 0.2	7.0 ± 0.2	6.7 ± 0.4
	Yeast biomass 2 [b]	7.5 ± 0.5	6.6 ± 0.3	6.3 ± 0.5	6.6 ± 0.4	6.4 ± 0.6
NCYC 1332	Yeast biomass 1 [a]	10.0 ± 0.5	10.5 ± 0.5	8.1 ± 0.8	10.6 ± 0.6	8.3 ± 0.5
	Yeast biomass 2 [b]	9.2 ± 0.6	9.6 ± 0.4	8.5 ± 0.5	9.8 ± 0.3	8.5 ± 0.5
M2	Yeast biomass 1 [a]	8.6 ± 0.6	8.4 ± 0.5	7.5 ± 0.4	8.1 ± 0.7	6.3 ± 0.6
	Yeast biomass 2 [b]	8.4 ± 0.4	7.9 ± 0.7	7.6 ± 0.3	7.5 ± 0.6	5.9 ± 0.5

[a] Data compromises un-allocated carbon proportion of the total carbon consumption, assuming that this percentage of carbon was attributed to yeast biomass, most likely as an artefact of cell maintenance, growth and division (Cutaia, 2007).

[b] Data was calculated from the increase in yeast dry weight at the end of each set of fermentations, based on the assumption that a carbon content comprises in 48% of the dry yeast biomass of 48 % (Van Hoek *et al.*, 1998).

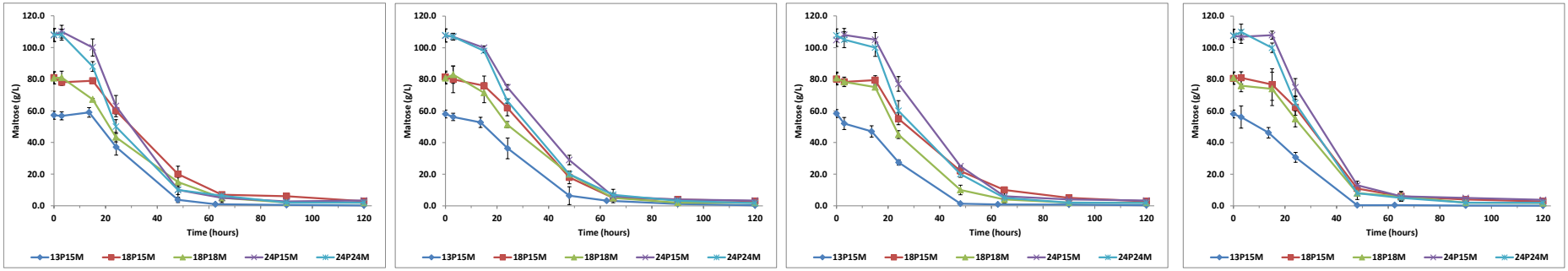
Supplementary data:

Concentrations of fructose (A), glucose (B), maltose (C) and maltotriose (D) during fermentations. Data points represent the mean of triplicate samples ± standard deviation from independent experiments. Sucrose was present in unfermented wort (2.1 ± 0.5 g/L at 13ºP, 3.0 ± 0.8 g/L at 18ºP and 5.6 ± 1.7 g/L at 24ºP), but not detected during subsequent analyses. This is most likely because sucrose was hydrolysed prior to the first sampling point at approximately 3 hours, leading to the transient increase in fructose and glucose.



Lager 1 **W34/70** **NCYC1332** **M2**

C



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