invited paper

G.G. Stewart

Brewer's yeast research – a keynote paper – part 1

This is the first part of a two-part article that considers the research activities associated with brewer's yeast strains. Initially, this part discusses factors that have directly influenced such activity such as financing, qualified and experienced researcher availability, the advent of novel technology/instrumentation and also developments in brewing fermentation procedures. Subsequently, this part reviews specific areas such as ale/lager yeast similarities and differences, wort fermentation with an influence on the uptake and metabolism of sugars and free amino nitrogen and the production of beer flavour metabolites during fermentation (metabolomics). Finally, stress effects on yeast with an emphasis on the fermentation of high gravity wort are discussed. In the second part of this article, yeast flocculation, centrifugation, genetic manipulation and the spontaneous mutation of brewer's yeast strains are considered.

Descriptors: ale/lager yeasts, beer flavour metabolites (metabolomics), brewing procedures, high gravity worts – stress effects, sugar and free amino nitrogen uptake and metabolism

1 Introduction

Comprehensive review papers considering the current situation regarding brewer's yeast research abound [98, 99, 111]. As a consequence, the question is: "Why are additional ones required?" Although there are a number of answers to this question, the two keynote papers focus on recent phenotypic, metabolic and genotypic studies that support our understanding of brewer's wort fermentation employing ale, lager and non-*Saccharomyces* yeast strains [37, 123]. However, it is not these papers' objective to provide a comprehensive review of research in this subject area dating from Pasteur's and Hansen's contributions and even earlier [38, 69]. The objectives of these papers are to consider recent developments on this subject and to reflect how yeast will influence contemporary brewer's wort fermentation practices.

Although brewer's yeast research has been extensively conducted since the end of World War II, and earlier, a large number of relevant peer reviewed papers, review articles and books have been published. The last 50 years have experienced an escalation in active brewer's yeast research. This has led to the publication of an increasing number of relevant papers in brewing focussed, open access and pure science journals. The principal reasons for this increase in yeast research can be summarised (not in order of priority) as follows:

https://doi.org/10.23763/BrSc19-03stewart

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- Financial support for yeast research from both the private and public sectors has increased due, in large part, to the acknowledgment that yeast is a model eukaryote [85] as well as being a very important microorganism economically [117]. Nevertheless, the consolidation of a number of international major brewing and distilling companies is resulting in some concern about their commitment to basic brewing research, particularly yeast. However, the advent and growth of the craft brewing sector in North America, Europe, Australia/New Zealand and elsewhere is beginning to fill this growing vacant hiatus.
- Increase in the number of research workers such as postgraduate students, postdoctorial scientists, research associates and technicians focusing on yeast research.
- Development of a large number of relevant analytical techniques, supported by novel instruments and techniques, many of which are automated. These techniques and instruments include: gas chromatography (GC), high performance liquid chromatography (HPLC), mass spectroscopy (MS), nuclear magnetic resonance (NMR), electron microscopy (EM) including scanning electron microscopy (SEM), scintillation counting, flow cytometry, confocal microscopy, electrophoresis including DNA fingerprinting and protein separation, polymerase chain reaction (PCR) [63] and a plethora of other molecular biology analytical methods. All of these instruments and techniques have played a major role to assist the progress of yeast research.
- Novel molecular biology methodology has been employed to enhance our overall knowledge of yeast genetics. There are a number of genetic manipulation methods which include: hybridisation, spheroplast fusion, rare mating, mutation and recombinant DNA and transformation. Also, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has recently been introduced. This latter research method has been modified in order to edit yeast genomes by allowing existing genes to be removed and/or new ones added [118].
- Developments in brewing fermentation procedures have also

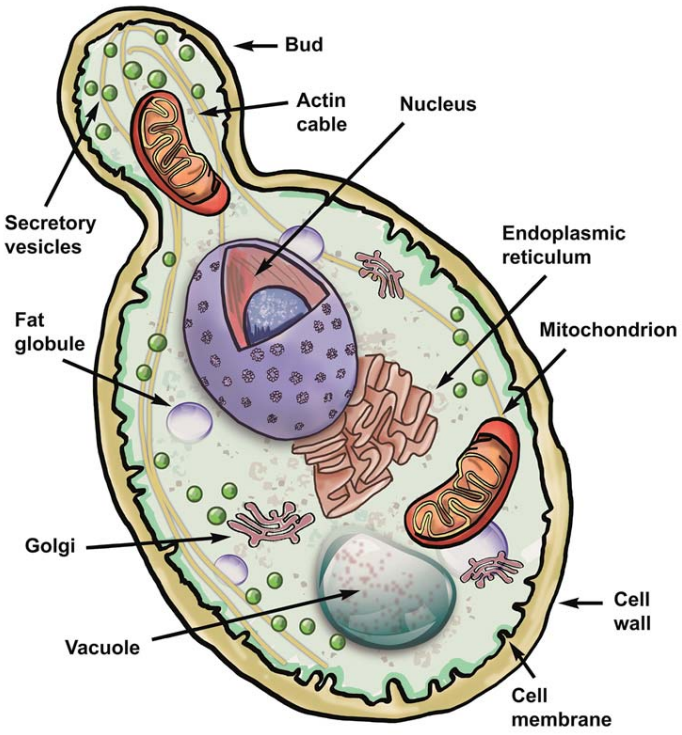

Fig. 1 Main intracellular features of a typical budding brewer's yeast cell

stimulated yeast research, along with the analytical methods already described. These include:

– Continuous fermentation [82]
– Fermentation of high gravity worts [108]
– Yeast culture collection, storage and propagation [97]
– Large scale vertical fermentation [64]
– Use of centrifuges to crop yeast [13].

Beer flavour and aroma is, in large part, determined by the yeast strain employed together with the composition of the wort fermented. In addition, yeast properties such as flocculation, fermentation ability (this includes the metabolism of wort sugars, amino acids, small peptides, ammonium ions, metal ions, etc., - details later), stress effects on yeast (for example, osmotic pressure, ethanol tolerance, mechanical stress, etc), and oxygen requirements all have a critical impact on fermentation performance. In addition, the production of a plethora of metabolites, many of which influence beer flavour, is a critical parameter. Also, proprietary yeast strains, belonging to individual breweries or brewing corporations, are also employed and usually jealously guarded and conserved [97, 110].

No satisfactory definition of yeasts that extensively encompasses its commonly encountered fermentation properties such as alcohol fermentation, sedimentation and growth characteristics (budding or fission) exists. Although nearly all (not all – some are fission yeasts [103]) yeast cells multiply by budding and are single cells (Fig. 1). Brewer's yeast cultures are fungi that predominantly belong to the genus *Saccharomyces*. There are also a minority of non-*Saccharomyces* cultures employed in brewing which will be discussed later. Yeast is propagated [10] in wort (an acidic aqueous malt extract sugary solution) of medium concentration (gravity – 10–12 °Plato) which permits the development of biomass. The propagated yeast culture is then pitched (inoculated) into a static fermentation containing wort (usually 10–16 °Plato initial gravity) – details of high gravity brewing (HGB) later. During fermentation, the cells absorb the dissolved sugars, simple nitrogenous matter (amino acids, ammonium ions and simple peptides), vitamins and ions through their plasma membrane (Fig. 1). Subsequently, they employ a series of reactions known as metabolic pathways (glycolysis, biosynthesis of cellular constituents, etc.) and use these nutrient materials for growth and fermentation. It is important to emphasise that the primary glycolytic products are: ethanol, carbon dioxide and glycerol [15] together with a number of secondary metabolites, many of which contribute to beer flavour potential (Fig. 2) [106].

Saccharomyces cerevisiae (ale yeast) has the ability to take up and metabolise a wide range of sugars, for example, glucose, fructose, mannose, galactose, sucrose, maltose, maltotriose and raffinose (in part). In addition, as will be described later, a subspecies of *S. cerevisiae*, *Saccharomyces cerevisiae* var. *diastaticus* is also able to utilise dextrins (partially hydrolysed starch). In addition, *Saccharomyces pastorianus* (lager yeast) is able to utilise the disaccharide melibiose (glucose-galactose) as well as the sugar spectra taken up by *S. cerevisiae*. This melibiose utilising property can be used as a diagnostic test to distinguish between ale and lager yeast strains. The other distinguishing test is that

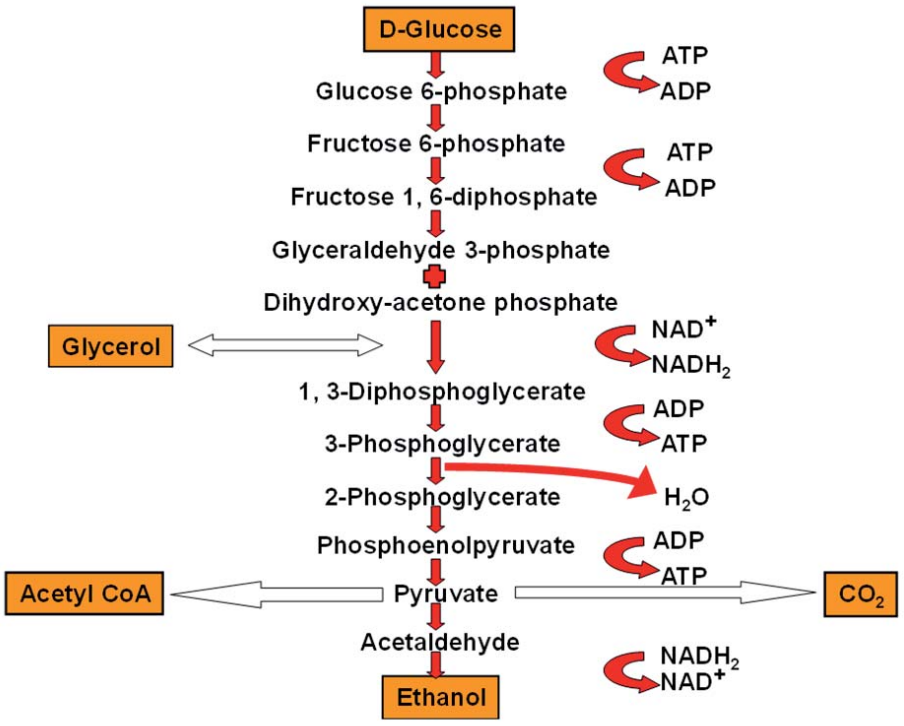

Fig. 2 The Embden-Meyerhof-Parnas glycolytic pathway

ale strains will grow at 37 °C (and above) whereas lager strains are unable to grow above 34 °C. Enzymatic hydrolysis of starch, as would occur during typical brewer's mashing of barley malt procedures, leads to wort consisting of a number of fermentable sugars – glucose, fructose, sucrose, maltose and maltotriose. The predominant sugars in most brewer's worts are: glucose, maltose and maltotriose (Fig. 3).

The other major group of wort constituents is free amino nitrogen (FAN). FAN is the sum of nineteen individual wort amino acids, ammonium ions and small peptides (di- and tripeptides). FAN is an important general measure of yeast nutrients which constitute yeast assimilable nitrogen during wort fermentations [54].

As well as wort constituents, the wort concentration (gravity) is critical. The whole question of HGB and the influence of concentrated wort on fermentation and yeast function will be discussed later. Another major factor that influences brewer's yeast activity is its cell wall structure and its influence on flocculation – this will also be discussed in part 2.

2 Wort fermentation

The objectives of brewing wort fermentation are to consistently metabolise wort constituents, in a sterile environment, into ethanol, carbon dioxide and glycerol and a number of other fermentation products in order to produce beer with satisfactory quality and stability [110]. Another objective is to produce yeast crops that can be confidently re-pitched into subsequent brews [107]. It is noteworthy that brewing is the only major fermentation process that recycles its yeast culture from one fermentation to another. Fermentation systems in the production of most potable and industrial ethanol products such as: wine, saké, whisky, brandy, gin, vodka, cider and industrial ethanol only use a yeast culture once. Recently, the multiple use of a yeast culture in the production of fuel alcohol, particularly in Brazil, is increasing [122]. Also, all these fermentations are conducted in non-sterile conditions. As a consequence, acid washing of some yeast cultures, particularly cultures being used for fuel alcohol production, is increasing [95]. The management of a yeast culture between fermentations is a critical part of the brewing process. It is important to jealously protect the quality of cropped yeast because it will be used to pitch a subsequent wort fermentation and will, therefore, have a profound effect on fermentation efficiency and the quality of the resulting beer [113].

Over time, considerable attention has been devoted to studies focussing on brewer's yeast (together with industrial yeast strains in general). The objectives of these studies can be summarised as two-fold:

- To learn more about the yeast's taxonomy, biochemistry, genetics and molecular biology and
- To improve the overall wort fermentation performance of such strains with particular emphasis being placed on more efficient substrate utilisation capabilities, increased ethanol production rates, and improved tolerance to environmental stress conditions such as temperature, elevated osmotic pressure, acetaldehyde and ethanol and finally to understand the mechanism(s) of flocculation [98].

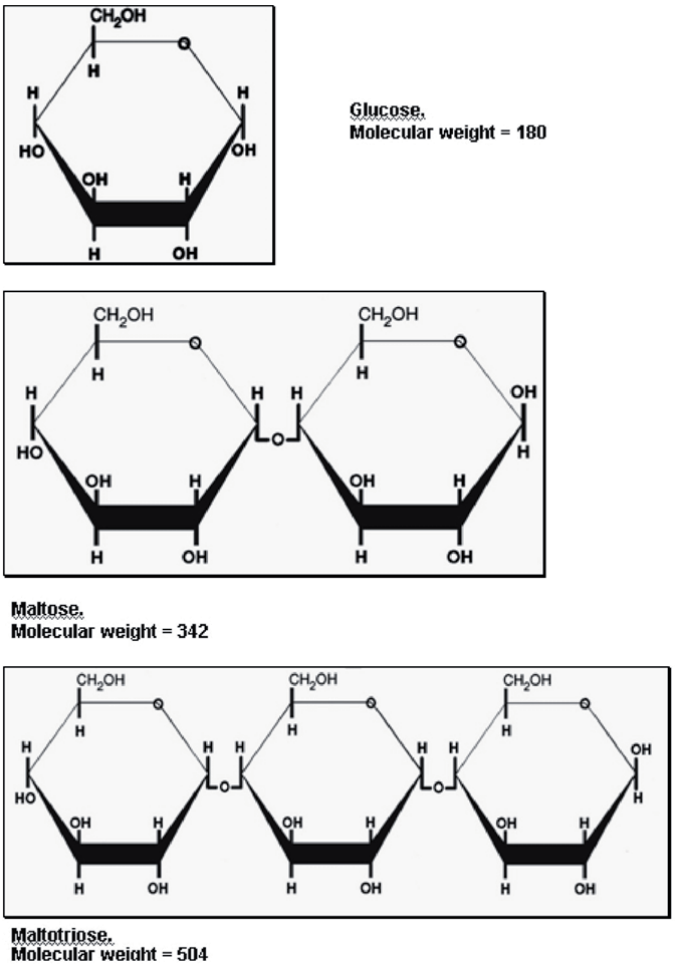

Fig. 3 Glucose, maltose and maltotriose structures

3 Basic differences between ale and lager yeast strains

There are a number of differences in the production of ale and lager beers with the major ones being the characteristics of the ale and lager strains employed and the fermentation temperature used. Extensive studies by many breweries, research institutions and universities on this topic have been conducted [101] and typical differences between ale and lager yeast strains established (Table 1, see page 34). The advent of molecular biology-based methodologies including gene sequencing of the ale and lager brewing strains has shown that they are interspecies hybrids with homologous relationships to one another and also to *Saccharomyces bayanus*, a yeast species used in wine fermentation and identified as a wild yeast in brewing fermentations [56]. The gene homology between *S. pastorianus* and *S. bayanus* strains is high at 72 %, whereas the homology between *S. pastorianus* and *S. cerevisiae* is considerably lower at 50 % [70]. It is worthy of note that the homology between human DNA and orangutan DNA is 96 %!

In 2011, a paper entitled "Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast" was published

Table 1 Basic differences between ale and lager yeast strains

Ale Yeast	Lager Yeast
Saccharomyces cerevisiae (ale type)	*Saccharomyces carlsbergensis*
Saccharomyces cerevisiae (ale and distiller's yeast)	*Saccharomyces uvarium* (carlsbergensis)
	Saccharomyces cerevisiae (lager type)
	Saccharomyces pastorianus (current taxonomic name)
Fermentation temperature (18–25 °C)	Fermentation temperature (8–15 °C)
Cells can grow at 37 °C or above	Cells cannot grow above 34 ºC
Cells cannot metabolise the dissacharide melibiose (galactose-glucose)	Cells can metabolise melibiose
Strains develop a distinctive colonial morphology on wort-gelatin medium	Strains do not have a distinctive morphology on wort-gelatin medium
"Top" fermentation	"Bottom" fermentation

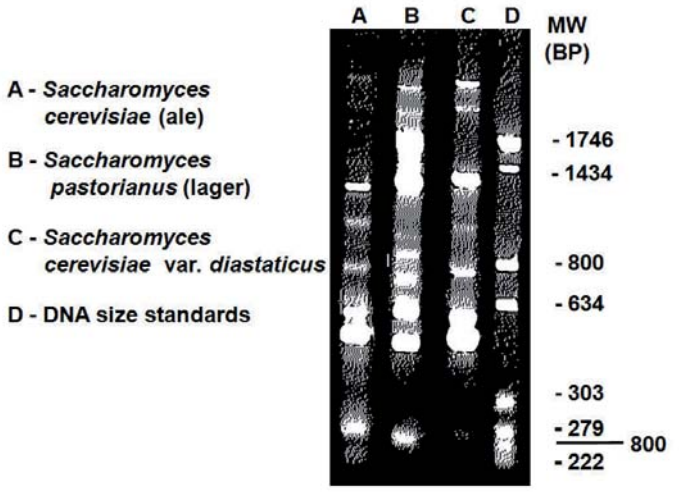

Fig. 4 Fingerprint patterns using polymerase chain reaction (PCR) technology and electrophoresis to differentiate brewer's and wild yeast strains

[56]. This study confirmed that *S. pastorianus* is a domesticated yeast species created by the fusion of *S. cerevisiae* with a previously unknown species that has been designated *Saccharomyces eubayanus* because of its close relationship to *S. bayanus*. This paper proposed that *S. eubayanus* only exists in the forests of Patagonia, South America.

Since this publication, *S. eubayanus* has also been isolated and identified in Tibet [9], the USA [51] and New Zealand [12]. It has not been found in Europe. It can tolerate lower incubation temperatures (8–12 °C). The initial publication from Argentina contains a draft genome sequence of *S. eubayanus* [56]. It is 99.5 % identical to the non-*S. cerevisiae* DNA fragment of the *S. pastorianus* genome sequence. This suggests specific changes in wort sugar [116] and sulphur metabolism [93], together with fermentation temperature optima [56], compared to ale strains, that are critical for determining lager beer characteristics. A detailed DNA sequence of *S. eubayanus*, compared to *S. pastorianus*, has also been published [4].

Traditional methods for differentiating brewing yeast strains are relatively simple. These tests are designed to detect differences in such phenotypic properties as colony morphology, flocculation, sensitivity to a number of relevant chemicals (ethanol, acetaldehyde and acetic acids) and their reaction to a variety of stresses. However, these tests exhibit a number of disadvantages because of their:

- Lack of objectivity – the results can be open to misinterpretation;
- Poor sensitivity – it is often difficult to detect differences between closely related strains;
- Lengthy response times – one week or more;
- Poor reproducibility – the test can have a profound influence on the conclusions [40].

Yeast strains vary from one another because of differences in their genetic make-up. As a consequence, the most direct and rapid approach to distinguish yeast strains involves DNA (and protein) analysis. There are a number of such methods (some of which have already been discussed) in this paper's Introduction:

- Denaturing gradient gel electrophoresis [76];
- Karyotyping, analysis of whole chromosomes [40];
- Mitochondrial DNA – RFLP (Restriction Fragment Length Polymorphism [84];
- Mass-spectrometric methods [120];
- Protein fingerprinting [49, 58];
- Polymerase chain reaction (PCR) for in vitro amplification of DNA fragments [81].

PCR is an in vitro method for amplifying very small amounts of selected nucleic acids (DNA or RNA) by several orders of magnitude over short time periods. This technique permits the detection of specified DNA fragments by making multiple copies. Employing this technique, a specific fragment of DNA from a particular yeast strain can be isolated, amplified (increased in amount – over a million-fold) and subsequently used to produce a fingerprinting pattern of different yeast species, as is shown in figure 4, PCR was developed by Kary Mullis and Michael Smith who jointly received the 1993 Nobel Prize for Chemistry to recognise this achievement [63].

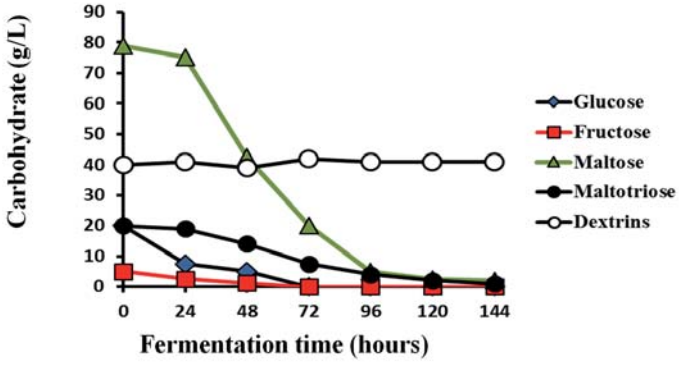

Fig. 5 The fermentable sugars in wort and their uptake order by a brewer's yeast culture

4 Uptake and metabolism of wort sugars

It is very important that wort sugars (and the FAN complex) are efficiently taken into the cell. In the normal situation, brewing yeast strains (both ale and lager) are capable of utilising wort sucrose, glucose, fructose, maltose and maltotriose in this approximate sequence (or priority), although some degree of overlap does occur. Characteristically, glucose, maltose and maltotriose are the predominant wort sugars (Fig. 5). The majority of brewing yeast strains leave maltotetraose (G4) and larger dextrins unfermented. However, *S. cerevisiae* var *diastaticus* is able to utilise dextrin material, albeit inefficiently. Wort dextrin utilization is possible by this yeast sub-species due to the secretion extracellularly of glucoamylase. However, this enzyme, produced by *S. diastaticus*, is incapable of hydrolysing the α-1,6 bonds of dextrins whereas it is able to hydrolyse the α-1,4 bonds [32].

The initial step in the utilisation of any wort sugar is either its passage intact across the yeast plasma membrane, or its hydrolysis outside this membrane, followed by entry into the cell of some or all of the hydrolysis products (Fig. 6). Maltose and maltotriose are examples of the sugars that pass intact across the cell membrane [131], whereas sucrose and dextrins are hydrolysed by extracellular enzymes and the resulting simple sugars are taken into the cell.

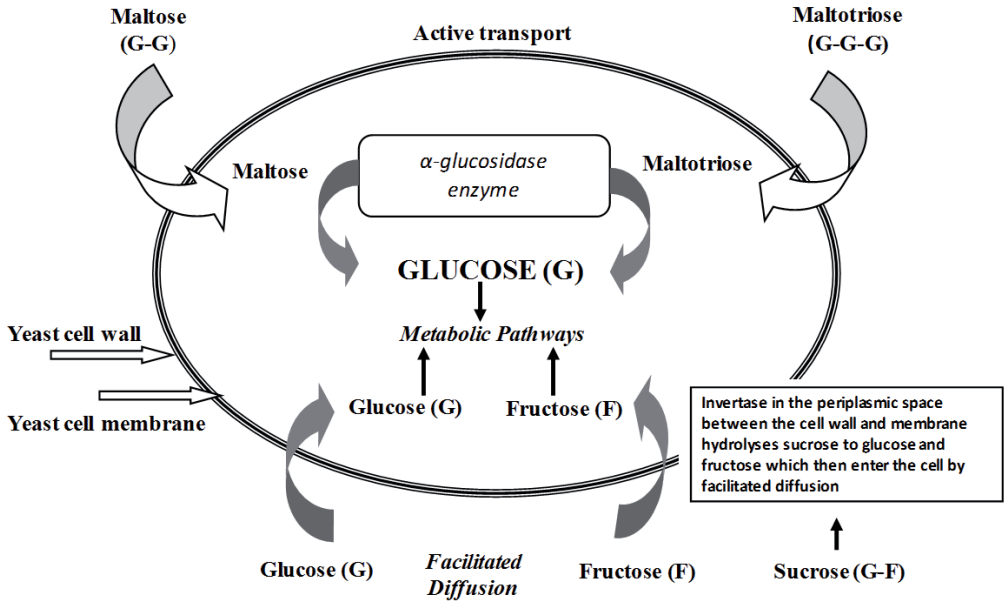

Fig. 6 Uptake of wort sugars by a yeast culture

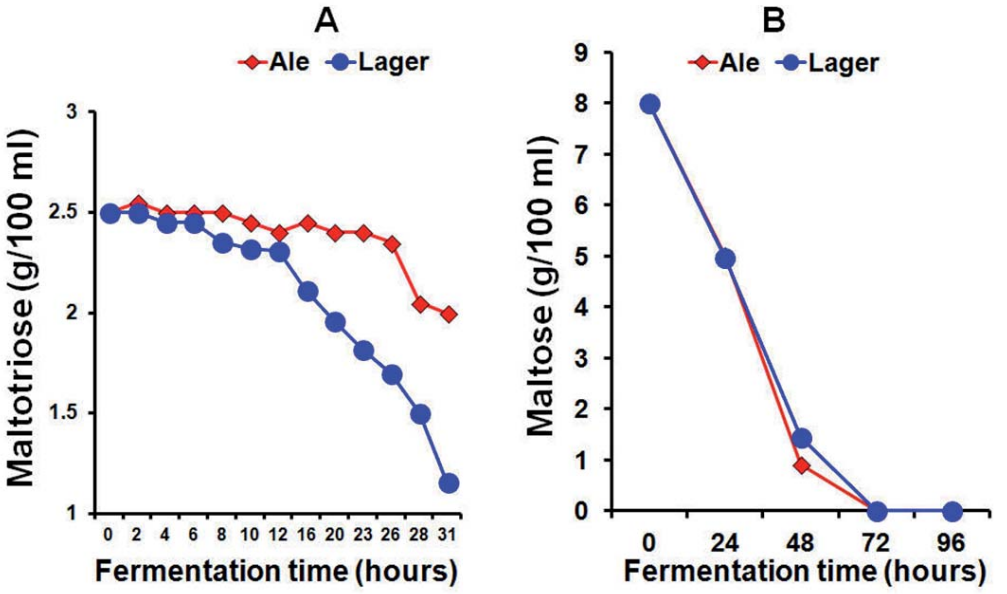

Fig. 7 Maltotriose (A) and maltose (B) uptake profiles from 16 °Plato wort with ale and lager yeast strains (results are means of fermentations with 8 ale and 8 lager yeast strains)

An important metabolic difference between the uptake of monosaccharides such as glucose and fructose and disaccharides/trisaccharides such as maltose and maltotriose is that energy (ATP conversion to ADP) is required for maltose and maltotriose uptake (active transport) whereas glucose and fructose are taken up passively with no energy necessary [29]. As maltose and maltotriose are the major sugars in brewer's wort, the ability of a brewing yeast strain to use these two sugars is critical and depends on the strain possessing the correct genetic complement. Brewer's yeast strains possess independent uptake mechanisms for maltose and maltotriose that transport their sugars across the cell's plasma membrane into the cell [104]. Once inside the cell, both sugars are hydrolysed to glucose units by the α-glucoside system (Fig. 6). The transport, hydrolysis and maltose/glucose interaction fermentation systems are particularly important in brewing, distilling and baking since maltose is the major sugar component of brewer's wort, spirit mash and wheat dough [89]. Maltose fermentation by brewing, distilling and baking yeast strains requires at least one of five MAL loci, each consisting of three genes encoding:

- The structural gene for α-glucosidase (maltase) (*MALS*);
- Maltose/maltotriose permeases (*MALT*);
- An activator whose product co-ordinately regulates the expression of the α-glucosidase and permease genes.

The expression of *MALS* and *MALT* is regulated by maltose induction and repression by glucose. When wort glucose concentrations are high (>10 mg/L), the *MAL* genes, in most brewer's yeast strains, are repressed and only when 40–50 % of the glucose has been taken up by the yeast culture from the wort will maltose and

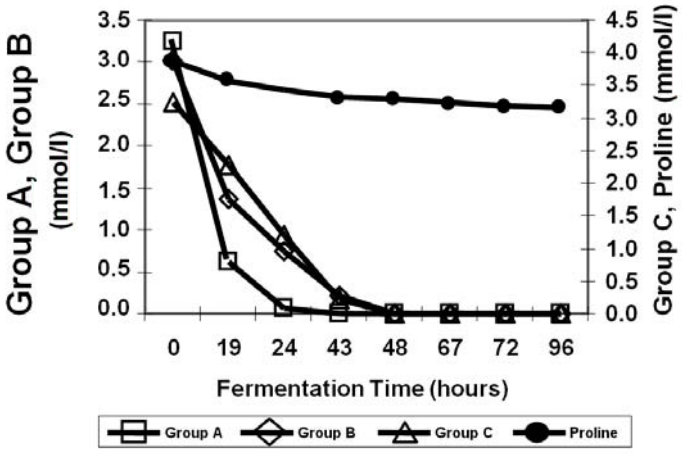

Fig. 8 Amino acid adsorption patterns during wort fermentation

maltotriose uptake commence (Fig. 5). The glucose repression of maltose uptake can be overcome in ale strains but not, in our experience, by lager strains [30]. However, the whole question of derepression of sugar uptake by ale and lager yeast strains is a complex, and largely undefined, problem.

A number of ale and lager yeast strains (eight strains from each species) have been used to explore maltose and maltotriose uptake mechanisms in wort. For example, the fermentation profile of a 16 °Plato all-malt wort, fermented in a 30 litre static vessel at 15 °C (Fig. 7), is the mean of eight results obtained. Under these conditions, lager strains utilised maltotriose more efficiently than ale strains, whereas maltose utilisation efficiency was not dependent on the type of brewing yeast species or the strain employed in this study [132, 86]. This supports the proposal that maltotriose and maltose possess independent but closely linked uptake (permease) systems [16]. In addition, this consistent difference between ale and lager strains supports the observation that ale strains appear to have greater difficulty than lager strains to completely ferment wort, particularly with high gravity worts (> 16 °Plato) [109].

5 Uptake and metabolism of free amino nitrogen (FAN)

FAN is a general measure of a yeast culture's assimilable nitrogen's metabolic efficiency. It is a good index of yeast growth and consequently its fermentation efficiency and sugar uptake [72]. Wort FAN is essential for the formation of new yeast amino acids, the synthesis of structural and enzymatic proteins, cell proliferation, and cell viability and vitality [43]. Lastly, FAN levels have a direct influence on the synthesis of beer flavour compounds (for example, higher alcohols, carbonyls and esters).

There are differences between lager and ale strains with respect to wort assimilable nitrogen uptake characteristics [44]. Nevertheless, with all brewing strains the amount of wort FAN content required by yeast under normal brewery fermentation conditions is directly proportional to yeast growth and certain aspects of beer maturation [50]. There has been considerable debate regarding the minimal wort FAN concentration required in order to achieve satisfactory yeast growth and fermentation performance in conventional gravity (10–12 °Plato) and HG worts which is considered to be 130 mg FAN/L for the former. Rapid attenuation of HG worts (> 16 °Plato) requires increased levels of FAN [55]. However, optimum wort FAN levels differ from fermentation to fermentation, from yeast strain to yeast strain and between the plethora of yeast species employed in the industry. Furthermore, optimum FAN values are dependent on different wort sugar levels and their type [112].

Studies on the uptake of wort nitrogen by brewer's yeast strains began over 50 years ago. During the 1960s, Margaret Jones and John Pierce, working in the Research Department of the Guinness Brewery in Park Royal, London, conducted notable studies on nitrogen metabolism during malting, brewing and fermentation [45]. They reported that the absorption and utilisation of exogenous nitrogenous wort compounds and their synthesis intracellularly are controlled by three principal factors:

- Total wort concentration of assimilable nitrogen;
- The concentration of individual nitrogenous compounds and their ratio;
- The competitive inhibition of these components uptake (mainly, but not exclusively, amino acids) via various permease systems [46].

A unique classification of wort's 19 amino acids has been established according to their rates of consumption during brewing. There are four groups of amino acids. Three groups of them are taken up at different stages of the fermentation cycle and the fourth group consists of only one amino acid, proline (the largest concentration amino acid in wort), which is not taken up during brewing fermentations (Fig. 8). This is because of the anaerobic conditions that prevail late in wort fermentations (oxygen is necessary for proline metabolism) [59]. When this classification was developed in the 1960s, the methodology employed liquid chromatography for measuring individual amino acids and was iconic! Similar measurements currently use automated computerized HPLC and it is difficult to envisage and appreciate the analytical challenges that were faced and overcome 50 years ago! Indeed, it has already been discussed that advances in analytical methods have been a primary reason for increasing our knowledge of wort fermentation control (both FAN and sugars).

Table 2 Order of wort amino acids and ammonia uptake during fermentation

Group A	Group B	Group C	Group D
Fast Absorption	**Intermediate Absorption**	**Slow Absorption**	**Little or No Absorption**
Glutamic acid	Valine	Glycine	Proline
Aspartic acid	Leucine	Phenylalanine	
Asparagine	Isoleucine	Tyrosine	
Glutamine	Histidine	Tryptophan	
Serine		Alanine	
Methionine		Ammonia	
Threonine			
Lysine			
Arginine			

The Jones and Pierce amino acid classification [46] continues to be the basis of our current understanding of the relative importance of individual wort amino acids during fermentation. This information has assisted manipulation of wort nitrogen levels by the addition of supplements such as yeast extracts or specific amino acids, particularly during HG brewing – details later. However, this assimilation pattern is often specific to the fermentation conditions and the nutritional preferences of a particular strain. The nutritional requirements, particularly the utilization of wort amino acids, has recently been re-examined [39] and the overall Jones and Pierce classification confirmed using contemporary analytical techniques (HPLC, etc.) with one exception. The order of methionine uptake has been transferred from Group B to Group A (Table 2) [55].

Approximately 30 % of incorporated nitrogen compounds come from sources other than amino acids [53]. Although the utilisation of small peptides by brewing yeasts was confirmed over 50 years ago, an understanding of their role for yeast nitrogen requirements is still limited. Small peptides can be used as nutritional sources of amino acids as both carbon or nitrogen materials and precursors of cell wall peptides during growth. Although, growth is considerably slower when they are the sole nitrogen source [54]. Polypeptides may also be used as a substitute because yeast can generate proteolytic enzymes extracellularly to provide additional assimilable nitrogen to the culture [14].

Most brewing yeast strains transport peptides that consist of no more than three amino acid residues but this molecular size limit is strain dependent [126]. Nevertheless, small wort peptides are an important source of assimilable nitrogen and 20–40 % of wort oligopeptides are used during fermentation. In a similar manner to single amino acids, peptides probably contribute to beer character and flavour [53].

A method to measure small wort oligosaccharides has now been developed [54]. The sample is deproteinized and the supernatant ultrafiltered through a membrane with a 500Da exclusion limit. The filtrate is subsequently acid and alkaline hydrolysed and the hydrolysate subjected to amino acid analysis by HPLC [53].

6 Specific aspects of wort fermentation

The metabolism of wort sugars and FAN during fermentation has already been discussed but a plethora of other metabolic reactions also occurs which will not be discussed in their entirety here. They are covered in greater detail in the cited references [21,29]. As a consequence, only five reactions will be considered here:

The repressing effects of glucose on the uptake of maltose and maltotriose during wort fermentation have already been discussed but not in detail (Fig. 5). This repression is a major factor that influences wort fermentation together with overall rate and extent. Maltose uptake only commences when approximately 50 % (this is both yeast strain and wort composition dependent) [23] of the wort glucose (and fructose) has been taken up by the yeast cells (Fig. 5). In other words, with most strains of *S. cerevisiae* and *S. pastorianus*, maltose utilization is subject to control by carbon catabolite repression [17].

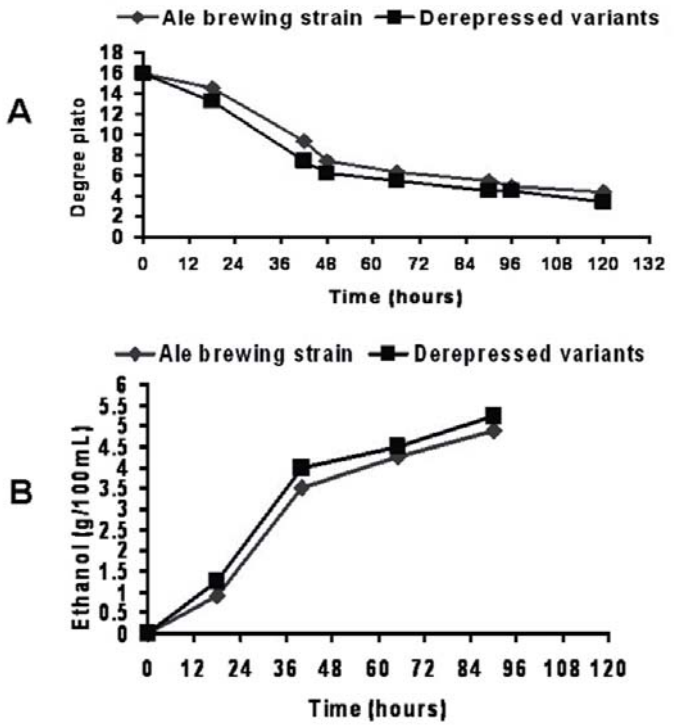

Fig. 9 Degree Plato reduction (a) and ethanol formation (b) by an ale brewing strain and its 2-DOG derepressed variant

In an attempt to overcome this repression, the glucose analogue 2-deoxy-glucose (2-DOG), has been successfully employed for the selective isolation of spontaneous mutants of yeasts [47] and other fungi. These 2-DOG mutants were derepressed for the production of carbohydrate-hydrolysing enzymes when selected employing this non-metabolyable glucose analogue. Derepressed mutants of brewing and other industrial yeast strains (such as baker's yeast strains) have been isolated and were able to metabolize wort maltose and maltotriose in the presence of glucose (Fig. 9) [92]. Fermentation and ethanol formation rates in 12 °Plato wort were also shown to have been increased in the 2-DOG mutants when compared to the parental strains [114].

All of these studies with 2-DOG mutants were conducted with *S. cerevisiae* ale and distilling yeast strains. These studies were unable to isolate 2-DOG mutants from the *S. pastorianus* lager strains screened. Since these studies (which were conducted in Canada in the late 1980s), a major Spanish brewing company with university collaborators, has examined the use of 2-DOG mutants to ferment a 25 °Plato wort, this time with a lager strain [73]. Stable spontaneous 2-DOG mutants of this lager yeast strain have been isolated. The fermentation characteristics of these 2-DOG lager mutants have been assessed with 25 °Plato wort employing 2L static fermenters at 13 °C. Improved fermentation capacity, where wort glucose did not repress maltose uptake, was achieved but

Table 3 Influence of wort gravity on beer ester levels

	12 °Plato Wort	20 °Plato Wort
Ethanol (v/v)	5.1	5.0
Ethyl acetate (mg/L)	14.2	21.2
Isoamyl acetate (mg/L)	0.5	0.7

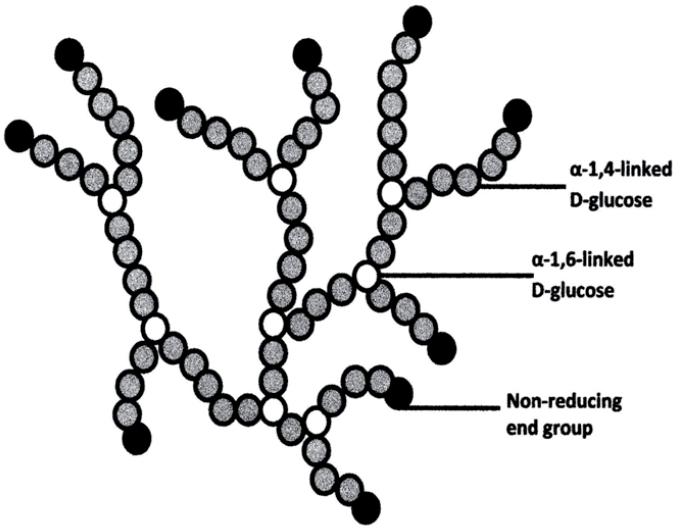

Fig. 10 Structure of glycogen

with changes in the beer flavour profile. Unfortunately, (similar to the situation with the Canadian trials employing ale strains), the increased wort fermentation rate was insufficient to introduce their 2-DOG lager yeast mutants into production brewing scale dimensions.

6.1 Yeast management

Yeast management includes:

- The initial stages of fermentation, where the intracellular storage carbohydrate glycogen (Fig. 10) is used in the presence of oxygen in order to synthesize unsaturated fatty acids (UFAs) and sterols which are incorporated into yeast membrane structures [83];
- The formation of diacetyl during the exponential stages of fermentation (Fig. 11) followed by its maturation (section 6.2).

6.2 Fermentation into maturation

The later stages of fermentation overlap into beer maturation (aging/lagering) [111]. This involves the management (reduction) of diacetyl and other vicinal diketones (VDK) (Fig. 11). Such compounds impart butterscotch/stale milk flavours to beer. There are a number of other beer flavour compounds produced during fermentation which will be discussed later.

- Also, the harvesting (cropping) of the yeast culture in the latter storage of wort fermentation for reuse either by flocculation for reuse either by flocculation or with a centrifuge – details later.

7 Beer flavour production as a result of fermentation – metabolomics

Beer aroma is the sum of several hundreds of flavour-active compounds produced during nearly every stage of the brewing process [6, 59]. The great majority (not all) of these substances are yeast secondary metabolites. They are produced during wort fermentation and consist of fermentation intermediates and byproducts (metabolites). Higher alcohols (also called fusel oils), esters, VDKs, other carbonyls and sulphur compounds (Fig. 12) are the key (not all) flavour elements produced by yeast. These compounds (plus malt and hop constituents) determine a beer's final quality, particularly when it is fresh [60]. Higher alcohols and esters are desirable volatile beer constituents, with a few exceptions [115]. Together with these compounds, yeast wort metabolism contributes to the biosynthesis of other beer flavour active compounds such as: organic acids, sulphur compounds (both organic and inorganic) and aldehydes (for example, acetaldehyde) [88]. This is an area of yeast activity that is currently termed metabolomics [22].

Metabolomics is: "the systematic study of the unique chemical fingerprints that specific cellular processes leave behind". It is the study of relatively small-molecule metabolite profiles [28]. Many of these small-molecules contribute to beer (and spirit) flavour profiles. Esters [and higher (fuel) alcohols] are major contributors to beer (and spirit) flavour profiles [57] along with a plethora of other metabolites [62].

Obviously, flavour-active compounds must be maintained within certain limits. Otherwise, a single compound or group of compounds (for example, VDK's) may predominate and prejudice a beer's flavour balance. Furthermore, flavour compounds such as esters often act in synergy with other components to affect beer flavour in concentrations well below their individual threshold values [130].

Each type of beer has its own unique aroma triggered (but not exclusively) either by the yeast strain employed during fermentation [83] or by a plethora of process parameters used during fermentation. Yeast strain, malt variety and type, fermentation temperature, adjunct (unmalted cereals and sugar) and percentage in the grist, fermenter design and geometry, wort pH, buffering capacity, wort concentration (gravity), etc., are all influencing factors [11]. In ad-

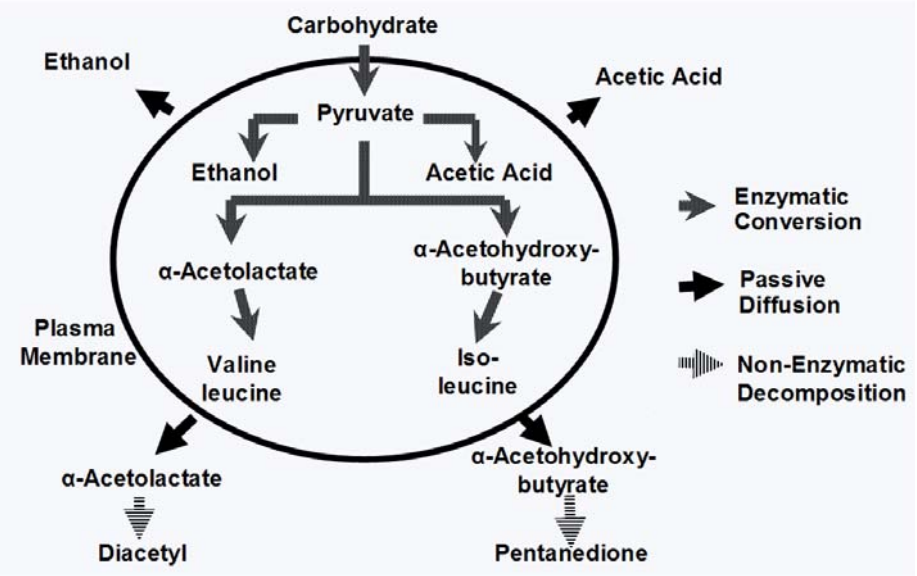

Fig. 11 Formation of diacetyl and 2,3-pentanedione

dition, hop variety and hopping procedures are important factors [87].

Only isoamyl acetate (banana-like aroma) concentration is usually above the threshold level in most lager beers, ales normally contain ethyl acetate (solvent-like aroma) and ethyl hexanoate (apple-like aroma) as supplementary flavouring compounds with levels above their taste threshold [5]. However, compounds such as diacetyl and other VDKs (with certain exceptions) should usually be below their flavour threshold values [100].

The biosynthesis and process variables of higher alcohols and esters during wort fermentation have been extensively reviewed by Pires and colleagues [75] and the following discussion is a summary of this publication (with permission). This also includes the effects of maltose concentration (compared to glucose) (Table 4) in a synthetic medium on ester formation [127]. Reduced ester formation in wort has also been documented. In addition, source material compiled from "An Introduction to Brewery Science and Technology" [110] published by the Institute of Brewing and Distilling has been employed here together with "Whisky: Technology, Production and Marketing" [89] – with permission.

The higher alcohols that occur in beer (and many spirits) are: n-propanol, isobutanol, 2-methyl-1-butanol, 3-methyl-butanol, 2-methyl-1-butanol, 3-methyl-butanol together with more than 40 other alcohols, have been identified. Regulation of the biosynthesis of higher alcohols is complex since they have been produced as by-products of amino acid metabolism or via pyruvate and ethanol produced from carbohydrate metabolism (Fig. 13).

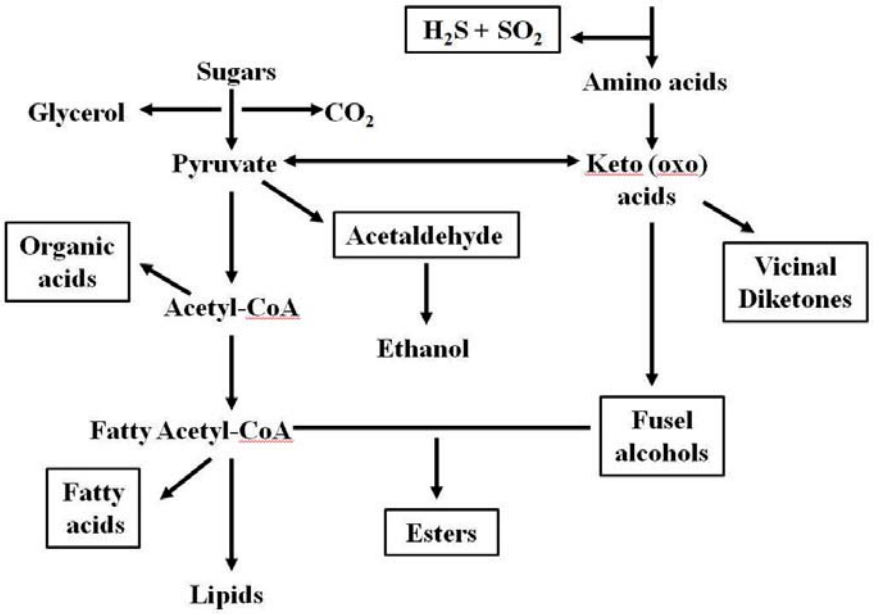

Fig. 12 Summary of the major metabolic routes by which brewer's yeast cultures produce higher alcohols, esters, sulfur compounds, VDK'S, acetaldehyde, glycerol, carbon dioxide and ethanol

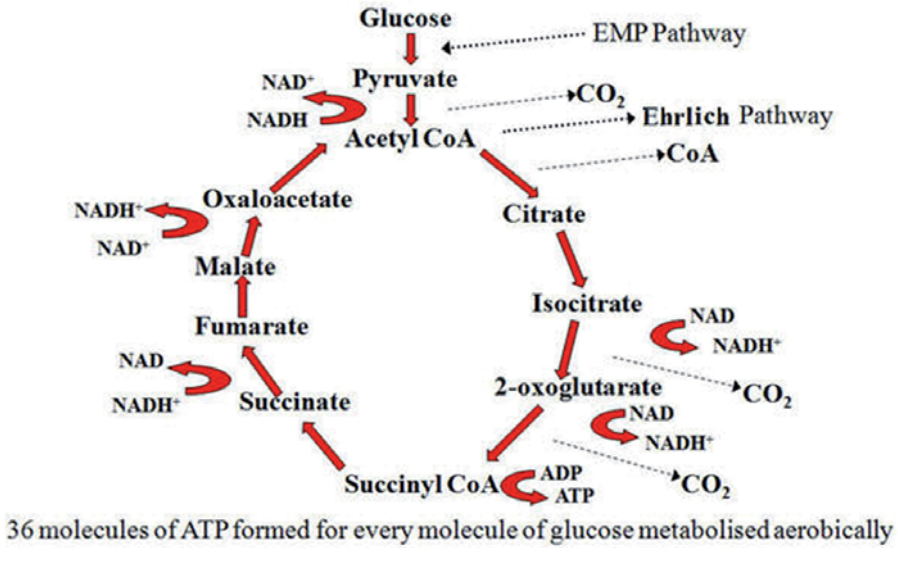

Fig. 13 The Tricarboxylic Acid Cycle. ~36 molecules of ATP formed for every molecule of glucose metabolised aerobically. The Ehrlich Pathway leads to the synthesis of higher alcohols

It has already been discussed that brewer's yeast strains absorb wort's spectrum of amino acids and small peptides in a distinct order (Fig. 8 and Table 2) from which they take the amino group (Ehrlich Pathway) so that they can be incorporated into their own structures. What remains from the amino acids are α-keto acids which enter into an irreversible chain reaction that will ultimately form higher alcohols. Ehrlich's research led to an investigation of the relationship between the amino acids isoleucine and amyl alcohol and isoamyl alcohol with leucine (Fig. 14) [27]. He also proposed that amino acids were enzymatically hydrolyzed to form the corresponding higher alcohol, along with ammonia and carbon dioxide. As ammonia was not detected in the reaction medium, it was assumed that it was incorporated directly into protein. Subsequently, a few intermediate steps to the Ehrlich Pathway have been proposed that completes this metabolic pathway [65].

Table 4 Percentage viability of brewing yeast strains after 96 hr fermentation of synthetic media[a]

	Glucose	Maltose
Ale 1	96	98
Ale 2	92	98
Ale 3	94	98
Lager 1	97	99
Lager 2	96	98
Lager 3	95	99

[a] Peptone – yeast extract – 4 % sugar medium
Methylene violet stain employed

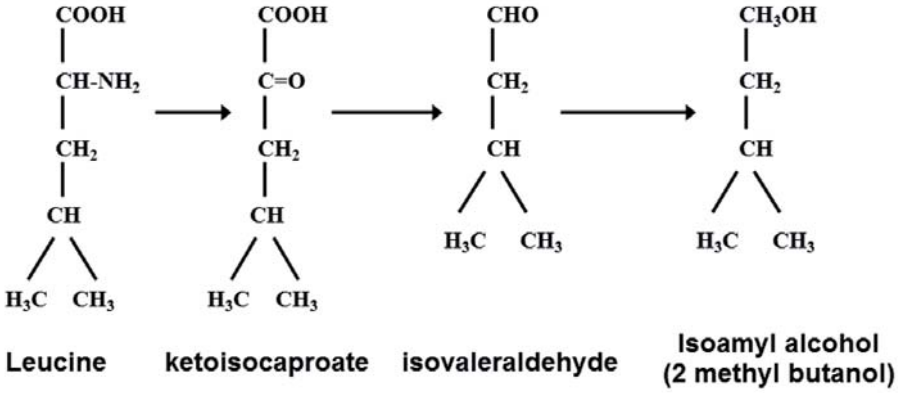

Fig. 14 Ehrlich Pathway – relationship between higher (fusel) alcohols and amino acids

Ethyl acetate is the most common ester produced by yeast :

$CH_3CH_2OH + CH_3COSCoA \rightarrow CH_3CH_2COOCH_3 + CoASH$

Ethanol acetyl CoA ethyl acetate

Fig. 15 Production of a typical ester during fermentation

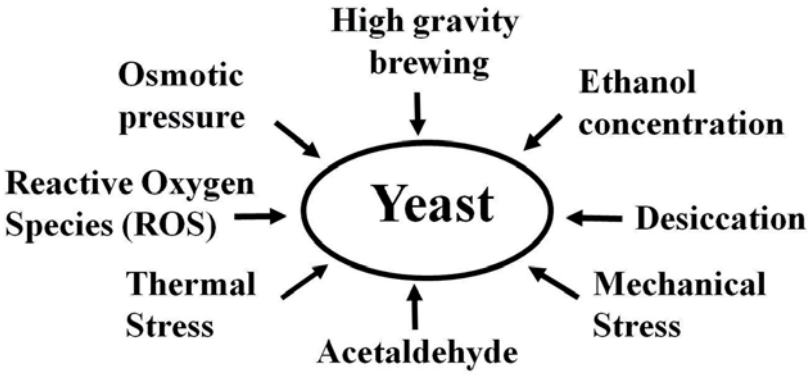

Fig. 16 Major stress factors that negatively affect yeast activity

Higher alcohols are also formed during the upstream (anabolic pathway) biosynthesis of amino acids [94]. In the brewing context, the de novo synthesis of branched-chain amino acids (BCAAs) occurs through the isoleucine-leucine (ILV) pathway (Fig. 14) [24].

Esters are largely formed during the active phase of primary fermentation by the enzymatic condensation of organic acids with alcohols. Volatile esters in beer are divided into two major groups: the acetate esters are synthesized from acetic acid (acetate) with ethanol or a higher alcohol (propanol, butanol, etc.) (Fig. 15). In the ethyl ester family, ethanol will form the alcohol and the acid side of the equation is an MCFA. Although many different esters can be formed during the fermentation stage of any beer or spirit, of major importance as the aroma constituents are: ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), isobutyl acetate (fruity aroma), phenylethyl acetate (roses and honey aroma), ethyl hexanoate (sweet apple aroma) and ethyl octanoate (sour apple aroma) [100]. Esters are synthesized in the yeast cell's cytoplasm, and they readily leave the cell because they are lipophilic. While small-chain acetate esters easily diffuse through the plasma membrane, MCFA ethyl esters may have their passage hindered [66].

Ester synthesis involves organic acids linked to a coenzyme module in order to form an acetyl-CoA molecule (Figs. 13 and 15). Acetyl-CoAs are highly energetic entities, which in the presence of oxygen can be oxidised (spliced) into smaller units in the yeast cell's mitochondria. The great majority of the acetyl-CoA, produced in the yeast cell, comes from the oxidative decarboxylation of pyruvate. Aerobic conditions inside mitochondria will induce acetyl-CoA to enter the TCA Cycle (Krebs Cycle) to form ATP. In the absence of oxygen, acetyl-CoA will be enzymatically esterified with alcohol to form acetate esters (Fig. 2). Also, MCFA ethyl esters are formed from longer chains of acyl-CoA with ethanol. Figure 12 summarises the major metabolic routes by which brewer's yeast strains synthesize both higher alcohols and esters.

Over 200 carbonyl compounds are reported to comprise the flavour of fresh beer and other alcoholic beverages [110]. These compounds influence beer flavour and are produced as a result of yeast metabolism during fermentation. They consist of various aldehydes (for example, acetaldehyde) and vicinal diketones, notably diacetyl, that has already been briefly discussed. Also, other carbonyl compounds (beyond the scope of this paper) exert a significant influence on beer flavour stability [61]. Excessive concentrations of carbonyl compounds are known to cause stale flavour in beer. The effects of aldehydes on flavour stability are reported to develop grassy notes [propanol, 2-methyl butanol, pentanol and a papery (cardboard) taste trans-2-nonenol, furfural]. A number of novel analytical methods have been developed to measure beer carbonyls [90, 91].

Acetaldehyde, is quantitatively the carbonyl present in highest concentration at the conclusion of primary fermentation (10–15 mg/L). It is produced by yeast as a result of the decarboxylation of pyruvate (Fig. 12) and is an intermediate in the metabolic formation of ethanol during glycolysis. It can be present in beer at concentrations above its flavour threshold (approximately 10 mg/L) and it imparts an undesirable "cut grass" or "green apple" character. This carbonyl accumulates during the active growth phase. As with higher alcohols and esters, the extent of acetaldehyde accumulation is determined by the yeast strain employed of the fermentation conditions. Although the yeast used is of primary importance, elevated wort oxygen concentrations, pitching rate, wort gravity and fermentation temperature all influence acetaldehyde accumulation [88]. In addition, premature (early) flocculation of a yeast culture

from suspension during wort fermentation [52] does permit the neutralisation of excreted acetaldehyde which is associated with the later stages of fermentation.

Acetaldehyde has long been known as a product of alcoholic fermentation (Fig. 12). Acetaldehyde, ethanol and acetate metabolism in *S. cerevisiae* is complex [13] because several enzymes are involved with a dependence on the cell's redox balance. The two enzymes ultimately responsible for their metabolic pathways are alcohol dehydrogenase (ADH) [1] and aldehyde dehydrogenase each of which consists of several isoenzymes. In addition, acetaldehyde can be metabolised by oxidation to acetate, by aldehyde dehydrogenase (ALDH) [8].

The presence of acetaldehyde is one of the stress conditions that yeast cells may encounter [2]. Several heat-shock protein (HSP) genes are induced that are also involved in response to other forms of stress (for example, ethanol) (Fig. 16) [74]. ALDHs play an important role in yeast acetaldehyde metabolism when the cells are growing on ethanol. Under several growth conditions, further addition of acetaldehyde (or ethanol) to wine yeasts induced the expression of some ALD genes and led to an increase in ALDH activity. This result is consistent with their need to obtain energy from ethanol during the biological ageing processes. Also, under certain conditions, acetaldehyde functions as a mutagen with respiratory deficient RD mutants as the primary result [31].

Diacetyl and 2,3-pendtanedione have already been briefly discussed in this paper [50]. They are produced during wort fermentation as by-products of specific amino acids synthesis (valine and isoleucine, respectively) by both ale and lager yeast strains (Fig. 11). Both of these vicinal diketones (VDKs) (especially diacetyl) have a significant effect on beer flavour, aroma and drinkability. Diacetyl imparts a butterscotch-like stale milk flavour with a threshold usually reported to be around 0.1 – 0.2 mg/L in lagers and 0.1 – 0.4 mg/L in ales [48]. However, diacetyl flavour thresholds as low as 1.7 mg/L have been reported. However, there is no doubt that the flavour threshold of diacetyl varies with a taster's geographical background, ethnicity and diet [48]. Diacetyl, at detectable concentrations, is acceptable in some beer styles such as Czech Pilsners and some (only some) English ales. Diacetyl metabolism during brewing has been the subject of significant polemic over the years, but a comprehensive review has discussed the history and current situation regarding diacetyl biosynthesis and subsequent metabolism during wort fermentation which are summarised in figure 11.

Although research on the metabolism of VDKs has been ongoing for over 50 years [121], a novel approach has been undertaken since the 1990s. The enzyme α-acetolactate decarboxylase (ALDC) catalyses the following reaction without the formation of diacetyl:

α-acetolactate → acetoin + carbon-dioxide

ALDC is not produced by brewer's yeast strains or any other yeast. However, it is produced by some bacteria that are generally regarded as safe (GRAS) (for example, the Gram-negative bacteria *Acetobacter aceti* which converts ethanol to acetic acid during the production of vinegar) [33]. ALDC has been isolated, purified and added to a brewer's wort fermentation and the total

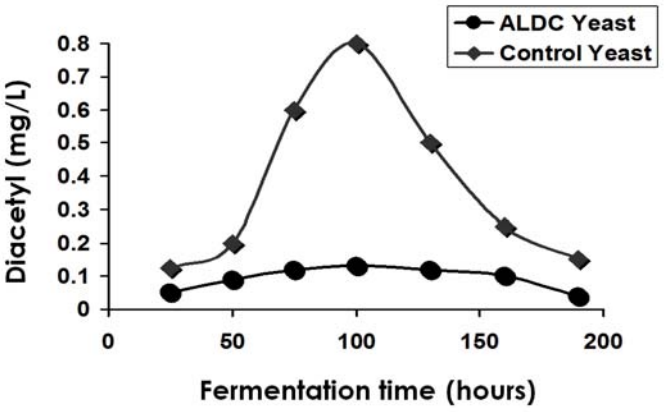

Fig. 17 Effect of α-acetolactate decarboxylase (ALDC) in a brewing yeast strain on diacetyl metabolism during wort fermentation

diacetyl concentration throughout the fermentation cycle determined (Fig. 17) [119]. Compared with an untreated control, little diacetyl was produced, and its concentration was inversely related to the activity of the ALDC added to the fermentation. It is worthy of note that commercial quantities of ALDC (under the brand name Maturex®) are produced by a genetically modified strain of *Bacillus subtilis* that has received the genetic coding for ALDC from a strain of *B. brevis* [125].

Sulphur compounds make a significant contribution to beer flavour and to fresh whisk(e)y spirit prior to being matured in oak casks [25]. Small amounts of sulphur compounds can be acceptable and even desirable in beer. In excess, they give rise to unpleasant off-flavours and special processes, such as purging with CO_2, use of a copper electrode [71] and prolonged maturation times are necessary to remove them [112]. Some of the sulphur compounds present in beer are not directly associated with fermentation and are derived from brewing raw materials (malt, hops, etc.) employed. However, the concentrations of hydrogen sulphide (rotten egg aroma) and sulphur dioxide (burnt match aroma) are dependent on yeast activity (Fig. 12).

The concentrations of hydrogen sulphide and sulphur dioxide, formed during fermentation, are primarily determined by the yeast strain used, although wort composition and fermentation conditions are important factors, particularly where levels of these compounds are abnormally high. The peak of hydrogen sulphide and sulphur dioxide production occurs during the second to the fourth days of fermentation (depending on the yeast strain, the wort gravity and the incubation temperature) [35, 41, 110]. The formation of excessive levels of hydrogen sulphide and sulphur dioxide, during fermentation is associated with conditions that restrict yeast growth. In this regard, the provision of adequate oxygen at the time of yeast pitching is critical! Since both hydrogen sulphide and sulphur dioxide are volatile, vigorous fermentation will promote their removal via carbon dioxide purging.

Sulphur dioxide, as well as contributing to beer flavour (burnt match), has a number of other functions in beer (and other alcoholic beverages). Although these functions are not directly associated with fermentation, it is worth discussing the fact that sulphur dioxide can act as an antimicrobial agent and an antioxidant [34] and it retards

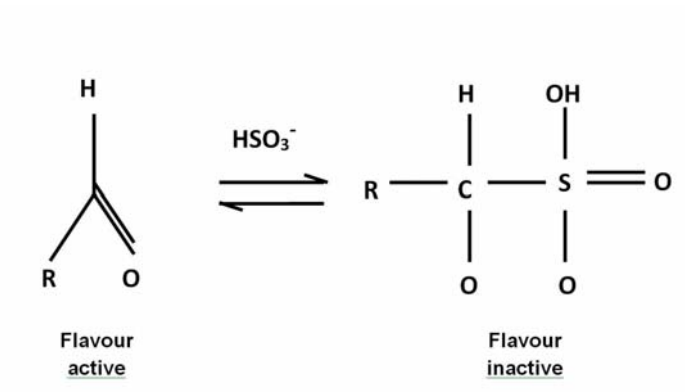

Fig. 18 Binding of bisulphate to carbonyls

the development of beer flavouring characteristics. Bisulphite's antimicrobial activity can only occur at concentrations in excess of 50 mg/L which is well above the permitted limit in beer for many countries (in the United States it is a maximum of 10 mg/L, Canada less than 10 mg/L and the United Kingdom less than 25 mg/L).

The effectiveness of bisulphite, besides its antioxidant properties, is also its ability to bind carbonyl compounds into flavour neutral complexes [7]. This can improve beer flavour stability. However, the efficiency of bisulphite binding to carbonyl compounds does vary. The reaction is reversible, so that an excess of bisulphite will increase the yield of the flavour inactive adjuvant (Fig. 18). The addition of bisulphites to fresh beer finings increases the acetaldehyde concentration during aging. Also, when added to stale beer, bisulphite lowers the concentration of free aldehyde and affects the removal of the cardboard flavour [42]. Over time, the bisulphite will be oxidized by oxygen in the beer, to sulphate, thus increasing the concentration of free aldehydes [7]. Recently, a *S. cerevisiae* mutant which produces higher levels of sulphur dioxide and glutathione but with lower levels of hydrogen sulphide and improved flavour stability has been reported [36]. This mutant has been developed by nongenetic engineering means and employed UV mutagenesis together with specific plate screening methods. The antioxidizing ability of this mutant was significantly improved and is safe for public use.

8 High gravity brewing – stress effects on yeast

High gravity brewing (HGB) is a procedure that employs wort at higher than normal concentrations. Consequently, it requires dilution with water (usually carbon filtered and deoxygenated) at a later stage in the brewing process in order to achieve a sales gravity beer [96, 99]. By using this process, increased standards can be met without expanding existing brewing, fermenting and storage facilities, producing savings in capital expenditure.

HGB can be regarded to be a type of brewing intensification which is a form of lean manufacturing [26]. Lean manufacturing ensures that the right improvement is chosen for the relevant reasons without sacrificing product quality and integrity. The advent and evolution of HGB has resulted in the publication of a number of specific fundamental papers concerning this process [105]. Critics of HGB maintain that this procedure waters down beer. This claim is inaccurate! HGB modifies the point of water addition from the beginning (during mash-in) to later in the process. Essentially, the same volume of water is employed as for a standard beer – maybe less! It should be emphasized that HG-brewed beers are different from high-alcohol undiluted beers. However, the effects of concentrated worts on yeast are very similar. Indeed, this section will predominantly focus the stress effects on yeast induced by concentrated wort.

Although there are many advantages to the use of HGB techniques there are also disadvantages. HG worts can influence yeast performance, with effects dependent upon fermentation and flocculation [99]. The increased osmotic pressure, elevated alcohol concentration and modified nutrient balance all have a profound influence on yeast performance during the fermentation of HG worts. Another negative effect of HG worts on yeast performance concerns the number of yeast cycles (generations) that can be fermented by a single yeast culture. The number of cycles that can be used by a yeast culture is reduced with increasing wort gravity. For example, a major Canadian brewery established the following specifications with their lager yeast strain: 12 °Plato > 20 cycles (generations); 14 °Plato > 15 cycles; 16 °Plato > 12 cycles and 18 °Plato > 8 cycles [102]. Significant strain-to-strain variation has been observed and, although there are exceptions, it appears that, in general, ale strains are more susceptible than lager strains to repeated repitching in HG worts (>16 °Plato) [79].

There is a relationship between yeast stress tolerance and growth, because a stress response enables yeast cells to continue growing and ferment under adverse conditions [20]. For this growth to occur, it requires various physiological adaptive changes and also produces distinct cellular morphological changes. The vacuole (Fig. 19) has been reported to function during periods of both osmotic and ethanol stress to ensure continued metabolic activity and yeast cell vitality [78]. Because both of these stresses are characteristic of HG wort fermentations morphological investigations have been conducted into the role of the yeast vacuole during HG fermentations [80].

The vacuole is an inherited organelle in yeast. Vacuolar volume changes with the cell growth phase and the growth conditions. Stationary phase yeast cells contain only one or two medium size vacuoles, whereas cells growing under stressful conditions have

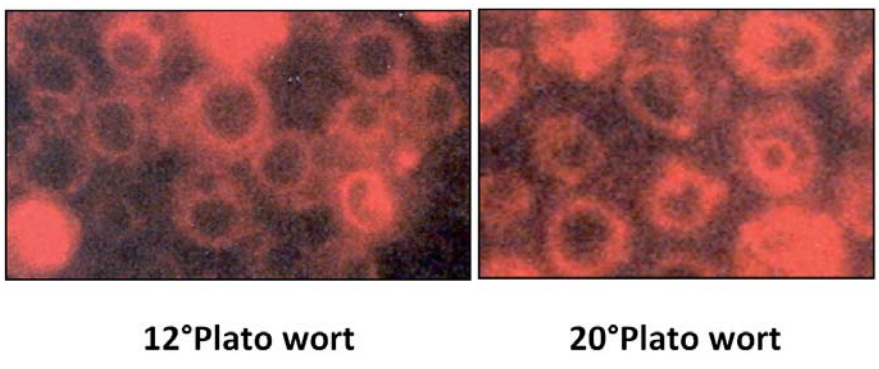

Fig. 19 Effect of wort gravity on vacuole size in a brewer's yeast strain

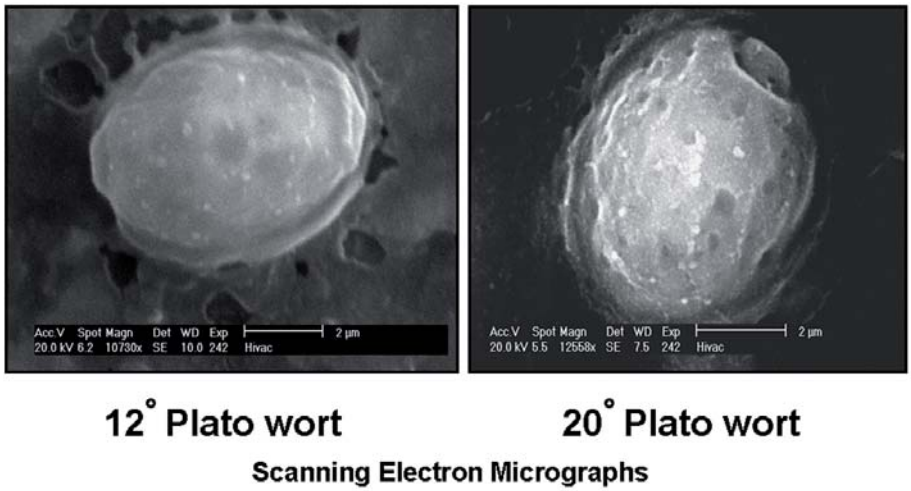

Fig. 20 Effect of wort gravity on the cell surface morphology of an ale yeast strain

very large vacuoles [124]. A lager yeast culture was harvested from both 12 and 20 °Plato worts and examined with scanning electron microscopy (SEM) in order to monitor morphological changes with vacuoles during fermentation. Changes associated with vacuoles are depicted in figure 19. The cells from the 20 °Plato wort fermentation exhibited greatly enlarged vacuoles when compared with vacuoles in cells harvested from the 12 °Plato wort fermentation. Morphological changes with yeast vacuoles from the lager yeast culture were also examined at specific times during fermentation in both 12 and 20 °Plato worts employing fluorescence microscopy and a fluorescent dye specific for vacuoles [77]. These changes are depicted in figure 20. These findings confirmed that the yeast vacuole plays an important role in the ability of yeast cells to successfully ferment HG worts and also to tolerate other stresses [78].

In addition to studies on vacuolar volume, the effect of wort gravity on cell surface morphology with ale and lager strains has been studied. To this end, the surface morphology of yeast strains was determined with SEM during the static fermentation of 12 and 20 °Plato worts. During the late stationary growth phase, cell surface features became apparent (Fig. 20). A more extreme effect of wort gravity on the yeast cell surface was observed in HG wort fermentation resulting in a wrinkly, prune-like, crenellated surface with numerous invaginations compared with yeast cells fermenting normal-gravity worts.

When yeast is first pitched into HG wort, passive diffusion of water out of the cell occurs, and this diffusion results in a decrease in cell viability (determined by methylene blue or methylene violet staining). Figure 21 illustrates experiments with 12 and 20 °Plato worts fermented with an ale strain [78]. Cell viability decreased in both ale and lager strains within the first 24 h of fermentation and the viability was exacerbated in the 20 °Plato wort. For reasons that are unclear, ale strains maintained higher vitalities than lager strains, particularly with 20 °Plato wort.

Saccharomyces and related species, including ale and lager brewing strains, contain two major intracellular storage carbohydrates: trehalose and glycogen. Trehalose is a disaccharide containing glucose units (Fig. 22). It protects the cell against stress (for example, osmotic pressure, ethanol, acetaldehyde, high and low temperatures, acid washing and desiccation (Fig. 16) [96]. It has been correlated with cell survival under adverse conditions and is an important indicator in brewing yeast cultures during HG wort fermentation. There was rapid synthesis of trehalose in 20 °Plato wort during the first 24 h of fermentation. As the cultures acclimatized to the stress conditions imposed by the 20 °Plato wort the intracellular trehalose levels decreased. It is interesting to note that lager strains maintained higher trehalose levels than ale strains.

Glycogen is an intracellular glucose polysaccharide with a structure similar to starch consisting of -1,4 linkages with 1,6 branch points (Fig. 10). It is the major reserve energy storage material in yeast cells of many other organisms (including the muscles of humans). Glycogen accumulates in yeast under nutrient-limited conditions. It has a role in providing carbon and energy for the maintenance of cellular activities. During the first 6–8 h of wort fermentation, there is rapid utilization of intracellular glycogen (Fig. 23, see page 44). This utilization is directly proportional to the synthesis of lipids (mainly unsaturated fatty acids and sterols such as ergosterol). These lipids are employed by the yeast culture to produce de novo membrane material during cell division. Once cell division begins to decrease, glycogen accumulates. It is important that maximum levels of intracellular glycogen are present in yeast cells when they

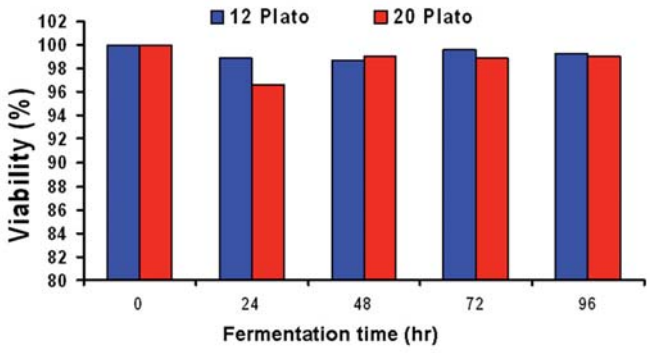

Fig. 21 Effect of wort gravity on the viability of an ale yeast strain during the intial stages of fermentation. Viability determined by the methylene blue staining method

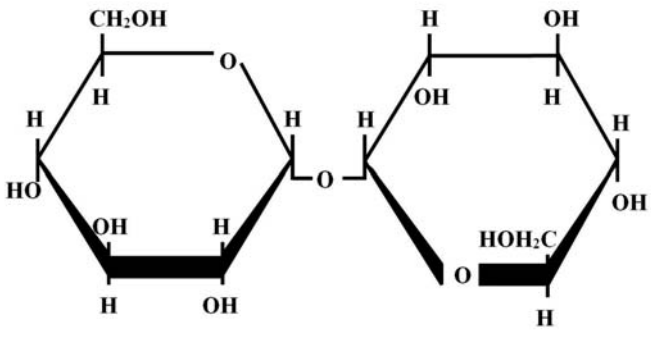

Fig. 22 Structure of trehalose

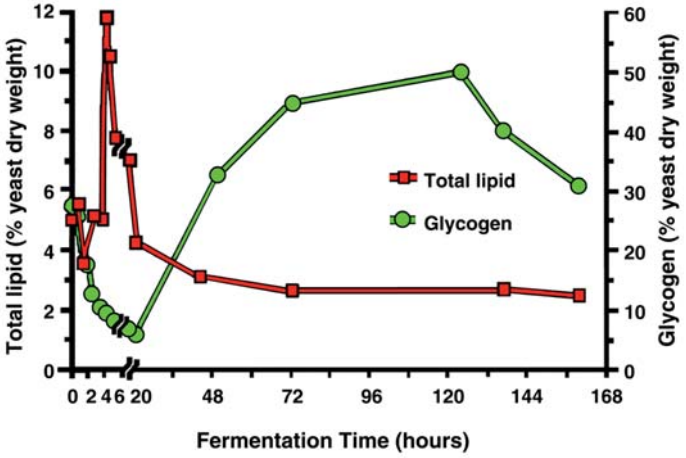

Fig. 23 Intracellular concentration of glycogen and lipids in a lager yeast strain during fermentation of a 15 °Plato wort

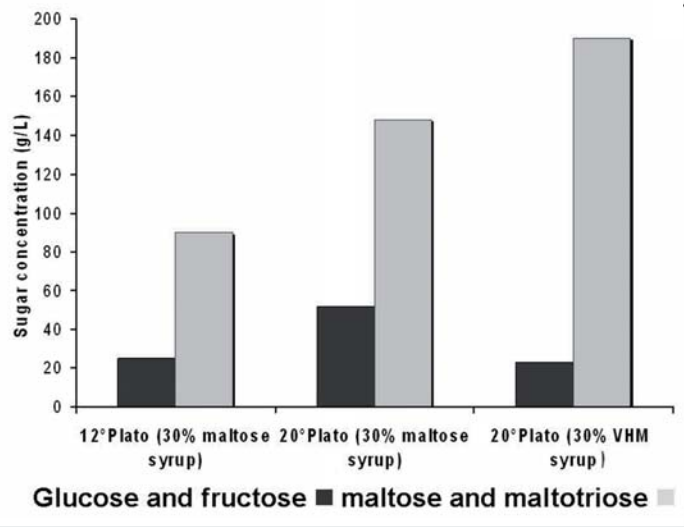

Fig. 24 Wort sugar profiles

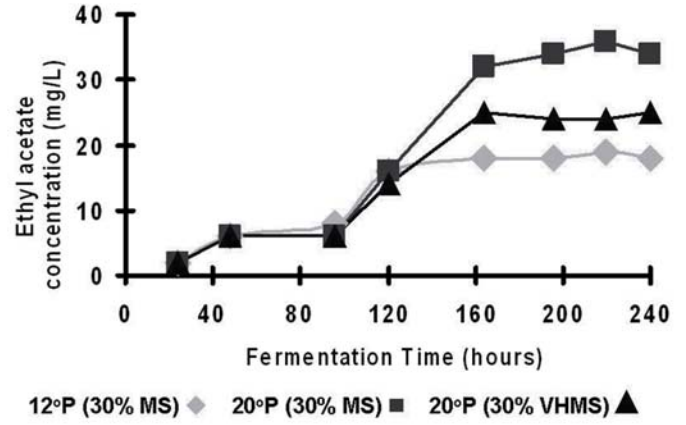

Fig. 25 Ethyl acetate concentration (mg/L) formed during the fermentation of worts with different gravities and sugar composition

are harvested for storage, prior to being repitched into a subsequent wort fermentation. It is critical that glycogen levels in yeast cells are conserved during storage because depleted glycogen levels will lead to incomplete fermentation.

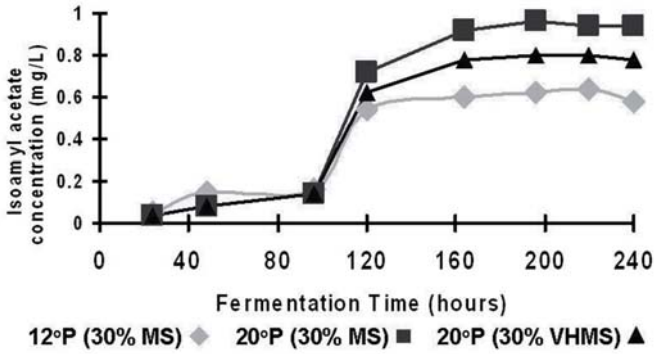

Fig. 26 Isoamyl acetate concentration (mg/L) formed during the fermentation of worts with different gravities and sugar composition

Another form of stress imposed upon brewer's yeast is the process of acid washing at pH 2.2. Since the studies of Pasteur [67, 68] it has been employed as an effective procedure by some brewers (not all) to remove bacterial contaminants from yeast slurries [3]. The physiological condition of the yeast culture in various environmental factors has been shown to affect the resistance of brewer's yeast to acid washing [95]. During HG brewing conditions [19], the adverse conditions of acid conditions are exacerbated. Acid washing affects yeast viability from a 20 °Plato wort fermentation, whereas yeast from a 12 °Plato wort fermentation was not affected to the same extent [18]. Strain variations have also been observed between lager yeast strains in their resistance to HG conditions and acid washing. The resistance to acid washing was also influenced by storage conditions between fermentations, particularly yeast that had been poorly stored which had the lowest yeast viability. Yeast management procedures must be optimized when repitching yeast from HG fermentation to ensure that the yeast is in good physiological condition and can maintain its resistance to acid washing. It is also important to emphasize that yeast from a high-alcohol environment in a HG fermentation [>6.5 % alcohol by volume (ABV)] should not be acid washed until the slurry is diluted (>5 % ABV) [18].

One disadvantage (there are others) of employing the HG brewing process is that fermentation of such worts induces the production of disproportionately high levels of beer esters (Table 3). Varying the wort sugar spectrum has been reported [29] to modify the level of many metabolites, including esters, although reasons for these differences are still unclear. It has already been discussed [29] that entry of hexose sugars (glucose and fructose) into the yeast cell is facilitated by similar protein transport systems. However, the utilization of glucose occurs more rapidly than fructose when the two sugars are fermented separately, probably because of the differing affinities of the two sugars for the transporter. The disaccharide maltose in wort is internalized by the yeast cell only when 40–50 % of the glucose has been removed from the wort [131] (details in section 4) and occurs by an active transport system, whereas the uptake of glucose and fructose is by a passive transport process [29].

Initial experiments with glucose and maltose were conducted employing a synthetic medium (yeast extract–peptone). Fermentations occurred separately with 4 % glucose and maltose as the carbon

Table 5 Vitality of brewing yeast strains after 96 hr fermentation of synthetic media[a]

	Glucose	Maltose
Ale 1	0.8	1.3
Ale 2	0.9	1.3
Ale 3	1.1	1.4
Lager 1	0.7	0.9
Lager 2	0.8	1.2
Lager 3	0.9	1.0

[a] Peptone – yeast extract – 4 % sugar medium
Acidification power test conducted

Table 6 Ethyl acetate and isoamyl acetate produced by brewing yeast strains during fermentation of synthetic media[a]

	Ethyl Acetate (mg/L)		Isoamyl Acetate (mg/L)	
	Glucose	Maltose	Glucose	Maltose
Ale 1	4.13	2.79	0.14	0.14
Ale 2	2.97	2.59	0.06	0.04
Ale 3	3.13	2.71	0.05	0.03
Lager 1	6.00	5.22	0.22	0.21
Lager 2	3.75	3.28	0.26	0.22
Lager 3	4.13	3.51	0.23	0.17

[a] Peptone – yeast extract – 4% sugar medium

Table 7 Sugar composition of brewing syrups

	Maltose Syrup (MS)	Very High Maltose Syrup (MS)
Glucose	15*	5
Maltose	55	70
Maltotriose	10	10
Dextrins	20	15

* % (w/v) composition

Table 8 Percentage viability of an ale and a lager brewing yeast strain after fermentation in 12 °Plato and 20 °Plato worts[a]

	12 °Plato Wort		20 °Plato Wort	
	MS [b]	VHMS [c]	MS	VHMS
Ale	95	98	93	96
Lager	94	97	95	98

[a] Methylene blue and methylene violet stains employed
[b] Maltose (55) syrup
[c] Very high maltose (70) syrup

source and the production of the esters ethyl acetate and isoamyl acetate monitored with a number of ale and lager strains [127]. All maltose-cultured cultures studied had higher viabilities and enhanced vitalities [129] when compared to their glucose-cultured counterparts (Tables 5 and 6). Reasons for these differences are not immediately apparent. It may be the result of slower initial uptake rates of maltose compared to glucose and consequent reduced growth rates. In addition, the fact that maltose uptake occurs by active transport and glucose by passive transport is no doubt relevant. Despite the apparent sturdiness of the maltose grown cells, the production of ethyl acetate and iso amyl acetate in the maltose medium was lower than in the glucose medium (Table 6).

It is generally agreed that a reduction in ester levels, particularly ethyl acetate and isoamyl acetate, from HG-brewed beers would be welcome by most brewers [128]. To study the influence of maltose and glucose levels in HG worts, two worts were prepared, one containing 30 % maltose syrup (MS) and the other containing 30 % very high MS (VHMS). The sugar composition of the two brewing syrups is shown in table 7. The two maltose syrups were used as kettle adjuncts and the sugar composition of the resulting three syrups (12 ° and 20 °Plato) is shown in figure 24. The three worts were fermented in a 2 hl/L pilot brewery with a lager yeast strain at 13 °C and the concentrations of ethyl acetate and isoamyl acetate were determined throughout the fermentation (Figs. 25 and 26). The profiles were similar for both esters. The concentrations of both esters in the 20 °Plato MS fermented wort were twice those observed in the 12 °Plato MS fermented wort. However, the ester concentration in the 20 °Plato VHMS wort was approximately 25 % reduced compared to the 20 °Plato MS wort [128]. This finding confirms the findings that employed synthetic media with single sugars (maltose and glucose) and maltose fermentations producing less ethyl acetate and isoamyl acetate than during glucose fermentations. In addition, similar to the synthetic media fermentations, the wort (both 12 ° and 20 °Plato) with elevated maltose concentrations produced yeast with higher viabilities than the cells produced in wort with lower maltose levels (Table 8).

9 Conclusion – in part 2 of this keynote paper

Acknowledgement

The author is grateful to Anne Anstruther for her support, patience, encouragement and guidance during the writing of this article.

10 References

1. Aranda, A. and del Olmo, M.: Response to acetaldehyde stress in the yeast *Saccharomyces cerevisiae* involves a strain-dependent regulation of several ALD genes and is mediated by the general stress response pathway, Yeast, **20** (2002), no. 8, pp. 747-759.
2. Aranda, A.; Querol, A. and del Olmo, M.: Correlation between acetaldehyde and ethanol resistance and expression of HSP genes in yeast strains isolated during the biological ageing of sherry wines, Arch. Microbiol., **177** (2002), no. 4, pp. 304-312.
3. Bah, S. and McKee, R.A.: Beer – spoilage bacteria and their control with a phosphoric acid – ammonium persulfate wash, Can. J. Microbiol., **11** (1965), pp. 309-318.
4. Baker, E.C.; Wang, B.; Bellora, N.; Peris, D.; Hulfachor, A.B.; Koshalek, J.A.; Adams, M.; Libkind, D. and Hittinger, C.T.: The genome sequence of *Saccharomyces eubayanus* with the domestication of lager-brewing yeast, Molec. Biol. Evol., **32** (2015), no. 11, pp. 2818-2831.
5. Bamforth, C.W.: Beer. A Quality Perspective. pub. Elsevier, 2009.
6. Bamforth, C.W.: Brewing materials and processes – a practical approach to beer excellence. Pub. Academic Press, 2016.
7. Barker, R.L.; Gracey, D.E.F.; Irwin, A.J.; Pipasts, P. and Leiska, E.:

Liberation of staling aldehydes during storage of beer, J. Inst. Brewing, **89** (1983), no. 6, pp. 411-415.
8. Beier, D.R.; Sledziewski, A. and Young, E.T.: Deletion analysis identifies a region, upstream of the ADH2 gene of *S. cerevisiae* which is required for ADR1-mediated derepression, Mol. Cell. Biol., **5** (2012), no. 7, pp. 225-232.
9. Bing, J.; Han, P.J.; Liu, W.Q.; Wang Q.M. and Bai, F.W.: Evidence for a Far East Asian origin of lager beer yeast, Curr. Biol., **24** (2014), no. 10, pp. R380-R381.
10. Boulton, C.A. and Quain, D.E.: A novel system for propagation of brewing yeast, Proc. 27th Cong. Eur. Brew. Conv., pp. 647-654, 1999.
11. Casey, G.P.; Chen, E.C.H. and Ingledew, W.M.: High-gravity brewing: Production of high levels of alcohol without excessive concentrations of ester and fusel alcohols, J. Am. Soc. Brew. Chem., **43** (1985), pp. 179-182.
12. Chen, P.; Dong, J.; Yin, H.; Bao, X.; Chen, L.; He, Y.; Chen, R.; Wan, X.; Zhao, Y. and Hou, X.: Genome comparison and evolutionary analysis of different lager yeasts (*Saccharomyces pastorianus*), J. Inst. Brewing., **122** (2016), no. 1, pp. 42-47.
13. Chlup, P.H.; Bernard, D. and Stewart.G.G.: Disc stack centrifuge operating parameters and their impact on yeast physiology, J. Inst. Brewing, **114** (2008), no. 1, pp. 45-61.
14. Cooper, D.J.; Stewart, G.G. and Bryce, J.H.: Yeast proteolytic activity during high and low gravity wort fermentations and its effect on head retention, J. Inst. Brewing, **106** (2000), no. 4, pp. 197-201.
15. Cori, C.F.: Embden and the glycolytic pathway, Trends Biochem. Sci., **8** (1983), no. 7, pp. 257-259.
16. Cousseau, F.E.M.; Alves, S.L. Jr.; Trichezy, D. and Stambuk, B.U.: Characterization of maltotriose transporters from *Saccharomyces eubayanus* subgenome of the hybrid of *Saccharomyces pastorianus* lager brewing yeast strain, Weihenstephan 34/70. Lett. Appl. Microbiol., **56** (2013), no. 1, pp. 21-29.
17. Cruz, S. H.; Batistote, M. and Ernandes, J. R.: Effect of sugar catabolite repression in correlation with the structural complexity of the nitrogen source on yeast growth and fermentation, J. Inst. Brewing, **109** (2003), no. 4, pp. 349-355.
18. Cunningham, S. and Stewart, G.G.: Acid washing and cereal repitching of brewing ale strains of *Saccharomyces cerevisiae* in high gravity wort and the role of wort oxygenation conditions, J. Inst. Brewing, **106** (2000), no. 6, pp. 389-402.
19. Cunningham, S. and Stewart, G.G.: High gravity brewing and acid washing in brewer's yeast, J. Am. Soc. Brew. Chem., **56** (1998), no. 1, pp. 2-7.
20. D'Amore, T.; Celloto, G. and Stewart, G.G.: Advances in the fermentation of high gravity wort. Proc. 29th Cong. Eur. Brew. Conv., pp. 337-344, 1991.
21. D'Amore, T.; Russell, I. and Stewart, G.G.: Sugar utilization by yeast during fermentation, J. Ind. Microbiol, **4** (1989), no. 4, pp. 315-324.
22. Daviss, B.: Growing pains for metabolomics, The Scientist, **19** (2005), no. 8, pp. 25-28.
23. Day, R.E.; Higgins, V.J.; Rogers, P.J. and Dawes, I.W.: Characterization of the putative maltose transporters encoded by YDL247W and YJR160c, Yeast., **19** (2002), no. 12, pp. 1015-1027.
24. Dickson, J.R. and Norte, V.: A study of branched-chain amino acid aminotransferase and isolation of mutation affecting the catabolism of branched-chain amino acids in *Saccharomyces cerevisiae*, J. Am. Soc. Brew. Chem., **70** (2012), pp. 195-204.
25. Duan, W.; Roddick, F.; Higgins, V. and Rogers, P.: A parallel analysis of H_2S and SO_2 formation by brewing yeast in response to sulphur-containing amino acids one ammonium ions, J. Am. Soc. Brew. Chem., **62** (2004), no. 1, pp. 35-41.
26. Dudbridge, M.: Handbook of Lean Manufacturing in the Food Industry, Wiley, New York. 2011.
27. Ehrlich, F.: Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweißaufbau der Hefe, Ber. Dtsch. Chem. Ges., **40** (1907), no. 1, pp. 1027-1047.
28. Ellis, D.I.; Dunn, W.B.; Griffin, J.L.; Allwood, J.W. and Goodacre, R.: Metabolic fingerprinting as a diagnostic tool, Pharmacogenomics, **8** (2007), no. 9, pp. 1243-1266.
29. Ernandes, J.R.; D'Amore, T.; Russell, I. and Stewart, G.G.: Regulation of maltose and glucose transport in strains of *Saccharomyces*, J. Indust. Microbiol., **9** (1982), no. 2, pp. 127-130.
30. Ernandes, J.R.; Williams, J.W.; Russell, I. and Stewart, G.G.: Effect of yeast adaptation to maltose utilization on sugar uptake during the fermentation of brewer's wort, J. Inst. Brewing., **99** (1993), no. 1, pp. 67-71.
31. Ernandes, J.R.; Williams, J.W.; Russell, I. and Stewart, G.G.: Respiratory deficiency in brewing yeast strains – effects on fermentation, flocculation, and beer flavour components, J. Am. Soc. Brew. Chem., **51** (1993), no. 1, pp. 16-20.
32. Erratt, J.A. and Stewart, G.G.: Fermentation studies using *Saccharomyces diastaticus* yeast strains, Devel. Indust. Microbiol., **22** (1981), pp. 577-586.
33. Godtfredsen, S.E. and Ottesen, M.: Maturation of beer with alpha-acetolactate decarboxylase, Carlsberg. Res. Commun., **47** (1982), no. 2, pp. 93-102.
34. Guido, L.F.: How do sulfites help to control beer ageing? Cerevisia, **30** (2005), pp. 132-137.
35. Hammerman, W.: Reducing beer maturation time and retaining quality, Tech. Quart. Master Brew. Assoc. Amer., **39** (2002), no. 3, pp. 149-155.
36. Hansen, J. and Kielland-Brandt, M.C.: Inactivation of MET2 in brewer's yeast increases the level of sulfite in beer, J. Biotechnol., **50** (1996), no. 1, pp. 75-87.
37. Hill, A.M. and Stewart, G.G.: A brief overview of brewer's yeast, Brewer and Distiller International, **5** (2009), pp. 13-15.
38. Holter, H.; Moller, K.M.: The Carlsberg Laboratory 1876-1976, Rhodos International Science and Art Publishers, 1976.
39. Horák, J. and Kotyk, A.: Energetics of L-proline uptake by Saccha*romyces cerevisiae*, Biochim. Et Biophys. Acta, **857** (1986), no. 2, pp. 173-179.
40. Hutzler, M.; Koob, J.; Riedl, R.; Schneiderbanger, H.; Mueller-Auffermann, K.; and Jacob, F.: Yeast identification and characterization, Woodhead Publishing, 2015.
41. Hysert, D.W. and Morrison, N.M.: Sulfate metabolism during fermentation, J. Am. Soc. Brew. Chem., **34** (1976), no. 1, pp. 25-31.
42. Ilett, D.R. and Simpson, W.J.: Loss of sulphur dioxide during storage of bottled and canned beers, Food Res. Int., **28** (1995), no. 4, pp. 393-396.
43. Jin, H.; Ferguson, K.; Bond, M.; Kavanagh, T. and Hawthorne, D.: Malt nitrogen parameters and yeast fermentation behaviour, Proc. 26th Conv. Inst. Brew. Asia Pac. Conv., pp. 44-50, 1996.
44. Jones, M. and Pierce, J.: Absorption of amino acids from wort by yeasts, J. Inst. Brewing, **70** (1994), no. 4, pp. 307-315.
45. Jones, M. and Pierce, J.: Nitrogen requirements in wort – practical applications, Proc. 10th Cong. Eur. Brew. Conv., pp. 182-194, 1965.
46. Jones, M.; Pragnell, M.J. and Pierce, J.S.: Absorption of amino acids by yeasts from a semi-defined medium simulating wort, J. Inst. Brew-

ing, **75** (1969), no. 6, pp. 520-536.
47. Jones, R.M.; Russell, I. and Stewart, G.G.: The use of catabolite derepression as a means of improving the fermentation rate of brewing yeast strains, J. Am. Soc. Brew. Chem., **44** (1986), no. 4, pp. 161-166.
48. Kluba. R.; de Banchs, N.; Fraga, A,; Jansen, G.; Langstaff, S.; Meilgaard, M.; Nonaka, R.; Thompson, S.; Verhagen, L.; Word, K. and Crumplen, R.: Sensory threshold determination of added substances in beer, J. Am. Soc. Brew. Chem., **51** (1993), no. 4, pp. 181-183.
49. Kobi, D.; Zugmeyer, S.; Potier, S. and Jaquet-Gutfreund, L.: Two-dimensional protein map of an "ale" – brewing yeast strain: proteome dynamics during fermentation, FEMS Yeast Research, **5** (2004), no. 3, pp. 213-230.
50. Krogerus, K. and Gibson, B.R.: 125th Anniversary review: diacetyl and its control during brewery fermentation, J. Inst. Brewing, **119** (2013), no. 3, pp. 86-97.
51. Krogerus, K.K.; Magalhães, F.; Vidgren, V. and Gibson, B.: Novel brewing yeast hybrids – creation and application, Appl. Microbiol. Biotechnol., **101** (2017), no. 1, pp. 65-78.
52. Lake, J.C. and Speers, R.A.: A discussion of malt-induced premature yeast flocculation, Tech. Quart. Master Brew. Assoc. Amer., **45** (2008), no. 3, pp. 253-262.
53. Lekkas, C.; Hill, A.E.; Taidi, B.; Hodgson, J. and Stewart, G.G.: The role of small peptides in brewing fermentations, J. Inst. Brewing, **115** (2009), no. 2, pp. 134-139.
54. Lekkas, C.; Stewart, G.G.; Hill, A.E.; Taidi, B. and Hodgson, J.: Elucidation of the role of nitrogenous wort components in yeast fermentation, J. Inst. Brewing, **113** (2007), no. 1, pp. 3-8.
55. Lekkas, C.; Stewart, G.G.; Hill, and A.; Taidi, B.: The importance of free amino nitrogen in wort and beer, Tech. Quart. Master Brew. Assoc. Amer., **42** (2005), no. 2, pp. 113-116.
56. Libkind, D.; Hittinger, C.T.; Valério, E.; Gonçalves, C.; Dover, J.; Johnston, M.; Gonçalves, P. and Sampaio, J.P.: Microbe domestication and the identification of the wild genetic stock of lager brewing yeast, Proc. Natl. Acad. Sci. USA, **108** (2007), no. 35, pp. 14539-14544.
57. Malcorps, P. and Dufour, J.P.: Short-chain and medium-chain aliphatic-ester synthesis in *Saccharomyces cerevisiae*, Eur. J. Biochem., **210** (1992), no. 3, pp. 1015-1022.
58. Manzano, M.; Cocolin, L.; Lacumin, L.; Cantoni, C. and Comi, G.: A PCR-TGGE (temperature gradient gel electrophoresis) technique to assess differentiation among enological *Saccharomyces* strains, Int. J. Food Microbiol., **101** (2005), no. 3, pp. 333-339.
59. Meilgaard, M.: Effects on flavour of innovations in brewery equipment and processing: a review, J. Inst. Brewing, **107** (2001), no. 5, pp. 271-286.
60. Meilgaard, M.: Flavour chemistry of beer. Part 1 flavour interaction between principal volatiles, Tech. Quart. Master Brew. Assoc. Amer., **12** (1975), no. 2, pp. 107-117.
61. Meilgaard, M.; Flavour chemistry of beer. Part II flavour and threshold of 235 aroma volatiles. Tech. Quart. Master Brew. Assoc. Amer. **12** (1975), no. 3, pp. 151-168.
62. Molina, A.M.: Swiegers, J.H.: Varela, C.: Pretorius, I.S. and Agosin, E.: Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds, Appl. Microbiol. Biotechnol., **77** (2008), no. 3, pp. 675-687.
63. Mullis, K.B.; Erlich, H.A.; Arnheim, N.; Horn, G.T.; Saiki, R.K. and Scharf, S.J.: Process for amplifying detecting and/or cloning nucleic acid sequences, Patent No. US4683195A.
64. Nathan, L.: Improvements in the fermentation and maturation of beers, J. Inst. Brewing, **36** (1930), no. 6, pp. 544-550.
65. Neubauer, O. and Fromherg, K.: Über den Abbau der Aminosäuren im bei der Hefegarung – De Gruyer, Hoppe-Seyler's. Z Physiol. Chem., **70** (1911), no. 4-5, pp. 326-350.
66. Nykänen, L. and Nykänen, I.: Production of esters by different yeast strains in sugar fermentation, J. Inst. Brewing, **83** (1977), no. 1, pp. 30-31.
67. Pasteur, L.: Etudes sur la biere. Gauthier-villars, Paris, 1876.
68. Pasteur, L.: Manufacture of beer and yeast. Patent No. US141,072, 1873.
69. Pasteur, L. and Faulkner, F.: Studies on Fermentation, The Diseases of Beer, Their Causes, and the Means of Preventing Them, Kessinger Legacy Reprints, 1879.
70. Pedersen, M.B.: Recent views and methods for classification of yeasts, Cerevisia – Belg. J. Brew. Biotechnol., **20** (1995), pp. 28-33.
71. Pfisterer, E.; Richardson, I. and Soti, A.: Control of hydrogen sulphide in beer with a copper electrode system, Tech. Quart. Master Brew. Assoc. Amer., **41** (2004), no. 1, pp. 50-52.
72. Pickerell, A.T.W.: The influence of free alpha-amino nitrogen in sorghum beer fermentations, J. Inst. Brewing, **92** (1986), no. 6, pp. 568-571.
73. Piña, B.; Carro, D.; Orive, M.; Fité, B.; Torrent, J. and Vidal, J.F: A new source to obtain lager yeast strains adapted to very high gravity brewing: 2-deoxy-D-glucose resistant colonies with fermentative capacity up to 25° Plato, Proc. 31st. Cong. Eur. Brew. Conv. Contribution 45.
74. Piper, P.; Talreja, K.; Panaretou, B.; Moradas-Ferreira, P.; Byrne, K.L.; Praekelt, U.; Meacock, P.; Récnacq, M. and Boucherie, H.: Induction of major heat-shock proteins of *Saccharomyces cerevisiae* including plasma membrane HspSO, by ethanol levels above a critical threshold, Microbiology, **140** (1994), no. 11, pp. 3031-3038.
75. Pires, E.J.; Teixeira, J.A.; Brányik, T. and Vicente, A.A. Yeast: the soul of beer's aroma – a review of flavour-active esters and higher alcohols produced by brewing yeast, Appl. Microbiol. Biotechnol., **98** (2014), no. 5, pp. 1937-1949.
76. Prakitchaiwattana, C.J.; Fleet, G.H. and Heard, G.M.: Application and evaluation of denaturing gradient gel electrophorsesis to analyse the ecology of wine grapes, FEMS Yeast Res., **4** (2004), no. 8, pp. 865-877.
77. Pratt, P.L.; Bryce, J.H. and Stewart, G.G.: The effects of osmotic pressure and ethanol on yeast viability and morphology, J. Inst. Brewing, **109** (2003), no. 3, pp. 218-228.
78. Pratt, P.L.; Bryce, J.H. and Stewart, G.G.: The Yeast Vacuole – A Scanning Electron Microscopy Study During High Gravity Wort Fermentations, J. Inst. Brewing, **113** (2007), no. 1, pp. 55-60.
79. Pratt-Marshall, P.L.; Brey, S.E.; de Costa, S.D.; Bryce, J.H. and Stewart, G.G.: High gravity brewing – An inducer of yeast stress, Brew. Guardian, **131** (2002), pp. 22-26.
80. Pratt-Marshall, P.L.; Stewart, G.G. and Bryce, J.H.: High gravity brewing – effects on yeast vacuolar morphology. Proc. 27th Conv. Inst. Brew. Asia Pac. Sect., pp. 1-10.
81. Pretorius, I.S.: Conducting Wine Symphonics with the Aid of Yeast Genomics, Beverages, 2 (2016), no. 4, pp. 33, MDPI AG.
82. Putman, R.: What goes around comes around, Brewer and Distiller International. **14** (2018), no. 3, pp. 42-49.
83. Quain, D.E.; Thurston, P.A. and Tubb, R.S.: The structural and storage carbohydrates of *Saccharomyces cerevisiae* changes during fermentation of wort and a role for glycogen metabolism in lipid synthesis, J. Inst. Brewing, **87** (1981), no. 2, pp. 108-111.
84. Rainieri, S.; Kodama, Y.; Nakao, Y.; Pulvirenti, A. and Giudici, P.: The inheritance of mTDNA in lager brewing strains, FEMS Yeast Res., **8** (2008), no. 4, pp. 586-596.

85. Rainieri, S.; Zambonelli, C. and Kaneko, Y.: *Saccharomyces* sensu stricto: systematics genetic diversity and evolution, J. Biosci. Bioeng., **96** (2003), no. 1, pp. 1-9.
86. Rautio, J. and Londesborough, J.: Maltose transport by brewer's yeasts in brewer's wort, J. Inst. Brewing, **109** (1993), no. 3, pp. 251-261.
87. Roberts, T.R. and Falconer, R.: Hops, Handbook of Brewing, 3rd edition. pub. Taylor & Francis, 2018.
88. Romano, P.; Suzzi, G.; Turbanti, L. and Polsinelli, M.: Acetaldehyde production in *Saccharomyces cerevisiae* wine yeasts, FEMS Microbiol. Lett., **118** (1994), no. 3, pp. 213-218.
89. Russell, I. and Stewart, G.G.: (eds) Whisky: Technology, Production and Marketing. 2nd edition. Pub. Academic Press. 2014.
90. Saison, D.; De Schutter, D.P.; Delvaux, F. and Delvaux, F.R.: Determination of carbonyl compounds in beer by derivatisation and headspace solid-phase microextraction in combination with gas chromatography and mass spectrometry, J. Chromatography, **A 1216** (2009), no. 26, pp. 5061-5068.
91. Saison, D.; De Schutter, D.P.; Uyttenhove, B.; Delvaux, F. and Delvaux, F.R.: Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds, Food. Chem., **114** (2009), no. 4, pp. 1206-1215.
92. Salema-Oom, X.; De Sousa, H.R.; Assunçao, M.; Gonçalves, P. and Spencer-Martins, I.: Derepression of a baker's yeast strain for maltose utilization is associated with severe deregulation of HXT gene expression, J. Appl. Microbiol., **110** (2011), no. 1, pp. 364-374.
93. Samp, E.J. and Sedin, D.: Important aspects of controlling sulphur dioxide in brewing, Tech. Quart. Master Brew. Assoc. Amer., **54** (2017), no. 2, 60-71.
94. Sentheshanmuganathan, S.: The purification and properties of the tyrosine – 2-oxoglutarate transaminase of *Saccharomyces cerevisiae*, Biochem. J., **77** (1960), no. 3, pp. 619-625.
95. Simpson, W.J. and Hammond, J.R.M.: The response of brewing yeast to acid washing, J. Inst. Brewing, **95** (1989), no. 5, pp. 347-354.
96. Stewart, G.G. High gravity brewing and distilling – past experiences and future prospects, J. Am. Soc. Brew. Chem., **68** (2010), no. 1, pp. 1-9.
97. Stewart, G.G.: Brewer's yeast propagation – the basic principles, Tech. Quart, Master Brew Assoc. Am., **54** (2017), no. 3, pp. 125-131.
98. Stewart, G.G.: Brewing and Distilling Yeasts, pub. Springer International, 2017.
99. Stewart, G.G.: Brewing Intensification. American Society of Brewing Chemists, 2014.
100. Stewart, G.G.: Esters – the most important group of flavour-active compounds in alcoholic beverages, Distilled Spirits. Production Technology and Innovation, Nottingham University Press., pp. 243-250, 2008.
101. Stewart, G.G.: Seduced by yeast, J. Am. Soc. Brew. Chem., **73** (2015), pp. 1-21.
102. Stewart, G.G.: Lean manufacturing including high gravity brewing. Handbook of Brewing. 3rd edition. Pub. Taylor & Francis, 2018.
103. Stewart, G.G.: *Saccharomyces* in the production of beer, Beverages, **2** (2016), no. 4, p. 34, MDPI AG.
104. Stewart, G.G.: Studies on the uptake and metabolism of wort sugars during brewing fermentations, Tech. Quart. Master Brew. Assoc. Am., **43** (2006), no. 4, pp. 265-269.
105. Stewart, G.G.: The Horace Brown Medal Lecture: Forty years of brewing research, J. Inst. Brewing, **115** (2009), no. 1, pp. 3-29.
106. Stewart, G.G.: The production of secondary metabolites with flavour potential during brewing and distilling wort fermentations, Fermentation, **3** (2017), p. 63, MDPI AG.
107. Stewart, G.G.: Yeast flocculation and flotation, Fermentation, **4** (2018), no. 2, p. 28, MDPI AG,.
108. Stewart, G.G. and Murray, J.: A selective history of high-gravity and high-alcohol beers, Tech. Quart. Master Brew. Association Amer., **47** (2010), 2-0416-01.
109. Stewart, G.G. and Murray, J.P.: Brewing intensification – Successes and failures, Tech. Quart. Master Brew. Assoc. Am., **49** (2012), no. 3, pp. 111-120.
110. Stewart, G.G. and Russell, I.: An Introduction to Brewing Science and Technology, Series III. Brewer's Yeast, The Inst. Brewing and Distilling, 2009.
111. Stewart, G.G.; Anstruther, A.M. and Russell, I.: (eds.), Handbook of Brewing, 3rd edition, pub., Taylor & Francis, 2018.
112. Stewart, G.G.; Hill, A. and Lekkas, C.: Wort FAN – its characteristics and importance during fermentation, J. Am. Soc. Brew. Chem., **71** (2013), pp. 179-185.
113. Stewart, G.G.; Hill, A.E. and Russell, I.: 125th anniversary review: developments in brewing and distilling yeast strains, J. Inst. Brewing, **119** (2013), no. 4, pp. 202-220.
114. Stewart, G.G.; Jones, R.M. and Russell, I.: The use of derepressed yeast mutants in the fermentation of brewery wort, Proc. 20th Cong. Eur. Brew. Conv., pp. 243-250.
115. Stewart, G.G.; Lyness, C.A. and Younis, O.S.: The control of ester synthesis during wort fermentation, Tech. Quart. Master Brew. Assoc. Amer., **36** (1999), no. 1, pp. 61-66.
116. Stewart, G.G.; Maskell, D.L. and Speers, A.: Brewing fundamentals – fermentation, Tech. Quart. Master Brew. Assoc. Amer., **53** (2016), pp. 2-22.
117. Stewart. G.G.: Industrial Uses of Yeast – brewing and distilling, New Food, **19** (2016), pp. 20-24.
118. Stovicek, V.; Holkenbrink, C. and Borodina, I.: CRISP/Cas system for yeast genome engineering: advances and applications, FEMS Yeast Res., **17** (2017), no. 5, pp. 1-16.
119. Suihko, M-L.; Penttilä, M.; Sone, S.; Home, S.; Blomqvist, K.; Tanaka, J.; Inove, T. and Knowles, J.: Pilot brewing with alpha-acetolactate decarboxylase active yeasts, Proc. 22nd Cong. Eur. Brew. Cong., pp. 483-490, 1989.
120. Timmins, E.M.; Quain, D.E. and Goodacre, R.: Differentiation of brewing yeast strains by pyrolysis mass spectrometry and Fourier transform infrared spectroscopy, Yeast, **14** (1998), no. 10, pp. 885-833.
121. Wainright, T.; Diacetyl – a review, J. Inst. Brewing, **79** (1973), no. 6, pp. 451-470.
122. Walker, G.M.: 125th Anniversary Review: fuel alcohol: current production and future challenges, J. Inst. Brewing, **117** (2011), no. 1, pp. 3-22.
123. Walker, G.M. and Stewart, G.G.: *Saccharomyces cerevisiae* in the production of fermented beverages, Beverages, **2** (2016), no. 4, p. 30, MDPI AG.
124. Wickner, W.: Yeast vacuoles and membrane fusion pathways, EMBO J., **21** (2002), no. 6, pp. 1241-1247.
125. Yamano, S.; Tomizuka, K.; Sone, H.; Imura, M.; Takeuchi, S. and Inoue, T.: Brewing performance of a brewer's yeast having alpha-acetolactate decarboxylase from *Acetobacter aceti* spp. *xylinum* in brewer's yeast, J. Biotechnol., **39** (1995), no. 1, pp. 103-111.
126. Yokata, H.; Sahara, H. and Koshino, S.: Fractionation and quantitation of oligopeptides in beer and wort, J. Am. Soc. Brew. Chem., **51** (1993), no. 2, pp. 54-57.
127. Younis, O.S. and Stewart, G.G.: Sugar uptake and subsequent ester

and alcohol production in *Saccharomyces cerevisiae*, J. Inst. Brewing, **104** (1998), no. 5, pp. 255-264.

128. Younis, O.S. and Stewart, G.G.: The effect of malt wort, very high gravity malt wort and very high adjunct wort on volatile production in *Saccharomyces cerevisiae*, J. Amer. Soc. Brew. Chem., **57** (1999), no. 2, 39-45.
129. Younis, O.S. and Stewart, G.G.: The effect of maltose content on volatile production and fermentation performance in brewing yeast, Brewing Yeast Fermentation and Performance, Pub. Blackwell Science Publications, pp. 170-176, 2000.
130. Zhang, C-Y.; Liu, Y-L.; Qi, Y-N.; Zhang, J.W.; Dai, L.H.; Lin, H. and Xiao, D-G.: Increased esters and decreased alcohols production by brewer's yeast strain, Eur. Food Res. Technol., **236** (2013), no. 6, pp. 1009-1014.
131. Zheng, X.; D'Amore, T.; Russell, I. and Stewart, G.G.: Factors influencing utilization during brewery fermentations, J. Am. Soc. Brew. Chem., **52** (1994), no. 2, pp. 41-47.
132. Zheng, X.; D'Amore, T.; Russell, I. and Stewart, G.G.: Transport kinetics of maltotriose in strains of *Saccharomyces*, J. Indust. Microbiol., **13** (1994), no. 3, pp. 159-166.