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125th Anniversary Review: Developments in brewing and distilling yeast strains

Graham G. Stewart,[1,2] Annie E. Hill[2] and Inge Russell[2,3]*

Progress during the past 25 years regarding our knowledge of brewer's yeast strains is considered. This is not a comprehensive review but rather focuses on some specific areas. These areas include a brief description of genomics, proteomics and metabolomics as applied to brewer's yeast strains. This review subsequently considers differences between ale and lager yeast strains, the uptake and metabolism of wort sugars and amino acids, yeast flocculation, yeast management between fermentations and yeast strain genetic stability. The question of process intensification, with particular attention to high-gravity brewing, is also addressed. Fermentation systems and processes are considered with an emphasis on novel procedures for stirred fermentations. **Copyright © 2013 The Institute of Brewing & Distilling**

Keywords: amino acids; centrifuges; fermenters; flocculation; wort sugars; yeast management

Introduction

In 1986, to mark the centenary of the Institute of Brewing, the authors published a review paper in this journal considering 'yeast research and development in the brewing industry' *(1)*. This review described the significant developments that had occurred in the research and development of brewer's yeast during the preceding 100 years. It also was emphasized that these developments had provided a considerable volume of information that had enriched both applied and fundamental information on yeast as a model eukaryote. It was highlighted that this research on yeast, as a fundamental eukaryote, had greatly assisted research on brewing and other industrial yeast strains. In this review, to commemorate the 125th Anniversary of the Institute, the synergy between fundamental fact-finding research and applied yeast research will be emphasized. The material covered here is not comprehensive and covers only select areas of research.

At the conclusion of the centenary review, Rainbow *(2)* was quoted: 'We need more information about the enzymatic make-up of the yeast cell, the genetic control of that make-up, the quantitative interplay of its metabolic pathways and the changes the latter undergo in response to changes in wort composition'. Since the publication of that centenary review many (but not all) of these requirements have been met. They come under the heading of three inter-related subject areas, namely genomics, proteomics and metabolomics *(3)*. Genomics concerns the study of organism genomes *(4)*. The field includes efforts to determine the entire DNA sequence of organisms and fine-scale genetic mapping efforts. The field also includes studies of intragenomic phenomena such as interaction between loci and alleles within the genome. Research of single genes does not usually fall into the definition of genomics. Proteomics *(5)* is the large-scale study of proteins, particularly their structure and function. Metabolomics *(6)* is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind – specifically, the study of their small-molecule metabolite profiles.

The objectives of brewer's and distiller's wort fermentations are to consistently metabolize wort constituents into ethanol and other fermentation products in order to produce products with satisfactory quality and stability. In addition, brewer's yeast should produce yeast crops that can be confidently re-pitched into subsequent brews. This review will largely discuss how this data has embellished our knowledge of brewing and distilling yeasts and how this information can be applied to enhance the efficiency and quality of brewing and distilling fermentation processes. This has resulted in improved product quality at lower cost.

The past 25 years have witnessed unprecedented developments in the molecular biology of yeast and many other organisms *(7)*. However, the expectation that genetically manipulated yeast strains would be extensively employed in brewing and distilling has not been achieved. In our centenary review we stated *(1)*: 'The use of manipulated yeast strains in brewing will become commonplace within the next decade with yeast strains specifically bred for such characteristics as extra-cellular amylases, β-glucanases, proteins, β-glucosidase production, pentose

* Correspondence to: Inge Russell, Alltech Inc., Nicholasville, KY 40356, USA. E-mail: irussell@alltech.com

[1] 13 Heol Nant Castan, Rhiwbina, Cardiff CF14 6RP, UK

[2] ICBD, Heriot–Watt University, Riccarton, Edinburgh EH14 4AS, UK

[3] Alltech Inc., Nicholasville, KY 40356, USA

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and lactose utilization, carbon catabolite repression, lower diacetyl and production of a plethora of heterologous proteins. There is no doubt that prior to the introduction of such strains at the production level, the environmental and legal impact of such a move will have to be assessed'. Twenty-five years later, genetically manipulated brewer's and distiller's yeast strains are still not routinely employed commercially, owing in large part because of opposition from public opinion. Whether this will change, only time will tell! Nevertheless, genetic techniques have been used to study the genetic composition and function of such strains. A number of examples of genetic manipulation of brewing and distilling strains will be cited below.

The requirements of an acceptable brewer's yeast strain can be defined as *(8)*: 'In order to achieve a beer of high quality, it is axiomatic that not only must the yeast be effective in removing the required nutrients from the wort, able to tolerate the prevailing environmental conditions (for example, osmotic and ethanol tolerance) and impart the desired flavour to the beer, but the microorganisms themselves must be effectively removed from the fermented wort by flocculation, centrifugation and/or filtration after they have fulfilled their metabolic role.' Essentially, the requirements of distiller's yeast are similar, except removal of yeast at the end of fermentation is not an issue, because the yeast is not recycled from one fermentation to another, the fermented wash (wort) plus yeast go directly into distillation. Details of the requirements of both brewing and distilling yeasts will be discussed during the rest of this review.

The yeast *Saccharomyces cerevisiae* has been used as a model organism for eukaryotic systems. It has a short generation time, can easily be cultivated in artificial media, can grow as a haploid, making genetic studies much simpler, and in 1996 was the first eukaryotic genome (haploid strain S288c) that was completely sequenced *(9)*. There are a number of databases that contain a wealth of information on the genes and proteins in this organism. One such site is the Saccharomyces Genome Database located at http://www.yeastgenome.org/. This site provides not only information on the budding yeast *S. cerevisiae*, but also has search and analysis tools allowing the researcher to explore the data. Currently, there are over 6000 protein coding genes identified. Recently an iphone App has been published to help researchers navigate and monitor the vast database of *Saccharomyces* for their direct genes of interest *(10)*.

Over the years, considerable effort has been devoted to a study of the biochemistry and genetics of brewer's yeast (and other industrial yeast strains). The whole genome sequence for over 30 *S. cerevisiae* strains has been published (Table 1) *(11)*. Industrial strains sequenced include the Japanese diploid saké yeast *S. cerevisiae* Kyokai no. 7, which was published in 2011 *(12)*, increasing our understanding of the functional and evolutionary genomics of this saké yeast. Borneman *et al. (13)* examined the whole genomes of a brewing ale yeast, a lager yeast and a number of wine yeasts. Babrzadeh *et al. (14)* published the whole-genome sequence of an industrial fuel ethanol strain widely used in Brazil (strain CAT-1) and Zheng *et al. (15)* used genome sequencing to examine the bioethanol *S. cerevisiae* strain YJS329.

Whole genomes are now being analysed *en masse* to answer specific questions *(16)*. Indeed, the explosion of research in this area has spawned an entire new area of investigation and the overwhelming amount of literature is almost beyond comprehension for the brewing and distilling scientist – in 2012 there were over 5792 papers listed as published in the area of yeast genomics on the yeast genome website.

The objectives of the studies by brewing scientists on industrial strains have basically been twofold: (1) to learn more about the biochemical and genetic characteristics of yeast strains; and (2) to improve the overall performance of such strains with particular emphasis being placed on broader substrate utilization capabilities, increased ethanol production, improved stress tolerance to environmental conditions such as high osmotic pressure, ethanol, temperature, salt, physical shear, and to understand the mechanisms of flocculation.

Understanding the substrate specificity of brewing and other industrial yeast strains is a major objective of many zymologists. Progress in achieving these objectives has been advanced by a detailed knowledge of wort composition that has been publicized during the past 25 years *(17)*. However, progress has been impeded for several reasons, including the fact that brewing and distilling yeast strains are not as readily amenable to genetic manipulation by classical techniques. Nevertheless, with the many newer methods that can now be employed in genetic research and development of industrial yeast strains, this is no longer the barrier it once was.

Industrial yeast strains possess stable and reproducible characteristics because they are usually polyploid or even aneuploid and, as a consequence, do not possess a mating type and have a low degree of sporulation and spore viability, rendering genetic analysis of such strains more difficult, but not impossible *(18)*.

Ale and lager yeast strains

One of the aspects of brewer's yeast that has intrigued many students of brewer's yeast, including these authors during the past 40 years, is the reasons for the differences between ale and lager yeast strains. Considerable research by many brewers and academic institutions has been conducted *(16)* and a number of typical differences between ale and lager yeast strains have been established and accepted (Table 2).

The availability of a wide range of whole genome sequences has enabled detailed comparison of industrial strains and has revealed that brewing strains are interspecies hybrids. The nomenclature arguments in terms of what brewing strains should be called and of their exact origin are long running, but a recent paper *(19)* has identified a new yeast isolated in Patagonia. Named *Saccharomyces eubayanus*, this yeast is thought to be an early ancestor of lager yeast and to have given yeast the capacity to ferment at cold temperatures. Lager brewers ferment wort at relatively cool temperatures, then condition beer under refrigeration. *Saccharomyces pastorianus* (the correct taxonomic name for the species that brewers used to refer to as *Saccharomyces carlsbergensis*) can tolerate lower temperatures than can ale-producing yeasts. This so-called cryotolerant lager yeast is a hybrid of the ale yeast *S. cerevisiae* and another yeast species that in the past has evaded conclusive identification. When the *S. eubayanus* genome was sequenced it was found to be distinct from any previously described species, but it was a 99.5% match with the missing piece of the hybrid lager yeast *S. pastorianus* – the part of the hybrid not accounted for by the well-studied, warm-fermenting ale yeast *S. cerevisiae (19)*. Nguyen *et al. (20)* also studied the hybridization history of lager *Saccharomyces* strains, finding mosaic genomes and patterns of introgression between *Saccharomyces bayanus*, *Saccharomyces uvarum* and *S. cerevisiae* (Fig. 1). The same novel species named *S. eubayanus* by Libkind *et al. (19)* was identified by Ngyuyen *et al. (20)* and called *Saccharomyces lagerae*.

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Table 1. Whole genome sequences of *S. cerevisiae* – table adapted from Engeland and Cherry *(11)*

Strain	Year	Provenance
S288C	1996	Laboratory strain
RM11-1a	2005	Haploid derivative of California vineyard isolate
YJM789	2007	Haploid derivative of opportunistic human pathogen
M22	2008	Italian vineyard isolate
YPS163	2008	Pennsylvania woodland isolate
AWRI1631	2008	Haploid derivative of South African commercial wine strain N96
JAY291	2009	Haploid derivative of Brazilian industrial bioethanol strain PE-2
EC1118	2009	Commercial wine strain
Sigma1278b	2009	Laboratory strain
Foster's O	2010	Commercial ale strain
Foster's B	2010	Commercial ale strain
VIN13	2010	South African white wine strain
AWRI796	2010	South African red wine strain
CLIB215	2010	New Zealand bakery isolate
CBS7960	2011	Brazilian bioethanol factory isolate
CLIB324	2011	Vietnamese bakery isolate
CLIB382	2011	Irish beer isolate
EC9-8	2011	Haploid derivative of Israeli canyon isolate
FL100	2011	Laboratory strain
Kyokai No.7	2011	Japanese sake yeast
QA23	2011	Portuguese Vinho Verde white wine strain
PW5	2011	Nigerian Raphia palm wine isolate
T7	2011	Missouri oak tree exudate isolate
T73	2011	Spanish red wine strain
UC5	2011	Japanese sake yeast
VL3	2011	French white wine strain
W303	2011	Laboratory strain
Y10	2011	Philippine coconut isolate
YJM269	2011	Austrian Blauer Portugieser wine grapes
BY4741	2012	S288C-derivative laboratory strain
BY4742	2012	S288C-derivative laboratory strain
CEN.PK 113-7D	2012	Laboratory strain
ZTW1	2012	Chinese corn mash bioethanol isolate
YJS329	2012	Chinese bioethanol strain *(15)*
BYZ1	2012	S288C-derivative laboratory strain *(15)*

Table 2. Traditional differences between ale and lager yeast strains

Ale yeast
Saccharomyces cerevisiae (ale type)
Fermentation temperature 18–22°C
Maximum growth temperature 37°C or higher
'Top' fermenter
Lager yeast
Saccharomyces uvarum (carlsbergensis)
Saccharomyces cerevisiae (lager type)
Saccharomyces pastorianus
Fermentation temperature 8–15°C
Maximum growth temperature 34°C
Ferments melibiose
'Bottom' fermenter

Further sequencing studies have shown that *Saccharomyces bayanus* is a complex hybrid of *S. eubayanus*, *Saccharomyces uvarum* and *S. cerevisiae* and to date has only been found in brewing environments. *Saccharomyces paradoxus* is a yeast species that lives on the bark of deciduous trees and its closest relative is *S. cerevisiae*. It is a phylogenetically distinct species, as the haploid gametes of *S. paradoxus* and *S. cerevisiae* cannot combine into a viable diploid hybrid organism *(21)*. Genetic hybridization, sequence and karyotypic analyses of natural *Saccharomyces* yeasts isolated in different regions of Taiwan revealed three biological species: *Saccharomyces arboricola*, *S. cerevisiae* and *Saccharomyces kudriavzevii (22)*.

Yeasts used in beverage production mostly belong to the genus *Saccharomyces*. There are various species of *Saccharomyces*, including *S. bayanus*, *S. cariocanus*, *S. cerevisiae*, *S. eubayanus*, *S. kudriavzevii*, *S. mikitae*, *S. paradoxus*, *S. pastorianus* and in some sources *S. uvarum*, which is usually considered as a subspecies of *S. bayanus*. The nomenclature and classification of species changes almost daily and therefore is not always uniform in the literature *(23)*. The species can be further classified into different strains and there are currently thousands of different strains of *S. cerevisiae*. Hybridization is common between the domesticated yeasts used in alcohol production. The yeasts used in the whisky industry are mostly *S. cerevisiae*, although various

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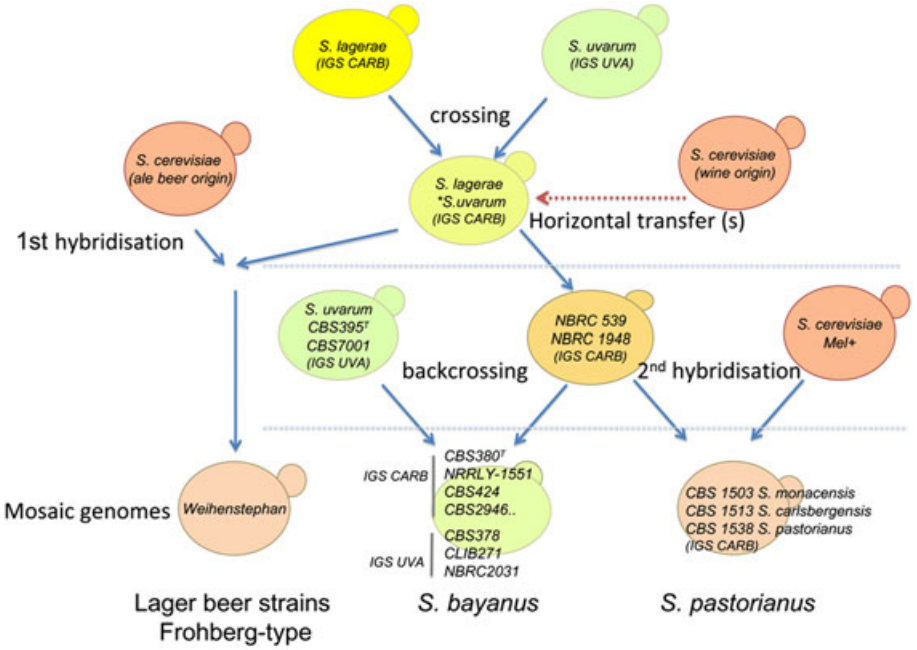

Figure 1. Proposal of evolution of *Saccharomyces* lager species through domestication. Graphic is reproduced from Nguyen *et al. (20)*.

secondary species are also used. Baker's yeast is usually *S. cerevisiae*, lager yeast is *S. pastorianus*, ale yeasts include *S. cerevisiae* and apparently some *S. bayanus* strains, rum is fermented primarily with *S. cerevisiae* and *Schizosaccharomyces* (with various wild yeasts), and the wine industry uses mostly *S. cerevisiae* and/or *S. bayanus*, together with various wild yeasts (for example *Kloeckera*, *Saccharomycodes*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Pichia* and *Torulopsis*).

Recent experiments by Piotowski *et al. (24)* have examined how selective pressure, in terms of temperature and ethanol, can lead to different genomic outcomes owing to interspecific hybridization as the yeast strains evolved over time. This is giving us better insights into how the current industrial strains may have adapted over time. This study concurs with the suggestion that the evolution of yeast may be due to the influence of brewers, who unknowingly applied selection pressures on the yeasts, for adaptation to a brewing environment *(25)*.

Studies on the differences between ale and lager yeast strains and their impact on beer flavour and overall beer characteristics have been conducted for many years. However, they have intensified during the last 25 years and, as already discussed, come under the three headings of genomics, proteomics and metabolomics *(26)*. Genetic studies on these two brewing yeast types have already been discussed. However, most of these studies have no direct brewing application.

A number of different studies are directly relevant to brewing. These studies include the following:

- There are differences in flocculation characteristics, which will be discussed in some detail below.
- There are subtle differences in the uptake of wort sugars particularly maltose and maltotriose *(27,28)*, and wort amino acids *(29)*. Lager strains appear to utilize maltotriose more efficiently than ale strains, with less residual maltotriose at the end of a lager fermentation *(30,31)*.
- Ale cultures are amenable to drying, whereas lager cultures are not easily dried *(32,33)*. Although the exact reason for this difference is not clear, it is thought that there is a relationship between intracellular trehalose levels, the ability to dry yeast cells and yeast viability *(34)*.
- Lager strains, under normal brewing conditions, produce considerably more sulphur dioxide than ale strains *(35)*. This difference is thought to be due to divergent sulphur metabolism pathways, and the lower fermentation temperature during lager production *(36)*.
- Ale/lager differences between diacetyl and other vicinal diketone (VDK) metabolites are also apparent *(37)*. Lager strains produce more α-acetolactate and subsequently diacetyl than ale strains. However, under similar environmental conditions, the rate of subsequent removal of diacetyl, late in the fermentation, is similar for both ale and lager yeast strains *(38,39)*.
- Lager strains also possess the *FSY 1* gene, which encodes a fructose transporter. This gene is not present in ale strains *(40)*.
- As already discussed, lager strains exhibit poorer temperature tolerance than ale strains *(41,42)*. Ale strains will grow at 37°C, whereas lager strains will not grow above 34°C. Plates containing lager yeast, and incubated at 37°C immediately after inoculation onto a nutrient media, will show no growth and any growth on the plates indicates the presence of contamination with an ale yeast or a wild yeast, and is a useful check for lager yeast purity in the pitching yeast *(43)*.

As detailed in Table 2, a 'traditional' difference between ale and lager yeast strains (*S. cerevisiae* and *S. pastorianus* respectively) is that lager strains are able to metabolize the sugar melibiose. The ability to metabolize melibiose depends on the presence of the enzyme melibiase (α-galactosidase), which is secreted into the periplasmic space of the yeast cell, and hydrolyses melibiose into glucose and galactose. These monosaccharides are subsequently taken up by the lager yeast culture. The production of melibiase is possible because of the presence in the lager strain genome of one or more *MEL* genes – a polymeric series of genes *(44)*.

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Ale yeast strains are unable to metabolize the sugar melibiose because they cannot produce melibiase owing to the absence of an active *MEL* gene. This ability to utilize melibiose has industrial relevance in the production of bioethanol from sugar beet *(45)*.

Metabolism of wort constituents

When yeast is pitched into wort, it is introduced into an extremely complex environment owing to the fact that wort is a medium consisting of simple sugars, dextrins, amino acids, peptides, proteins, vitamins, ions, nucleic acids and other constituents too numerous to mention *(43,46)*. Compared with other media employed for potable and industrial alcohol production – must, cane juice, molasses-based media, apple/pear juice, various syrups, etc. – wort is a very sophisticated medium. It functions as both a growth medium to develop new yeast cells and as a fermentation medium for the yeast to produce ethanol, carbon dioxide and other metabolic products, many of which influence the flavour of the beer and spirits *(47)*. Later it will be discussed that, without the correct genetic make-up (genomic aspects), the yeast strain cannot conduct effective growth (proteomic aspects) and complete wort fermentation will not occur. Consequently, unwanted metabolites will be produced, or required metabolites will not be produced (metabolic aspects). This will result in alcoholic products with atypical flavour characteristics.

Wort sugars

Wort contains the sugars sucrose, fructose, glucose, maltose and maltotriose together with dextrin material *(48)*. One of the major advances in brewing and distilling science during the past 40 years has been the elucidation of the mechanisms by which the yeast cell utilizes, in an orderly manner, the plethora of wort nutrients. In the normal situation, *S. cerevisiae* strains, including brewing strains, have the ability to take up and ferment a wide range of sugars, for example, sucrose, glucose, fructose, galactose, mannose, maltose and maltotriose, in this approximate sequence (or priority), although some degree of overlap does occur, leaving maltotetraose and the larger dextrins unfermented (Fig. 2). In addition, *S. diastaticus* (a sub-species of *S. cerevisiae*) is able to utilize some dextrin materials.

The initial step in the utilization of any sugar by yeast is either its passage intact across the cell membrane or its hydrolysis outside the cell membrane, followed by entry into the cell by some or all of the hydrolysis products (Fig. 3).

Maltose (a disaccharide formed from two units of glucose joined with an α-1,4 bond) and maltotriose (a trisaccharide formed from three units of glucose with α-1,4 bonds) are examples of sugars that pass intact across the yeast cell membrane. Sucrose and dextrins are hydrolysed by extracellular enzymes [invertase for sucrose and glucoamylase (amyloglucosidase) for dextrins] and the hydrolysis products are taken up into the cell *(40)*. An important metabolic difference between the uptake of monosaccharides, such as glucose and fructose vs maltose and maltotriose uptake, is that energy (ATP conversion to ADP) is required for maltose and maltotriose uptake (i.e. active transport) whereas glucose and fructose are taken up passively (no ATP required) via facilitated diffusion mediated by specific hexose permeases *(49)*.

Maltose and maltotriose are the major sugars in most brewer's worts, spirit mash and wheat dough, and as a consequence, a brewing yeast's ability to use these two sugars is vital and depends upon the correct genetic complement, which is a diverse and complex system. Competition for the same transporters for maltose and maltotriose, with maltose being the preferred substrate, result in maltose being utilized first, as transport is the rate-limiting step *(50,51)*.

Maltose fermentation in brewing, distilling and baking yeasts requires at least one of five unlinked polymeric (*MAL*) loci located in the telomeric regions of the different chromosomes (*MAL1–MAL4* and *MAL6*) . The genes for maltose and maltotriose fermentation are located in the *MAL* loci *(52,53)*. Each *MAL* locus consists of three genes encoding (1) the structural gene for α-glucosidase (maltase), (2) maltose permease and (3) an activator protein needed for regulation of the expression of the α-glucosidase and permease genes.

The expression of the maltase and the maltose transporter is also regulated by maltose induction and glucose repression. When glucose concentrations are high (greater than 10 g/L), the *MAL* genes are repressed and only when 40–50% of the glucose has been taken up from the wort will the uptake of maltose and maltotriose commence (Fig. 2).

Brewer's yeast strains possess independent uptake mechanisms (maltose and maltotriose permeases) to transport the

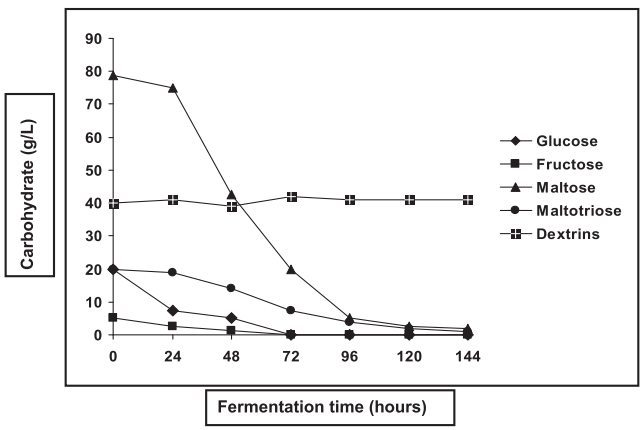

Figure 2. Order of uptake of wort sugars by brewing lager yeast at 15°C from a 16°P, 30% corn adjunct wort.

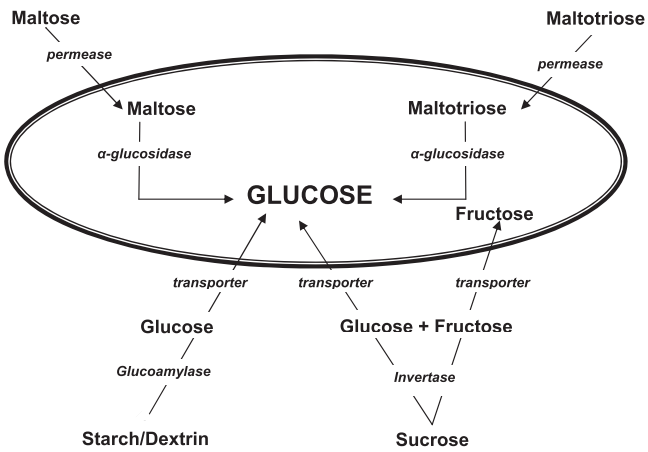

Figure 3. Uptake of wort sugars by the yeast cell.

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two sugars across the plasma membrane into the cell. Four different types of transporter have been identified, Malx1, Mtt1, Mphx and Agt1, and they differ in their distribution and in their substrate range *(50–52,54–56)*. Almost all brewing strains of *S. cerevisiae* strains examined have the AGT1 gene and an increased copy number of *MALx1* permeases. *MAL* transporter genes are generally regarded as specific for maltose *(53)*, but activity towards maltotriose has been claimed *(57)*. The *AGT1* (α-glucoside transporter) encodes the widest substrate specificity reported to date *(51)*. The Agt1 transporter can transport trehalose, turanose, α-methylglucoside and sucrose in addition to maltose and maltotriose *(51)*. Once inside the cell, both sugars are hydrolysed to glucose units by the α-glucosidase system.

As already discussed, maltose transport by ale and lager yeast strains has been compared. *S. pastorianus* yeast strains utilize maltotriose more efficiently than *S. cerevisiae* strains *(30)*. Crumplen *et al*. *(58)* found that glucose more strongly inhibited maltose transport by an ale strain than by a lager strain. Rautio and Londesborough *(59)* found that trehalose and sucrose strongly inhibited maltose transport by an ale strain, but only weakly inhibited maltose transport by a lager strain. Taken together, these results suggest that the dominant maltose transporters of the ale strains studied had a broader specificity than those of lager strains and were probably *Agt1* proteins.

However, hybridization studies showed that all of the ale and lager strains tested contained *AGT1* and several *MAL* genes *(50)*. This discrepancy has been partially resolved by the finding *(60)* that the *AGT1* genes contained stop codons rendering these genes defective. The same defect has also been found in other lager strains but not in ale strains *(61)*. Glucose was noted to repress the expression of the Agt1 gene in the ale strains *(62)*. Gibson *et al*. *(63)* recently examined transcription of the α-glucoside genes in *S. pastorianus*.

A strong temperature dependence for maltose transport has been found for ale yeasts; however, a markedly smaller temperature dependence for the transport of this sugar was observed with lager strains. The faster fermentation of lager yeast at the low temperatures may be the result of the different maltose transporters *(42)*. Vidgren *et al*. showed that, in the ale and lager strains studied, the strains used different maltose and maltotriose transporter types. For the ale strains, the Agt1 transporter was dominant, whereas for the lager strains the Malx1 and Mtt1-type dominated *(42)*.

A number of ale and lager yeast strains have been employed in order to explore the mechanisms of maltose and maltotriose uptake in wort. A 16°P all-malt wort was fermented in a 30 L static fermentation vessel. Under these conditions, lager strains utilized maltotriose more efficiently than ale strains, whereas maltose utilization efficiency was not dependent on the type of brewing strain *(30)*. This supports the proposal that maltotriose and maltose possess independent, but closely linked, uptake (permease) systems *(31)*. In addition, this consistent difference between ale and lager strains supports the observation *(30)* that ale strains appear to have greater difficulty completely fermenting wort (particularly high-gravity wort) than lager strains.

In order to investigate the MAL gene cassettes further, a strain with two *MAL2* and two *MAL4* genes copies was constructed, employing hybridization techniques. The wort fermentation rate was compared with a strain containing only one copy of *MAL2*. As expected, the overall fermentation rate with the strain containing multiple *MAL* genes was considerably faster than the strain containing a single copy of *MAL2*. The principal reason for this faster fermentation rate was due to an increased rate of maltose uptake and subsequent metabolism, compared with the yeast strain containing the single *MAL2* copy *(64)*.

The *S. pastorianus* lager yeast usually ferments maltose and maltotriose efficiently, and transport of these α-glucosides into the brewer's yeast cell is thought to be rate-limiting in the fermentation of these sugars from wort. A new maltotriose transporter gene in the *S. eubayanus* subgenome of an industrial lager brewing strain (Weihenstephan 34/70) has been reported, and it allows efficient maltotriose fermentation by yeast cells. The characterization of maltotriose transporters from industrial yeasts opens new opportunities to increase yeast fitness for the fermentation of brewer's wort, preventing incomplete or sluggish maltotriose fermentations *(65)*.

There are a number of considerations regarding the metabolism of wort sugars during brewing and distilling that have to be considered. The effects of high-gravity brewing are particularly important and some aspects of this area will be reviewed below.

Metabolism

It is well documented that nitrogen is an essential element for yeast growth and function *(66,67)*. It has already been emphasized that active yeast growth is critical for efficient wort fermentation. Active yeast growth involves the uptake of nitrogen and as yeast multiplication stops, nitrogen utilization also begins to decelerate. Not all nitrogen materials in the wort can be utilized by the yeast to conduct its metabolic activities.

Free amino nitrogen (FAN) is the grouping of nitrogenous compounds available for consumption by yeast. FAN is the sum of the individual wort amino acids, ammonium ions and small peptides (di-, tripeptides). FAN is an important general measure of the yeast nutrients, which constitute the yeast assimilable nitrogen during brewery fermentations *(68–70)*.

Research in this area during the past 25 years has confirmed that, even if attenuation of wort carbohydrates proceeds normally, the same quality of beer is not guaranteed to be produced. This confirms that the sugar content of wort alone is not a good indicator of yeast performance *(71)*. Indeed, the uptake of sugar is stimulated by higher nitrogen levels in high-gravity wort. Consequently, FAN is regarded as a better index for prediction of healthy yeast growth, viability, vitality, fermentation efficiency and hence beer quality and stability *(72)*. In addition, wort FAN is used by the yeast to accomplish its metabolic activities such as the *de novo* synthesis of amino acids and ultimately structural and enzymatic proteins *(73)*.

There are differences between lager and ale yeast strains with respect to wort-assimilable nitrogen uptake characteristics *(74)*. Nevertheless, with all brewing strains the amount of wort FAN content required by yeast under normal brewery fermentation is directly proportional to yeast growth and also affects certain aspects of beer maturation, for example diacetyl management *(38,75)*. There has been considerable polemic regarding the minimal FAN required to achieve satisfactory yeast growth and fermentation performance in normal gravity (10–12°P) wort and it is generally agreed to be around 130 mg FAN/L. For rapid attenuation of high-gravity wort (16°P), increased levels of FAN are required – details are given below *(76)*. However, optimum FAN levels differ from fermentation to fermentation and from yeast strain to yeast strain. Furthermore, the optimum FAN values change with different wort sugar levels and type *(70)*.

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During the 1960s, Margaret Jones and John Pierce conducted notable studies on nitrogen metabolism during malting, mashing and fermentation. They reported that the absorption and utilization of exogenous nitrogenous wort compounds and their synthesis intracellularly are controlled by three main factors: (1) the total wort concentration of assimilable nitrogen; (2) the concentration of individual nitrogenous compounds and their ratio; and (3) the competitive inhibition of the uptake of these components (mainly amino acids) via various permease systems *(74,77)*.

Jones and Pierce *(74)* established a unique classification of amino acids according to their rates of consumption during ale brewing wort fermentations (Table 3). When this classification was developed, the methodology employed (liquid chromatography for measuring individual amino acids, etc.) was iconic. Similar measurements today employ automated computerized high-performance liquid chromatography and it is difficult to envisage the challenges that were overcome 50 years ago! The Jones and Pierce amino acid classification *(74)* is the basis of today's understanding of the relative importance of individual wort amino acids during the fermentation and manipulation of wort nitrogen levels, by the addition of yeast extract on specific amino acids during high-gravity brewing. However, this assimilation pattern of FAN is often specific to the conditions employed. The yeast strain's nutritional preferences is perhaps most significant. Because of the differences in malting barley varieties, brewing conditions and the yeast strains employed in the brewing industry worldwide, a more detailed view is desirable *(28)*. Recent studies have confirmed the Jones and Pierce amino acid classification, but it has been suggested that methionine uptake be moved to Group A from Group B *(78)*.

A research project conducted to investigate the wort components that might play an essential role in brewer's yeast strain fermentation performance perhaps ended up posing more questions than it provided answers *(79)*. A quantitative and qualitative identification and determination of malt nitrogen compounds that affect yeast metabolic activity in terms of oligopeptides, ammonium salts and both total and individual amino acids was performed. Fermentation results indicated that wort FAN correlated well with at least three fermentation performance indicators *(28)*. First, high initial FAN content allowed a more efficient reduction of the wort gravity. Secondly, pH decrease during fermentation was proportional to the amount of FAN utilized, and thirdly, the FAN wort content was suggested to be a useful index towards the formation of total VDKs, esters and higher alcohols in the later stages of fermentation.

Wort FAN has a direct influence on beer quality, through its components and the metabolites surviving in the final product. These determine some key aspects of beer flavour and also impact on yeast performance. It has been suggested that the most useful index of tolerance is the flavour compounds that display the most sensitive reaction to a change in one or more wort FAN compounds. Consequently, there is a need for the development of models relating wort FAN composition and yeast quality to the production of metabolites that have a flavour impact. A model of this nature could assist the brewer in controlling wort FAN composition. Currently, most brewers rely on wort as an index of fermentation quality and yeast quantity, assuming the relative balance of nitrogenous materials remains constant. In terms of beer flavour, it is not only a matter of the initial wort FAN content, but also equally the amino acid and ammonium ion equilibrium in the medium, and a number of undefined fermentation parameters. Our knowledge of the roles of nitrogenous components of malt and wort in order to meet yeast requirements has substantially increased over the years. Nevertheless, optimization of the nitrogen content of wort is a very complex issue owing to the large number of nitrogen compounds found in the malt *(68)*.

Yeast flocculation and cell wall structure and function

The topic of yeast flocculation and sedimentation in brewing has been well reviewed in the 125th anniversary review by Vidgren and Londesborough *(62)*, by Speers *(80)*, and in the IBD 'Blue Book' *(81)*. These publications discuss the major determinants of flocculation, the effects of the environment on flocculation, as well as the phenomenon of premature yeast flocculation. Soares *(82)* has also published a comprehensive review of flocculation in *S. cerevisiae*. The flocculating property of a particular yeast culture is one of the major factors when considering important characteristics during brewing and distilling fermentations, particularly brewing *(83)*. Flocculation has many definitions *(84)*. However, the one that we have used for many years is: 'the phenomenon wherein yeast cells adhere in clumps and either sediment from the medium in which they are suspended or rise to the medium's surface'. This definition excludes forms of 'clumpy-growth' and 'chain formation', which will not be discussed further *(84)*. The importance of flocculation as a method to crop a yeast culture at the end of primary fermentation, in order that it can be re-used in a subsequent fermentation, cannot be overstated.

Table 3. The order of wort amino acid uptake during an ale fermentation as reported by Jones and Pierce *(77)*

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	Methionine	Phenylalanine	
Asparagine	Leucine	Tyrosine	
Glutamine	Isoleucine	Tryptophan	
Serine	Histidine	Alanine	
Threonine		Ammonia	
Lysine			
Arginine			

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There are currently three proposed mechanisms to explain yeast cell flocculation: zymolectin binding, hydrophobic interactions and surface charge neutralization *(85)*. However it seems very likely that more than one mechanism is at play during a brewing fermentation. For example, Speers *et al*. *(86)* have shown that fermentable sugar levels, as well as shear force, exert a major influence on yeast flocculation during brewery fermentations. Recent studies *(87)* have found that nitrogen starvation induces flocculation in bottom-fermenting yeast. Both fungi and malt have also been implicated in premature yeast flocculation *(88,89)*.

Genetic studies on yeast flocculation began over 50 years ago and in 1951 Thorne *(90)* and Gilliland *(91)* confirmed that this phenomenon was an inherited characteristic, with flocculence being dominant over non-flocculence. The first flocculation gene (*FLO* gene) to be studied in detail was *FLO1*. Employing traditional gene mapping techniques (mating, sporulation, micromanipulation, tetrad analysis, etc.), it was shown that *FLO1* is located on Chromosome I, 33 cM from the centromere on the right-hand side of the chromosome and this was later confirmed by cloning of the physical localization *(92,93)*.

Genome sequencing of a laboratory strain of *S. cerevisiae* has found five *FLO* genes, four located nearby the chromosome telomeres *FLO1*, *FLO5*, *FLO9* and *FLO11*, and one neither at the centromere nor at the telomeres – *FLO11 (87)*. These genes encode lectin-like proteins, which are also known as adhesins, zymolectins or flocculins. The widely accepted model for yeast flocculation describes it as the result of the interaction between adhesins and mannans, polysaccharides built up of mannose residues, present on mannoproteins in the cell wall *(94)*. In most laboratory strains (haploid and diploid), added mannose will block adhesin-binding sites and thus inhibit flocculation by preventing the adhesins from binding the mannose present on neighbouring cells *(95)*. A similar adhesin of considerable industrial importance responsible for the mannose-, glucose- and maltose-sensitive 'new flo' type of lager yeast strains has been identified *(96)*. In this case, competitive binding of such carbohydrates by this adhesin takes place and ensures that flocculation only occurs at the appropriate stage in the wort fermentation, namely, when all fermentable carbohydrates have been depleted and hopefully when the VDK levels (particularly diacetyl) are under control.

It has been reported that yeast strains that exhibit ideal properties on day 1 of a fermentation can rapidly change and evolve towards too early, or too late, flocculation onset and even lose the ability to flocculate altogether. A plethora of studies have described the presence of mutated sequences in the *FLO1* gene *(97)*. Frequent intragenic recombination events will typically result in the net loss or gain of tandem repeat units. Expansion of the *FLO1* tandem repeat domain size results in stronger flocculation *(98)*.

The genetic variability of flocculation genes has important consequences for studies and applications targeting these genes in brewing and distilling strains, which possess unknown genomes because of their polyploid and aneuploid characteristics. It has been revealed that there is considerable genetic variability in the chromosomal regions where the flocculation genes are located. As noted by van Mulders *et al*. *(99)*, genetic variation between flocculation genes hampers attempts to understand and control the flocculation behaviour in industrial brewer's yeasts. Christiaens *et al*. *(100)* state that the presence of highly variable tandem repeats within the *FLO* genes results in their capacity to evolve and diverge faster than other genes. As a result, each brewer's yeast strain possesses its own reservoir of FLO genes, which can change during consecutive fermentations. This variation is significant because it changes the adhesin structure and expression, and therefore alters flocculation onset and/or strength.

In the future, our knowledge of yeast flocculation mechanisms and control, particularly in the context of the brewing process, research and industrial application of it, should be adapted to the flocculation gene families present in brewing yeast strains.

Yeast management

Yeast management in the brewing context refers to yeast handling between fermentations. This category includes: yeast storage, acid washing and recycling and propagation. The whole question of yeast management has assumed much greater importance during the past 25 years, as the significance of yeast quality and the influence of procedures between fermentations has been recognized. In addition, the control of bacteria in the brewing process has recently been reviewed by Vriesekoop *et al*. *(101)*.

The critical parameter for all stages of yeast management is to maintain the viability and vitality of cultures in order to ensure that, when the yeast is pitched into wort, the lag phase is kept to a minimum. In order for this to occur, the intracellular glycogen level must be maintained. At the beginning of fermentation, synthesis of unsaturated fatty acids and sterols, which are essential cell membrane components, occurs at the expense of glycogen (Fig. 4). This must occur for a normal growth pattern of the yeast population during the wort fermentation process.

For many years it has been known that yeast are unable to synthesize unsaturated fatty acids and sterols under strictly anaerobic conditions *(102)*. Consequently, oxygen (by aeration or increasingly by the use of gaseous oxygen) is supplied during yeast pitching. The question of the optimum oxygen content of the wort in the early stages of fermentation has been the subject of considerable discussion over the last 25 years *(103)*. This question will be discussed again below in the context of high-gravity brewing.

The best conditions for yeast storage are as follows:

- reduced temperature, but not too low in order to prevent freezing (2–4°C);
- yeast suspended in fermented wort with a 4–5% alcohol (v/v) concentration (some breweries do employ water);
- slow agitation in order to maintain the yeast cells in suspension.

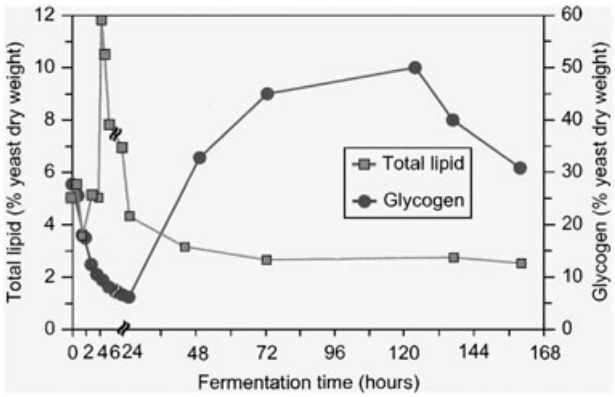

Figure 4. Intracellular concentrations of glycogen and total lipid in a lager yeast strain during fermentation of a 15°P wort.

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Vigorous agitation has been associated with loss of viability, glycogen breakdown and poor fermentation performance *(104)*. It can also result in cell wall mannan being released, which can yield unfilterable hazes *(105)*. Too much agitation during storage can cause the secretion of intracellular hydrolytic enzymes (particularly proteases) into the medium *(106)*. This can negatively affect beer foam stability owing to the hydrolysis of hydrophobic polypeptides *(107)* – more details are provided below.

Acid washing is routinely employed by some (not all) breweries. Acid washing of distiller's yeast is not routinely used. Acid washing of yeast slurries between fermentations is used to eliminate bacterial infections. The pH is normally 2–2.2 and the acid most commonly employed is phosphoric acid. The principle of the procedure is that most bacteria, particularly those that contaminate a brewery fermentation, are less acid tolerant than the production yeast (including wild yeast).

Nevertheless, acid washing does exert considerable stress on a yeast culture and can result in a yeast culture with reduced viability and vitality, resulting in an inability to completely ferment wort, together with changes in such parameters as flocculation, fining, size of yeast crop and variation in metabolites (flavour compounds). The combination of high-gravity brewing and acid washing can exacerbate the stress effects and yeast management procedures must be optimized when repitching yeast from high-gravity fermentations. It is important to ensure that the yeast is in good physiological condition and can maintain its resistance to the acidic conditions *(108)*.

Studies during the past 25 years have defined the conditions necessary for effective acid washing *(109)* and divided them into the do's and do not's of the process.

The Do's of acid washing as given by Simpson and Hammond *(109)* are:

- use food-grade acid and chill it before use;
- add the yeast slurry and maintain the temperature at <5°C throughout the wash;
- wash the yeast as a beer slurry or as a slurry in water for no longer than 2 h;
- ensure constant stirring whilst the acid is added to the yeast and preferably throughout the wash to avoid 'hot spots';
- verify the pH of the yeast slurry;
- pitch the yeast immediately after the 2 h wash.

The do not's of acid washing are:

- do not wash for more than 2 h;
- do not store washed yeast;
- do not wash unhealthy yeast, that is, cultures with a high dead cell count;
- avoid washing yeast from a high-gravity fermentation prior to dilution – details are provided below.

There are a number of options to acid washing brewer's yeast:

- Some never acid wash their yeast but rather maintain a low cycle (generation) specification and discard the yeast culture when there is evidence of contamination (bacteria and/or wild yeast).
- Some acid wash every cycle. As already discussed, this procedure can have an adverse effect on yeast quality. However, there is at least one international brewing company that maintains that, unless their yeast is acid washed, their beer does not possess the appropriate flavour characteristics.
- Some only acid wash when a bacterial infection warrants the procedure. Many brewers frown on this policy, but as long as the appropriate quality control microbiological tests are in place, it can be a very effective procedure.

Yeast propagation

Yeast propagation is a traditional and well-established process in most large breweries *(110)*. Nevertheless, development is constantly ongoing and questions remain to be answered. The requirement for a freshly propagated yeast cultures is that it is non-stressed, highly vital and viable, and that the yeast is free from contaminating organisms. The way to this objective involves a carefully designed sanitary propagation plant with an aeration (oxygenation) system that is able to supply sufficient oxygen to all cells in the propagator, without causing mechanical stress to the cells, which are in a wort of the right nutrient composition.

No matter how much these conditions are optimized, it is still only possible to obtain relatively low cell numbers (approximately 100–200 million cells/mL, equivalent to 2.5–5.0 g dry matter per litre). In order to avoid losing time during the wait for the yeast to consume all of the wort sugars, another process must be employed. This process has been adapted from the baker's yeast industry and is conducted in a fed batch reactor, whereby the sugar concentration is maintained at a consistently low level, but not too low a level, in order to avoid the yeast growing purely aerobically and thereby potentially losing some of its fermentation characteristics during the propagation. Consequently, a hybrid process between traditional brewery propagation and the purely aerobic yeast propagation process used for baker's yeast production appears to be the preferred solution *(111)*. Thirteen *Saccharomyces* yeasts were used to study the effect of temperature, pH and density of industrial media (wort or must of white grapes) on yeast growth/biomass production. Lowering the pH of the wort up to an industrial acceptable value of 4.8 had almost no effect on yeast growth for the different densities of the wort. Lowering the density of the wort or must to about 10°P extract of malt or grapes always had a positive effect on the yeast growth. This suggests that such a medium contains sufficient nutrients for industrial yeast growth during propagation or hydration/revitalization. Where industrially necessary, a higher yeast growth temperature can be used. At 30°C, the same final cell number can be obtained in half of the time compared with at room temperature. Methods such as flow cytometry can be used to monitor the propagation process by measuring the cell, cycle, cell size, growth rates, glycogen, DNA and protein content *(112–116)*.

Fermentation systems and procedures

The predominant fermentation systems employed by most breweries and distilleries during the past 25 years have been batch systems. However, in the 1960s and 1970s considerable effort and expense was devoted to the development of continuous fermentation, but in the early 1980s most brewing companies decided to reject continuous fermentation and returned to batch processes. In the 1990s, there was a return to consideration of continuous fermentation systems, but now immobilized yeast cell systems were studied. Fermentation using an immobilized cell system, compared with a free cell

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system, offers a number of advantages such as protection of cells from the build-up of toxic metabolites (such as ethanol), potential for reuse, lower operating costs (no centrifugation or filtration required) and high volumetric productivity. A number of support materials have been utilized for bioethanol fermentation using immobilized microorganisms with cell entrapment within hydrogel polymers, especially calcium alginate gels, which have been the most widely studied *(117–120)*. Final ethanol concentrations of as high 13.6% can be tolerated by immobilized yeast and they can be reused seven or eight times *(121,122)*. A review of the 'continuous fermentation story' in the brewing of beer would be enlightening, but too detailed for this review *(123)*. New Zealand is the only country that still employs a multivessel continuous fermentation system for beer production. The use of continuous fermentation in grain distilling in Scotland is discussed below.

Most breweries (large and small) employ vertical fermenters, most with conical bottoms for yeast cropping *(124)*. A large number of these fermenters employ static fermentation techniques, that are multibrew and many are over 5000 hL in capacity. Stirred fermentation techniques in brewing were employed by a few brewers in the 1960s and 1970s. Schlitz in the USA was an example of this development with a process they called the 'accelerated batch fermentation' process that was later re-defined as the 'advanced balanced fermentation' *(125)*. The key element of the accelerated batch fermentation system was the use of large low-speed stirrers during fermentation, which ensured that the yeast culture would be uniformly distributed. However, considerable beer quality problems were encountered (e.g. poor foam and cloudy beer) and the stirred technique was abandoned. Reasons for these difficulties were that the stirring was too vigorous; the fermentation temperatures were too high and inappropriate processing aids were used. Recently, stirred fermentation has been trialled again using a pump loop mixing system in cylindro-conical fermenters *(126)*. However, these trials have recently been abandoned.

The advantages of cylindro-conical fermenters are:

- slender-shaped vessels that occupy little ground area with reduced real estate and capital costs;
- faster fermentation owing to more vigorous agitation resulting from a long bubble path;
- greater hop utilization because of reduced top crop and less adsorption of hop resins;
- improved cleaning and reduced beer losses resulting from excellent draining and rinsing characteristics of the vessel;
- ease of cooling because of the proximity of the beer to the cooling surface;
- product flexibility – lagers and ales (with appropriate yeast strains) can be produced in the same vessel;
- vessel geometry that makes it easier to apply top pressure and assists in purging and CO_2 collection, and venting during maturation/ageing – unitank operation.

An aspect of these large vertical vessels that has recently received attention leading to renewed interest in stirred systems is the question of stratified fermentation *(127)*.

Kapral *(127)* has defined a stratified fermentation as follows: two distinctly separate fermentations occurring in the same fermenter at the same time. The fermentations are layered. They are marked by differences in density and/or temperature and/or cell count.

There are a number of common conditions that favour the development of stratification:

- addition of fresh, unyeasted wort to an active fermentation;
- all of the yeast added in the first brew of a multibrew fermenter;
- employment of the multibrew fermenter as a propagator – then 'topping up' with wort;
- low fill velocity of pitched wort coupled with the addition of unyeasted wort;
- wort gravity and/or temperature differences between added wort and actively fermenting wort.

During the initial stages of stratification, new wort displaces the actively fermenting wort rather than mixing with it. Consequently, the added wort picks up a low yeast count (approximately 4–6 million cells/mL) from incidental contact with the active fermentation. A stratification line forms separating the fresh high-gravity low cell count wort, from the active fermentation above the stratification line.

A brewer suspecting stratification has several methods that can be used to assist in the detection of this phenomenon:

- A sudden temperature change that occurs approximately 18–30 h after the last brew signals the possibility that stratification exists.
- Changes in the CO_2 generation rate at approximately 18–30 h after addition of the last brew implies that stratification has occurred.
- Unusual fermentation or yeast performance and behaviour may also indicate a stratification problem.

Although there are a number of ways to correct stratification, all stratifications centre on the simple need to assure that new unyeasted wort mixes with the actively fermenting wort. This can be achieved with velocity, agitation, circulation or other mixing procedures *(127,128)*. A basic suggestion would be to find a solution that fits all fermenters rather than modify all fermenters in the brewery to solve one problem.

Impact of process intensification on yeast

Over the past 30 years and longer, process optimization and increased efficiencies have been the priority for many brewing and distilling companies (large and small). Process intensification has become part of this endeavour and has focussed on the following:

- reduced capital expenditure;
- increased rates of fermentation and final attenuation;
- high-quality yeast (viability and vitality);
- decreased maturation times;
- more efficient stabilization and filtration;
- enhanced beer quality and stability;
- high-gravity brewing.

All of the areas listed above have recently been reviewed in detail *(84,129)* and only specific areas will be discussed below.

Gibson *(130)* reviewed the area of high-gravity brewing and wort enrichment and supplementation in 2011 as one of the 125th anniversary reviews. In addition, very high-gravity brewing (VHGB) has also recently been reviewed *(47)*. High-gravity brewing was developed in the 1960s, 1970s and 1980s primarily to reduce capital expenditure. Recently, this process has received a 'new lease of life' because of its other

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advantages, which include a reduction in water, energy, labour, cleaning and effluent costs. Consequently, the sustainability advantages are enhanced. This review will focus on four specific aspects of high-gravity brewing:

- effect of proteinase A secretion and wort gravity on beer foam;
- influence of wort sugar spectrum and gravity on ester formation;
- influence of high-gravity wort (wash) on the production of grain whisky;
- high-gravity brewing and yeast centrifugation.

Effect of Proteinase A secretion and wort gravity on beer foam stability

Beers brewed at higher gravities followed by dilution have poorer foam stability compared with similar beers brewed at lower gravities *(131)*. Specific hydrophobic polypeptides play an important role in foam formation and stability *(132,133)*. The level of hydrophobic polypeptides was determined throughout the brewing and fermentation of high- and low-gravity worts (20° and 10°P, respectively). During brewing, there was a proportionately greater loss of hydrophobic polypeptides from the 20°P wort than from the 10°P counterpart (Fig. 5) *(134)*.

When the high-gravity fermented beer was diluted to 4.5% alcohol by volume, equivalent to the low-gravity beer, it contained 50% of the hydrophobic polypeptides present in the low-gravity beer *(135)*. In addition, the head retention of the diluted high-gravity brewed beer was less than that of the low-gravity brewed beer *(136)*. Hydrophobic polypeptides are lost during brewing and fermentation. In brewing, foam positive hydrophobic polypeptides are lost as a result of hot and cold break formation. Fermentation is a key stage during which hydrophobic polypeptides are reduced. At least three factors during fermentation account for this *(135)*:

- hot and cold break precipitation;
- foaming in the fermenter-enhanced adhesion of foam active compounds to the side of the fermenter;
- yeast 'secretes' proteolytic enzymes into the fermenting wort, and Proteinase A (PrA) enzyme activity has been shown to increase throughout fermentation.

Interest in PrA during wort fermentation, and its secretion into the fermenting medium, has increased recently because of the development of analytical methods for PrA. This includes the use of a specific peptide containing a fluorescent tag *(137)* and flow cytometry *(138)*. The effect of the deletion of the gene that encodes for PrA(*PEP4*) in a lager yeast strain has been examined *(139,140)*. PrA activity in wort fermented with this mutant was significantly lower than in the parent strain, and the resulting beer exhibited improved foam stability.

The overall question of yeast stress during a brewing and distilling fermentation is an important area of current research *(141,142)*. However, it is too vast a subject area to be discussed in detail in this review. There are a number of other factors, such as thermal and mechanical stress that can promote the release of yeast PrA. The question of mechanical stress will be considered below in the context of centrifuge operations for cropping yeast.

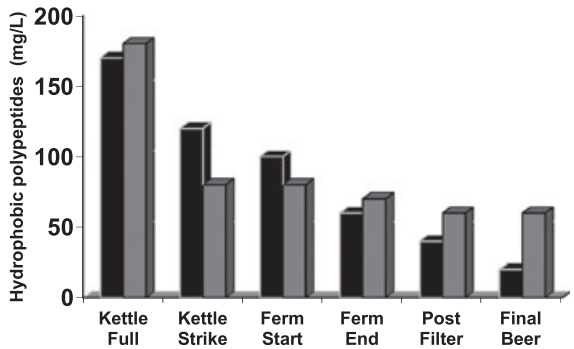

Figure 5. Changes in hydrophobic polypeptide levels in (black bars) high-gravity 20°P and (grey bars) low-gravity 10°Plato wort from kettle full to final beer (final beers were both diluted to 4.5% alcohol by volume).

Influence of wort sugar spectrum and gravity on ester formation

It has been reported many times since the 1970s that one of the disadvantages of high-gravity brewing is that it induces the production of disproportionately high levels of esters *(143)*. Varying the wort sugar source has been reported to modify the levels of many metabolites, including esters, although the reasons for these differences were unclear *(144)*. Some differences between glucose and maltose metabolism have already been discussed. Another difference is the production of ethyl acetate and isoamyl acetate in maltose grown cells, the production of which has been shown to be lower than in glucose grown cells *(143)*.

It is generally agreed that a reduction in ester levels, particularly ethyl acetate and isoamyl acetate, from high-gravity brewed beers would be welcome. Two adjunct brews at 20°P were prepared: one containing 30% maltose syrup (MS) and the other containing 30% very high maltose syrup (VHMS) *(144)*. The sugar composition of the two brewing syrups is shown in Table 4.

In addition, a 12°P wort containing 30% (w/v) MS was prepared and used as a control. The maltose plus maltotriose concentration in the 20°P VHMS was increased compared with the 20°P wort, with a corresponding decrease in the concentration of glucose plus fructose.

The three worts were fermented with a lager strain, at 13°C in the ICBD 2 hL pilot brewery, and the concentrations of ethyl acetate and isoamyl acetate determined throughout the fermentation. The profiles were similar, consequently only ethyl acetate results are shown (Fig. 6).

The concentration of both esters in the 20°P (MS) fermented wort was twice the level of those in the 12°P (MS) fermented wort. However, the ester concentration in the 20°P (VHMS) wort was reduced by approximately 25%, compared with the 20°P (MS) wort *(144)*. Indeed, this VHMS is currently employed as an

Table 4. Percentage sugar composition of brewing syrups

	Maltose syrup (MS) (%)	Very high maltose syrup (VHMS) (%)
Glucose	15	5
Maltose	55	70
Maltotriose	10	10
Dextrins	20	15
Total	100	100

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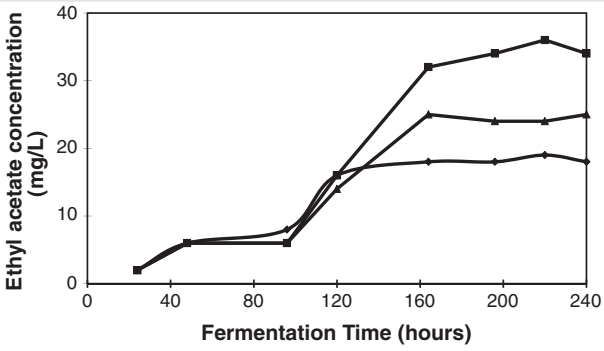

Figure 6. Ethyl acetate concentration in fermenting worts of differing gravities and sugar compositions (square, 20°P, 30% maltose syrup; triangle, 20°P, 30% very high maltose syrup; diamond, 12°P, 30% maltose syrup).

adjunct in high-gravity wort by some breweries as a way to reduce ester levels, and at the same time, obtain enhanced production capacity without significant capital expenditure, and also to yield a more sustainable process.

Influence of high gravity wort (wash) on the production of grain whisky

There are essentially three types of Scotch whisky: malt, grain and blended. Grain whisky is produced by both batch and continuous processes. A schematic diagram of a continuous fermentation process, with a wort composition of 90% grain and 10% malted barley, is shown in Fig. 7.

Yeast is purchased (cream, cake or dried form) from a yeast supplier. The yeast is usually grown on a molasses medium where the principal sugar is sucrose. To acclimatize (and liven) the yeast to a cereal-based fermentation environment, where the principal sugars are maltose and maltotriose, the yeast is incubated in the grain wort in a 'bub' vessel for 24 h. The acclimatized yeast is then incubated in grain wort (also termed wash) in a continuous fermenter (also termed a wash back) in flow-through mode at 30–32°C for 36–48 h. When a steady state has been established, the rate of wort addition is in balance with the rate at which the fermented wort leaves the fermenter.

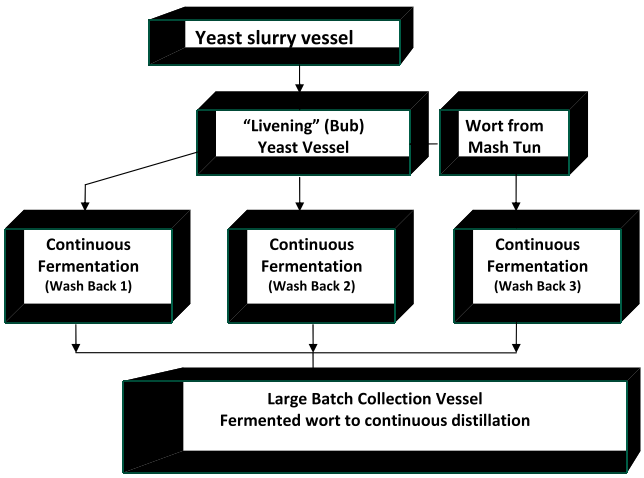

Figure 7. Schematic of an example of a continuous fermentation process that is used in a grain whisky production facility in Scotland.

The fermented wort is then placed in a holding tank prior to distillation in a continuous process still.

In 2005, a grain distillery in Scotland successfully employed a continuous fermented 21°P (1084 SG) grain wort that yielded 11% (v/v) alcohol in the fully fermented wort. This situation continued until mid-2006, but in late 2006, problems began to occur, with a decrease in alcohol yield to 9.6% (v/v) owing to incomplete utilization of maltose and, particularly, maltotriose (Fig. 8). This equated to a reduction in alcohol yield from 385 to 370 L/t of grain. In addition, because of the residual wort maltose and maltotriose, the resulting distiller's dried grain had a sticky consistency and was not acceptable for use as an animal feed.

In an attempt to overcome this problem, the Original Gravity (OG) of the wort was reduced to 19°P (1076 SG) (Table 4). This resulted in complete fermentation of the wort with no residual maltose and maltotriose and improved the consistency of the distiller's dried grain. However, the distillery's overall alcohol yield was reduced below budgeted productivity levels. The reasons for the deterioration in yeast efficiency regarding maltose and maltotriose uptake are still unclear, although it would appear that the 21°P (1084 SG) wort exerted stress effects on the pitching yeast, with inhibitory effects exhibiting as maltose and maltotriose uptake problems. Stress effects on maltose and maltotriose uptake have already been discussed in this review. The exact reasons for this particular inhibition were unclear but involvement of the active transport of both sugars (already described) cannot be ignored *(53)*.

High-gravity brewing and yeast centrifugation

The use of centrifuges has become an established way to increase brewery throughput and decrease environmental costs by optimizing beer production clarification times. Centrifugation can play a number of roles within a brewery *(145)*:

- cropping of non-flocculent yeast at the end of primary fermentation;
- reduction of the yeast quantity from 'green' beer before the start of secondary fermentation;
- beer recovery (barm beer) from cropped yeast;
- separation of hot break (trub) after wort boiling;
- removal of cold break (trub) at the end of maturation.

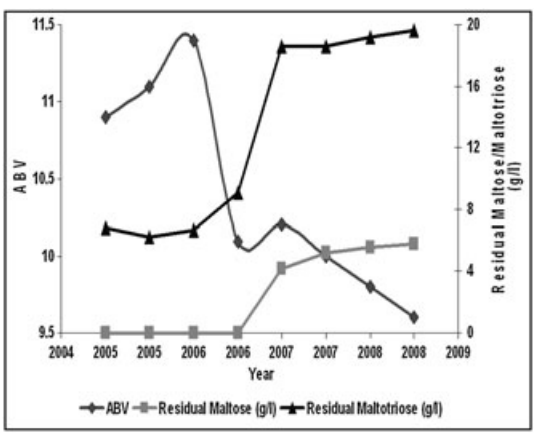

Figure 8. Fermentation trends (alcohol by volume and residual maltose and maltotriose) in a distillery from 2005 to 2008 with a 21°P grain wort.

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Passage of yeast through a centrifuge creates mechanical and hydrodynamic shear stress and can cause a decrease in cell viability and flocculation, cell wall damage, increased extracellular PrA levels, hazier beers and reduced beer foam stability *(146)*. Despite evidence of cell damage, little has been reported until recently regarding the effect of repitching yeast that had been cropped using a centrifuge on yeast and beer quality, especially in a high-gravity wort environment *(147)*.

Two series of fermentations were conducted in the ICBD 2 hL pilot brewery. One series was a 12°P all-malt wort and the other series a 20°P all-malt wort. A lager yeast strain was used and the fermentation temperature was 13°C. The yeast cellular characteristics, before and after 12° and 20°P wort fermentations, showed that the centrifugation stress with the high-gravity wort resulted in more deleterious effects on the yeast in terms of viability, damaged cells, intracellular pH, bud index and intracellular glycogen than with the lower gravity wort. Scanning electron microscope analysis provided visual evidence of yeast damage and the release of cell wall components (probably mannan) as a result of disc stack centrifugation at high *g*-force (Fig. 9).

Although centrifugation can produce negative effects, the positive effects of centrifugation on brewery production and effluents cannot be overstated. However, numerous factors associated with centrifugation do individually or collectively impose stress on yeast cells. The effect of environmental conditions on beer production equipment may have been underestimated or ignored in the past. An understanding of yeast biological responses to interactions between cell physiology and brewing equipment is an important criteria for maintaining beer quality.

Genetic stability of brewer's yeast strains

As already discussed, yeast cells are assaulted by a number of different stresses during brewery and distillery fermentations

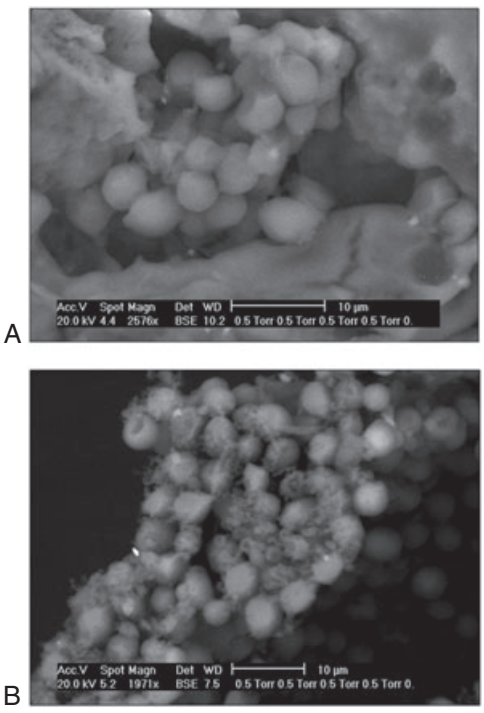

Figure 9. Scanning electron microscope analysis of a centrifuged lager yeast culture. (A) Before centrifugation and (B) after centrifugation.

(141), rendering the yeast genome susceptible to modification. Spontaneous yeast mutations are a common occurrence throughout the growth and fermentation cycle, but they are usually recessive, owing to functional loss of a single gene *(148)*. Because of the polyploidy/aneuploid nature of most brewing strains, the dominant gene will function adequately in the strain, and it will be phenotypically normal. Only if the mutation takes place in all complementary genes will the recessive character be expressed. However, if the mutation weakens the yeast, the mutated strain will be unable to compete and will soon be outgrown by the non-mutated yeast population. The characteristics that are routinely encountered resulting from mutation that can be harmful to a wort fermentation are:

- the tendency of yeast strains to mutate from flocculent to non-flocculent *(149)*;
- the loss of ability to ferment maltotriose *(150)*;
- the presence of respiratory deficient (RD, 'petite') mutants *(151)*.

The RD or 'petite' mutation is the most frequently identified mutant found in brewing yeast strains. This mutation arises as a result of mitochondrial DNA (mt DNA) damage. Petite mutants are respiratory deficient and require fermentable forms of carbohydrate for survival. The inability of the cells to respire reduces their growth rate, and colonies growing on agar plates are typically smaller than respiratory competent (also called respiratory sufficient) colonies (hence the term 'petite'). This mutation can have a significant effect on brewery fermentations, with studies reporting reduced fermentation rates (the uptake of maltose and maltotriose is specifically retarded), poor flocculation and atypical flavour development, particularly with an effect on diacetyl management *(152)*.

Mitochondrial petite mutants are also typically more sensitive to stresses, including ethanol stress *(153)*. In addition, RD mutant cultures are difficult to store on nutrient slopes. Liquid nitrogen at −196°C and −70°C refrigeration have both been found to be the most effective storage methods *(154)*. Flocculation, cell wall, plasma membrane structure and cellular morphology are all affected by this RD mutation *(155)*, as is longevity *(156)*.

The effect of centrifugation and temperature on RD levels in an ale production yeast strain was studied. Fermented ale wort (16°P OG) was centrifuged in order to crop the yeast. This cropped non-flocculent yeast was repitched into wort, and slow, and eventually 'hung' fermentations occurred with increasing yeast cycles (generations) following yeast cropping by centrifugation. The 'hung' fermentations were largely due to residual wort maltose and particularly maltotriose. Also, VDK (especially diacetyl) formation and particularly reduction rates later in the fermentation were reduced, resulting in residual diacetyl above the taste threshold levels in the beer. Further studies revealed that when the centrifuge exit temperature was 30°C, the yeast culture after 10 cycles contained 28% RDs (determined by the triphenyl tetrazolium overlay method) *(157)* and the culture's viability was 72% (determined by methylene blue staining; Table 5). When the centrifugation exit temperature was reduced to 20°C, after 10 cycles, the RD level was reduced to 8%, and the cell viability increased to 84%. The wort fermentation rate and extent returned to normal because of complete maltose and maltotriose uptake. The VDK profile also returned to normal.

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Table 5. Effects of centrifugation and temperature on respiratory deficient (RD) levels and viability of an ale production yeast strain

	Freshly propagated culture	Centrifugation with 20°C exit temperature (10 cycles)	Centrifugation with 30°C exit temperature (10 cycles)
RD (%)	<1	8	28
Viability (%)	98	84	72

Improving yeast strains

The prediction that genetically modified yeast strains would be in widespread use by the start of the twenty-first century has not come to fruition. The major objectives of yeast strain development are to improve the efficiency of the process, that is yield and obtaining a good-quality product *(7)*. More recently within brewing, the concept of the creation of novel strains to produce 'healthier' beers, such as those with less alcohol and/or sugar, has gained increased attention. The genetic improvement of brewer's yeast has recently been reviewed by Saerens *et al*. *(7)*. As they describe, concerns about genetically modified organisms have steered research to the isolation of natural mutants with the desired properties, which are based on the knowledge of genotype–phenotype linkage.

Genome shuffling is one approach available to accelerate evolution. It is commonly achieved using multiparental protoplast fusion, however Hou *(158,159)* has created a method to generate useful mutations and enlarge population diversity using ethyl methane sulphonate (EMS) to mutate diploid yeast cells. By using several rounds of selective media with increasing concentrations of ethanol, new *S. cerevisiae* strains may be obtained with optimized fermentation characteristics under very high-gravity conditions *(158)*. Zheng *et al*. *(15)* used a similar approach, with additional screening for 'elite' strains, to create a new *S. cerevisiae* strain capable of yielding 10.55% more ethanol than the parent strain under increased heat and very high-gravity conditions.

Ekberg *et al*. *(152)* generated new hybrids using spore clones of lager yeast and *S. cerevisiae* and complementation of auxotrophies of the single strains upon mating. The hybrids were improved on several parameters, including growth at elevated temperature and resistance against high osmolarity or high ethanol concentrations. All of these hybrid strains showed improved stress resistance as seen in the ale parent, including improved survival at the end of fermentation. Importantly, some of the strains showed improved fermentation rates using 18°P at 18–25°C.

An adaptive evolution method to obtain stable *S. pastorianus* brewing yeast variants with improved fermentation capacity has been described by Guadalupe-Medina *et al*. *(160)*. They were selected for rapid growth resumption at high osmotic strength. It was applied to a lager strain and also to a previously isolated ethanol-tolerant strain. The fermentation performance of the strains was compared using a 15°P wort. A selected osmotolerant variant of the ethanol-tolerant strain showed a significantly shorter fermentation time than the parent strain, producing 6.45% alcohol by volume beer in 4–5 days, with mostly similar organoleptic properties to the original strain. However, the diacetyl and pentanedione levels were 50–75% higher and 3-methylbutyl acetate and 2-phenylethyl acetate were 50% higher than with the original strain, leading to a small flavour change. The variant contained significantly less intracellular trehalose and glycogen than the parent. It is suggested that an attenuated stress response contributed to the improved fermentation performance.

A non-mating, non-sporulating lager strain was mutagenized with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine *(161)*. Mutants in the biosynthesis of isoleucine were isolated by resistance to 5,5,5-trifluoro-DL-leucine. The mutants formed higher levels of amyl alcohol and similar or lower amounts of iso-amyl alcohol and isobutanol. Similar mutagenesis experiments with EMS generated mutants resistant to 5,5,5-trifluoro-DL-leucine or to fluoro-DL-phenylalanine, with an overproduction of leucine or phenylalanine, respectively, have been obtained from a lager strain *(162)*. These mutants produced greater of the corresponding alcohols and esters. However, they fermented slower than the parent strain, although final attenuations and ethanol yields were the same. EMS at low levels was also used to induce non-deleterious genetic changes. After selection pressure in VHGB conditions, well performing mutants were obtained by Huuskonen and Londesborough *(163)*.

In 2007, Blieck *et al*. *(164)* used UV to mutagenize a lager brewing strain together with selective pressure under VHGB conditions. Mutants were obtained with better fermentation performance than their parental strain under these stressful conditions. James *et al*. *(165)* also applied EMS and selective pressure under VHGB conditions and high temperatures, at the same time, to isolate mutants with good fermentation behaviour under these stresses.

Little has been announced about these new strains being used at a production brewing scale. However, it is possible that they are in use as they are legally accepted.

Spontaneous mutants resistant to a glucose-like component, 2-deoxy-glucose (2-DOG), are de-repressed mutants. They have the capability to use maltose and maltotriose in the presence of glucose, and therefore should theoretically have increased fermentation performance. An approach to isolate very-high-gravity adapted 2-DOG resistant yeasts rendered a mutant capable of good performance at 25°P in 2 litre trials *(166)* but not good enough to be employed on a production scale.

Sometimes strategies combine several improvement methods *(167,168)*. Jones *et al*. *(169)* used spontaneous mutants of both brewer's yeast strains and *S. cerevisiae* var. *diastaticus* strains resistant to 2-DOG and thus these were de-repressed for maltose and starch metabolism in the presence of glucose, respectively. The mutants were fused from protoplasts. Stable mutants of haploid, diploid and polyploid chromosome number were obtained that could use maltose or starch in the presence of glucose.

Thevelein and Dumortier *(170)* inserted the AGT1 gene into a stable, well-conserved position in the genome of production strains of *S. cerevisiae* in order to generate a marker for rapid identification in industrial fermentations. The resulting strains were found to have improved fermentation performance on starch hydrolysates and other maltose and/or maltotriose containing media.

Blasco *et al*. *(140)* devised a method for improving beer foam. They identified a gene in brewer's yeast that prolongs beer foam lifespan by making a protein that protects the bubbles. They reported brewing beers with foam heads that are stable for several hours.

It has already been discussed that wort contains unfermentable dextrins. These dextrins remain in the finished

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beer and consequently give it mouth feel and contribute to its calorific value *(171)*. In order to produce a low-calorie beer, dextrins must be reduced. There are a number of techniques to reduce dextrins *(171)*. One method would be to employ a yeast strain that possesses the ability to metabolize some of the wort dextrins. The fact that there is a grouping of yeast – *S. cerevisiae* var. *diastaticus* – that is taxonomically closely related to brewer's yeast strains has already been discussed in this review. These strains contain the genetic ability to produce an extracellular glucoamylase that can hydrolyse the dextrins to glucose, which will be taken up by the yeast during wort fermentation. These genes have been identified as *STA1/DEX1*, *STA2/DEX2* and *STA3/DEX3 (172)*. A yeast strain incorporating these genes was constructed using classical hybridization techniques and its fermentation characteristics assessed during a wort fermentation. The amylolytic yeast exhibited a faster fermentation rate and a lower final wort degree Plato than the control yeast, which was not able to metabolize the wort dextrins *(173)*.

The extracellular glucoamylase produced by this group of yeast was thermotolerant, probably because it was heavily glycosylated (a mannoprotein) *(174)*. As a consequence of this, the glucoamylase was not inactivated during pasteurization (12 PU) of the low dextrin beer and the resultant beer produced with this amylolytic yeast became sweeter and sweeter in the bottle over time. Also, this enzyme does not possess branching activity *(173)*. A similar low-dextrin beer was produced with a glucoamylase-containing yeast (where the gene was incorporated using cloning rather than traditional hybridization) and this beer was produced on a semi-production scale. It was called 'Nutfield Lyte' and was part of a collaborative project between the Brewing Research Foundation (now Campden BRI, Brewing Division) and Heriot–Watt University. This particular strain was approved for use on a production basis by the UK's Novel Food Products and Processes *(175)*. However, it is not currently being employed on a production basis for brewing.

Kilonzo *et al. (176)* produced a recombinant *S. cerevisiae* strain for the study of glucoamylase production and plasmid stability. A number of similar constructions are reported in the literature by researchers such as Wang *et al. (177)*, who constructed an industrial brewing strain to produce low-calorie beer by incorporating a number of genes for both reducing off flavour compounds, in addition to reducing residual maltotriose in the fermentation.

Conclusions

During the past 25 years, our knowledge of brewer's yeast strains and fermentation processes has advanced considerably. These advances have been possible, in large part, by development of analytical techniques in the areas of genetics (genomics), protein chemistry (proteomics) and yeast metabolism (metabolomics) together with process engineering. In our centenary review, published in 1986, we predicted that genetically manipulated brewing strains would be employed in beer production in the ensuing decade. Owing mainly to adverse public opinion, this has not been the case. Currently, there are a number of genetically manipulated yeast strains that could be used and, in the next ten years or so, we could see some of these strains employed in production, particularly non-beverage processes such as fuel ethanol.

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