CENTENARY REVIEW

ONE HUNDRED YEARS OF YEAST RESEARCH AND DEVELOPMENT IN THE BREWING INDUSTRY

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The effect that yeast research and development on yeasts has had on the brewing industry is traced from the time of Pasteur to the present time.

Key words: Saccharomyces diastaticus, Saccharomyces cerevisiae, Saccharomyces uvarum, *fermentation, hybridisation, genetic engineering, flocculation, ethanol-tolerance, alcohols, esters, carbonyl compounds, diacetyl, sulphur compounds, immobilised systems.*

Prologue

The most logical title for a microbiology review honouring the centenary of the Institute of Brewing would be, quite naturally, 'A Century of Brewing Microbiology' but that was the title chosen by the late Dr Cyril Rainbow for his excellent Horace Brown Memorial Lecture in 1977.[92] Consequently, 'One Hundred Years of Yeast Research and Development in the Brewing Industry' is something of a compromise. Nevertheless, it is to be hoped that the reader will be able to gain an appreciation of the significant progress that has occurred during this period and obtain some insight into the prospects for the future.

Early Research and Development

Although both yeast and bacteria were first reported to have been observed microscopically by the draper Antonie van Leeuwenhoek in 1680, it was not until the year 1837 that Cagniard-Latour, following observations made by Spallanzani in 1776, suggested that the process of fermentation was intimately associated with the budding of yeast cells. The work of Cagniard-Latour was considerably expanded by Schwann in 1839, who further suggested that the growth of yeast cells was dependent upon the presence of sugar which acted as their food material. The view that yeast as a living agent played any part in the transformation of sugars into alcohol was debated by van Liebig and many other leading chemists of the day, who envisaged the production of alcohol from sugar as a purely chemical process dependent upon the post-mortem decomposition of dried yeast. This notion was generally accepted until 1857, when Pasteur presented convincing proof concerning the association of fermentation with living matter.

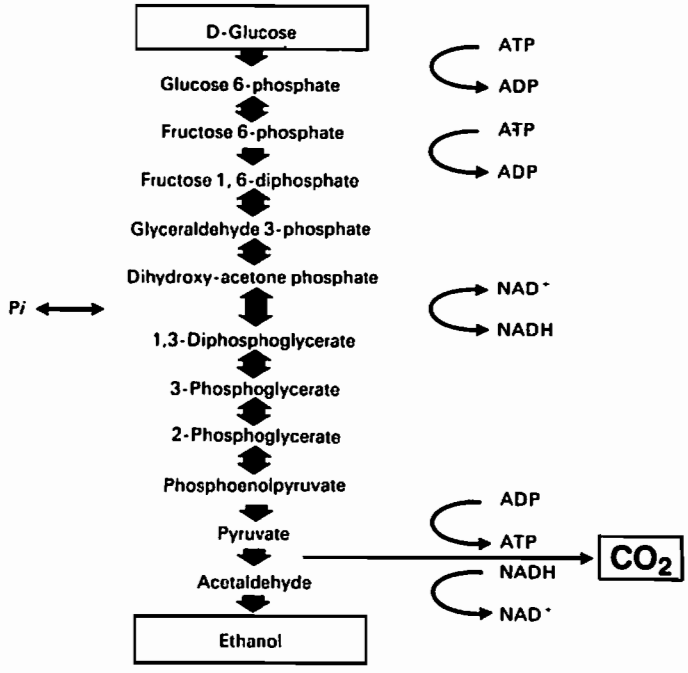
A hundred years ago, the microbial spoilage of beer was causing significant financial losses. In the UK, the large stocks of ale brewed between October and May for sale in the summer months were especially prone to spoilage by a bacteria causing first a 'silky' turbidity and then lactic acidification. By the last quarter of the nineteenth century, the life history of the causative organism, later named *Saccharobacillus pastorianus*, was documented by Horace Brown.[13] The prevailing experimental difficulties were significant, techniques were lacking and methods had to be devised for the isolation and growth of microorganisms; the forcing test that was devised for predicting spoilage is still in use today.

Almost concurrently with Brown, Pasteur was also studying acetification of alcohol beverages, particularly wine. In '*Etudes sur le Vin*'[84] which was followed by '*Etudes sur le Vinaigre*'[85] and '*Etudes sur la Biere*',[86] Pasteur ascribes acetification to the film-forming organism *Mycoderme aceti*, which can be recognised as the agent by which atmospheric oxygen was transferred to alcohol in the formation of acetic acid.

In his treatise on beer, Pasteur relates that he was 'permitted to go through a large London brewery where the microscopical study of yeast was unknown'. He was furthermore allowed to make 'certain experiments in the presence of the managers' and he detected and made diagrams of microorganisms found in the pitching yeast and also present in 'diseased' beer. His demonstrations so impressed the directors that eight days later they acquired a microscope. Pasteur's insight began a revolution in the fermentation industry, the impact of which may still be felt today. As a method of controlling microbial spoilage, he ultimately rejected the addition of harmless antiseptics, such as sulphite, in favour of the heat treatment now universally known as pasteurisation, the invention of which illustrates just one aspect of his particular genius. In retrospect, he was a most remarkable man, although formally trained as a chemist, he became a microbiologist, applied the techniques of chemical engineering, and in the qualification of his results by groups of tasters he even approached modern techniques employed for the sensory evaluation of beverages and foods.

Both Pasteur and Brown were familiar with the phenomenon of 'ropiness'. They recognised the causative organisms as a 'viscous ferment', but neither appear to have proceeded far in its study. Brown was also well aware of the role of 'wild' yeasts in production, which, in his experience, caused more beer spoilage than did bacteria. Horace Brown's own research efforts were gargantuan, and he is truly the 'Father of British Brewing Science'. He has recorded his endeavours in a lecture entitled 'Fifty Years Experience of Science in Brewing Practice', published in 1916.[13] Perhaps one of the most interesting sections in this document relates to his study of microscopic methods and in his own words he related his reaction to reading Pasteur's 'Etudes sur le Vin': '*The immediate effect was that of a ray of light piercing the darkness and illuminating a new path into the unknown. It is true that the work dealt only with wine, but it was at once evident that the new principles must be equally applicable to beer brewing, and from that moment I turned my attention with renewed energy to microscopical work, fully confident that I should thereby obtain an answer to the many questions that I had been asking myself for the past three or four years.*'

Pasteur considered that fermentation was tied to the whole cell; however, in 1897 Buchner showed that the cell-free juice expressed from yeast cells by high pressure can ferment glucose and other sugars to ethanol and carbon dioxide. This chance encounter precipitated a whole series of studies directed towards unravelling the complicated fermentation pathway; many brilliant researchers—Harden, Young, Robinson, Neuberg, Kluyver, Warburg, Meyerhof, Embden and Parnas—have been associated with this endeavour. The formation of ethanol occurs in *Saccharomyces* and a number of other yeasts via the Embden–Meyerhof–Parnas Pathway (Fig. 1) where, theoretically, 1 g of glucose will yield 0·51 g of ethanol and 0·49 g of CO_2. However, because of biomass



Theoretical yield:
1·00 g ($C_6H_{12}O_6$) → 0·51 g (C_2H_5OH) + 0·49 g (CO_2)
Practical yield:
1·00 g ($C_6H_{12}O_6$) → 0·46 g (C_2H_5OH) + 0·44 g (CO_2 + 0·10 g (Biomass)

Fig. 1. Formation of ethanol from glucose by the Embden–Meyerhof–Parnas Pathway.

production, an ethanol yield of 0·46 g and 0·44 g of CO_2 from 1 g of hexose is more realistic.

It is interesting to note the extent to which these early findings on yeast and other microorganisms have later been related to the mammalian cell. As a consequence of this, as will be discussed in greater detail later, the use of yeast as a 'model experimental eucaryote' is still a valid concept today. Such parallel observations in unicellular and multicellular systems have served to emphasise the unity of life. A few years ago it would have been acceptable to paraphrase Jacques Monod, 'What is true of *Saccharomyces cerevisiae* is true for elephants, except more so'. However, with the advent of more sophisticated molecular biological techniques, particularly gene splicing and sequencing, the similarities at the nuclear level are not as close as was originally postulated. Nevertheless, studies on yeast have and will continue to provide valuable information that is pertinent to all eucaryotes.

The scientific findings of the late nineteenth and early twentieth centuries were hotly debated by word of mouth and print, and emotions ran high on many occasions. It is interesting to note that for much of this century whilst scientific knowledge was growing almost exponentially, the habit of discussing new scientific ideas (in print at least) waned and only rarely did one find critical comment on articles and papers. However, in recent years, with the advent of the 'new biotechnology' with its potential for commercial development and environmental impact, polemical discussions of scientific results and their implications has returned to both the serious and popular scientific literature.

Until the middle of the nineteenth century bottom-cropping yeasts were employed exclusively by Bavarian brewers, notably in Munich, while the rest of the world used top-cropping yeasts. However, in 1842 the yeast and fermentation techniques of Bavaria were smuggled to Czechoslovakia and only three years later, bottom fermentation techniques were taken to Copenhagen. About the same time, bottom-cropping yeasts were introduced into Pennsylvania and spread throughout the USA largely due to the immigation of German, Dutch and Danish brewmasters. After the spread of bottom fermentation (i.e., lager brewing), the traditional top fermentation (i.e., ale brewing) techniques were largely discarded except in the British Isles and some of its colonies. However, a portion of the beer produced today in Australia, Belgium, Canada, the United States, New Zealand and West Germany is of this type.

At the end of the last century favourable conditions for research and development existed in the Carlsberg Brewery in Denmark and one of the research topics under active study by Emil Christian Hansen was that of brewing yeasts. He successfully isolated four strains of bottom fermenting yeast. When he studied them from the standpoint of brewery use, only one of the strains proved to be suitable for beer fermentation. The strain, described as '*Carlsberg Yeast No. 1*' was introduced into the Carlsberg Brewery for use on a production scale on 13 May 1883 and pure strain brewing can be said to have commenced on this date.[56] Due to the origin of '*Carlsberg Yeast No. 1*' it was named *Saccharomyces carlsbergensis* Hansen 1883.

Hansen soon found that it was tedious and inconvenient for the laboratory to regularly furnish the Carlsberg Brewery with pure cultures and that it would be easier to obtain a specific apparatus for this purpose. With the assistance of a coppersmith, W. E. Jansen, Hansen started to construct such an apparatus; and at the beginning of 1886, the apparatus was working perfectly at the Carlsberg Brewery. Jansen began to sell the apparatus and Heineken was one of the first breweries to purchase the equipment.

As a result of Hansen's work, the practice of employing a pure strain in lager production was soon adopted. Ale-producing regions however, met this 'radical innovation' with severe opposition! The method was merely regarded as a means to reduce infection by wild yeasts and bacteria. Over the years, however, the use of pure strains has increased in ale-producing areas. It is interesting to note that in 1959 it was reported[42] that of 39 yeast cultures in use commercially in Britain, 12 contained a single strain, 16 had two major strains and the rest contained three or more components. If a similar study were undertaken today, the percentage of pure strains being employed for ale production would be considerably higher.

Another 'milestone' in the history of brewing microbiology occurred in 1935. Winge, working in the same laboratory as Hansen in the Carlsberg Brewery, showed that stained vegetative cells of *Saccharomyces* were diploid, being produced by the copulation of two spores or of two gametes derived from spores. The diploid nuclei undergo reduction division at spore formation to produce haploid spores of either *a* or α mating type (Fig. 2). Winge established the basic

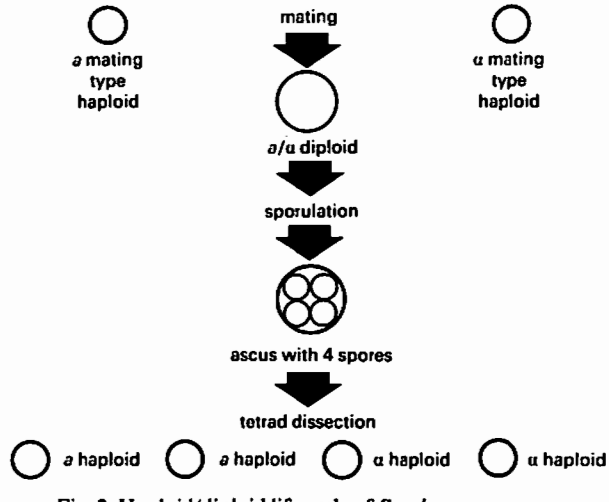


Fig. 2. Haploid/diploid life cycle of *Saccharomyces sp.*

facts of the life cycle in a classical series of observations on the germination of ascospores and the formation of haploid cells. When spores of *Saccharomyces* are planted on a culture medium each spore germinates to produce a cluster of round haploid cells which undergo a period of vegetative growth. There may be an extended haplophase before copulation begins and cell fusion reconstitutes the diplophase.[145] Winge's studies, therefore, presented the possibility of breeding new yeast strains for specific purposes.[146] He began an area of research and development, namely yeast genetics and molecular biology, that is vogue today. A discussion of current developments in this field is to be found later in the document.

Traditional brewing fermentation systems were batch methods. Ale fermentation occurred in open circular or rectangular vessels of limited depth and, by rising to the surface, the yeast was cleared from the fermented wort and skimmed off. The vessels were constructed of wood, stone or slate. Some of the wooden vessels were lined with copper or lead, but the harmful effects of lead occurring in the beer appears to have been a constant concern and it was quickly replaced with copper. In addition to this basic design, there were a number of localised traditional ale fermentation systems (some still in limited use) such as the Burton Union System (wherein a characteristically flavoured beer was produced by fermenting with a non-flocculent 'powdery' yeast that would not clear from the beer by traditional means) and the Yorkshire Stone Square System (which utilised a flocculent yeast which was maintained in suspension by continual rousing of the fermentation). From circa 1880 extensive use was made of live steam, since it was clear by this time that such treatment was the only way to combat the 'ferments of disease', a phrase use by Pasteur.

Traditionally, lager fermentation differed from that of ale fermentation by the use of bottom-cropping yeast and lower fermentation temperatures. The traditional lager fermentation was affected by a wide variety of vessels similar to those for ale. However, the lack of skimming has given lager fermentations more flexibility and consequently has tended to give them an edge over ale fermentations in technological advances in design.

Shortly after the turn of the century, a Swiss engineer named Nathan[76] designed new cylindro-conical fermentation vessels. He combined them with a maturation system whereby undesirable flavour constituents were purged from green beer with carbon dioxide. Until 1930 Nathan campaigned, to no avail, to have his vessels adopted. It was not until tower vessels were being advocated in the 1960s that the merits of Nathan's designs were finally acknowledged.

Hudson[43] has rightly stated that brewers have only limited control over yeast and fermentation. The major means of control lies in altering the composition of wort through choice of grist materials and mashing conditions at brewing, and by pitching rate and temperature adjustments in the fermentation cellars. There was great expectation in the 1950s and 1960s that significantly improved control would be gained by turning to continuous fermentation. Continuous fermentation for the production of beer was first attempted before 1900. Indeed, by 1906 at least five separate systems had been proposed including simple stirred tanks, multiple arrangements of such vessels and towers packed with supporting materials upon which a culture of yeast was maintained. The reasons why these systems at that time failed to gain a foothold in commercial operations are obscure, but it is likely that inability to guard adequately against contamination and also resistance to change were major factors.

A re-awakening of interest was stimulated in the late 1950s when multivessel systems were in operation in Canada and in New Zealand. This was shortly followed by a novel system in the UK which exploited the ability of flocculent strains to sediment, thus enabling a high concentration of cells to be held within the system.[89] This opened up the possibility of much more rapid fermentation than had been hitherto possible. In the decade between 1960 and 1970 substantial interest arose in the brewing industry in the field of continuous fermentation. Increases in knowledge of brewing science, together with the relatively advanced engineering and electronic control equipment which was becoming available, offered real hope that continuous fermentation could be developed into a viable process. It was anticipated that the following advantages would result from the use of continuous fermentation of beer products: (i) reduced capital cost as a result of faster throughput; (ii) less beer tied up in process as a result of faster throughput; (iii) reduced labour costs due to less down time and therefore less cleaning and automatic control of steady state; and (iv) lower product cost resulting from the production of more ethanol and less yeast, reduced beer losses, improved hop utilization and reduced detergent usage. The major economic gains were therefore in respect of capital investment, labour costs and value of the product in process. Today, this view has substantially changed. With the exception of two brewers in New Zealand, no company is dependent upon continuous fermentation. An increase in its use in the UK for ale products in the late 1960s has proved transitory; continuous fermentation never proved acceptable for lager production.

Why did the brewing industry fail to make a commercial success of continuous fermentation? Essentially batch fermentation is simple; a vessel is cleaned, sterilised and rinsed; it is filled with wort and the required quantity of yeast is inoculated. The cycle can be pre-programmed and little further attention is required until it is necessary for further processing, 4 to 7 days later for an ale, 7 to 10 days for a lager. Operation by trained but not highly qualified staff is straightforward. On the other hand, continuous fermentation requires constant laboratory monitoring and complex automatic control of flow rates, temperature gradients, yeast recycle and oxygen levels. Cell morphology and fermentating wort gravity require regular checking. Engineering support to correct possible faults in control systems, pumps, heat exchangers and pasteurisers is required. All these must be available 24 h a day, 7 days a week.

The much more rapid flow rate from continuous fermentation is, in part, an illusion. It is necessary to have a reservoir of wort to feed the fermenter. Because other types of beer are likely to be produced in the same plant, a beer reservoir to accumulate the output in suitable batches for further processing is required. Although the residence time within the fermenter may be short, this is not the economic factor that should be considered. It is the residence time in the plant that matters and this may be in excess of 24–36 h. The use of continuous fermentation significantly reduces the flexibility of a brewery. Not all consumers drink the same beer, they drink more in summer than winter, more on a hot dry weekend than on a cold wet one. An ability to provide the required diversity of products in varying and unforeseeable amounts is a prerequisite of a successful brewing operation. Batch fermentation can meet this need for flexibility far better than a continuous process which is best suited for the production of a high volume product at an unvarying rate.

Portno[90] is of the view that any change in the process which results in it becoming increasingly complex and demanding must offer corresponding advantages before it will be accepted. Continuous fermentation in the brewing industry tends to create more problems than it offers solutions. Continuous fermentation is scientifically and technically viable and can give rise to products of excellent quality, although not always a match of existing brands; however, for the reasons discussed above, it is most unlikely to be the fermentation method of choice in the brewing industry. A variation on the overall concept is the use of immobilised yeast systems in brewing and these developments will be discussed later in this review.

RECENT RESEARCH ON BREWER'S YEAST STRAINS

Introduction

Over the past 30–40 years research in the brewing industry has been going through a period of seeking scientific reasons for empirical knowledge. Fermentation and yeast research have been no exception to this generalisation. Efforts have been concentrated on 'what the cell does and how it does it' attempting to discover the reasons and mechanisms behind the empirical facts of yeast growth, fermentation and flocculation. Early research concentrated upon biochemical and physiological aspects. However, over the past decade this has been expanded to include a consideration of genetic and molecular biological aspects leading to the development of yeast strains genetically constructed for a specific purpose.

The yeast genus *Saccharomyces* has often been referred to as 'the oldest plant cultivated by man'. Indeed, the history of beer, wine and bread making, with the fortuitous use of yeast, is as old as the history of man himself; however, recent years have seen the transition of yeast from being solely the 'workhorse' in the traditional food and beverage industries to being, in addition, one of the organisms of choice for gene manipulation involving the 'new genetics'. No other group of microorganisms has been more intimately associated with the progress and well-being of the human race than the yeasts. Their contribution to man has been based very largely on the capacity of certain yeasts to effect a rapid and efficient conversion of sugars into ethanol and carbon dioxide, and to effect an alcoholic fermentation of sugary liquids such as grain extracts, grape juice and milk. It is the opinion of some biotechnologists, an opinion not shared by this laboratory, that the future industrial importance of this group will diminish due to the fact that they produce few secondary metabolites of commercial interest. However, many species of yeast (particularly of the genus *Saccharomyces*) are of GRAS (*G*enerally *R*egarded *A*s *S*afe) status and produce two very important primary metabolites—ethanol and carbon dioxide. The ethanol is used in both beverages and for industrial or fuel purposes. The carbon dioxide is employed for leavening in baked goods, for carbonation of beverages, as a solvent in its liquid state and in the culturing of vegetables in greenhouses under controlled environmental conditions.

In the production of fermentation ethanol (be it for beer, wine, potable spirit or industrial ethanol), the microorganism being employed should possess a number of important characteristics: (i) rapid and relevant carbohydrate fermentation ability, (ii) appropriate flocculation and sedimentation characteristics, (iii) genetic stability, (iv) osmotolerance (i.e., the ability to ferment concentrated carbohydrate solutions), (v) ethanol tolerance and the ability to produce elevated concentrations of ethanol, (vi) high cell viability for repeated recycling, and (vii) temperature tolerance. Most of the developments that are currently occurring in industrial yeast strains, are to improve their efficiency to produce ethanol e.g., broadened substrate specificity, increased fermentation efficiency, greater ethanol, osmo- and temperature tolerance and appropriate sedimentation characteristics.[118]

There is a plethora of literature describing the genetics and biochemistry of laboratory strains of *Saccharomyces cerevisiae* but there is a general lack of knowledge regarding the genetics and biochemistry of industrial *Saccharomyces* strains. The haploid strain that the molecular biologist employs in the university laboratory as the organism of choice, for reasons already stated in this document, is usually totally unsuitable for use in the industrial world. Brewing yeasts, and many other industrial yeasts, have been selected over time for exactly those characteristics which render them so unamenable to easy genetic manipulation in the laboratory. They are usually polyploid or aneuploid, lack a mating type characteristic, sporulate poorly, if at all, and the spores that do form are usually not in fours and exhibit poor spore viability, rendering tetrad analysis difficult.[123] It would appear that the widespread use of polyploid yeasts is no accident. Owing to their multiple gene structure, polyploids are genetically more stable and less susceptible to mutational forces and thus can be recycled with a higher degree of confidence than either haploid or diploid strains.

In recent studies in this laboratory[9] a marked improvement in the sporogenic ability of a polyploid/aneuploid brewing strain of *Saccharomyces cerevisiae* has been observed by the cultivation of cells in liquid rather than agar culture media. An analysis of the effects on ascosporogenesis of temperature and presporulation carbon source (acetate *versus* glucose) led to the identification of culture conditions for improved sporulation production. Sporulation at 21°C, instead of the usual 27°C, gave significant increases in ascus formation. Substitution of glucose with acetate as the presporulation carbon source gave a further increase in total sporulation and a very high rate of tetrad induction was noted. This increase in sporulation and tetrad formation has permitted the isolation of an array of segregants from the brewing strain in question.

Brewer's Yeast Strains

The two main types of beer—lager and ale—are fermented with strains of *Saccharomyces uvarum (carlsbergensis)* and *Saccharomyces cerevisiae*, respectively.

Traditionally, lager yeast strains at the end of a fermentation flocculate and collect at the bottom of the fermenter. Ale yeasts tend to be less flocculent and loose clumps of cells are carried to the fermenting wort surface, adsorbed to carbon dioxide bubbles. Consequently, top yeasts are collected for reuse from the surface of the fermenting wort (a process termed skimming) whereas, bottom yeasts are collected (or cropped) from the fermenter bottom. The differentiation of lagers and ales, on the basis of bottom and top cropping, is becoming less and less distinct with the advent of vertical conical bottom fermenters and centrifuges. With centrifuges, non-flocculent yeast strains ae required for both lagers and ales, where, as soon as fermentation is complete and certain parameters such as diacetyl and α-acetolactate are under control and before the yeast has had the opportunity to sediment, the fermented media is passed through a centrifuge in order to separate the yeast from the 'green' beer. With conical bottom fermenters, in the absence of a centrifuge, a more sedimentary yeast (lager or ale) settles into the cone of the fermenter at the completion of fermentation. It is then removed and a portion of this yeast reused as an inoculum for a subsequent fermentation. Taxonomically, the two brewing yeast species have been distinguished by *Saccharomyces uvarum (carlsbergensis)* ability to produce the extracellular enzyme, α-galactosidase (melibiase) and thereby ferment the disaccharide melibiose whereas *Saccharomyces cerevisiae* is negative in this regard.[61] In addition, strains of *Saccharomyces cerevisiae* are able to ferment at much higher temperatures (approximate maximum 38°C) than *Saccharomyces uvarum (carlsbergensis)* (approximate maximum 34°C). However, in two recent texts on yeast taxonomy[6,58] these two groups of yeasts have been consolidated into one species, *Saccharomyces cerevisiae*. Indeed, it has been proposed that the number of species within the genus *Saccharomyces* be reduced from 41, as described by Lodder[61], to either six[6] or seven.[58] Whether or not these proposals will receive acceptance by scientists active in the field of yeast research will have to await the test of time. It is at the strain level that interest in brewing yeast centres. At the last count there were at least 1000 separate strains of *Saccharomyces cerevisiae*—these strains may be brewing, baking, wine, distilling or laboratory cultures. There is a problem classifying such strains in the brewing context; the minor differences between strains that the taxonomist dismisses are vitally important to brewers.

TABLE I. Order of Absorption of Amino Acids From Wort By Brewer's Yeast[a]

Group A Immediately absorbed	Group B Absorbed gradually during fermentation	Group C Absorbed after a lag	Group D Only absorbed slowly after 60 hours
Arginine Asparagine Aspartate Glutamate Lysine Threonine	Histidine Isoleucine Leucine Methionine Valine	α-Alanine Ammonia Glycine Phenylalanine Tryptophan Tyrosine	Proline

[a] Adapted from[52].

The behaviour, performance and quality of a yeast strain is influenced by two sets of determining factors, collectively called *nature-nurture* effects.[129] The nurture effects are all the environmental factors (i.e., the phenotype), to which the yeast is subjected from inoculation onwards. On the other hand, the nature influence is the genetic make-up (i.e., the genotype) of a particular yeast strain.

When yeast is pitched into wort, it is introduced into an extremely complex environment due 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. One of the major advances in brewing science during the past 25 years has been the elucidation of the mechanisms by which the yeast cell, under normal circumstances, utilises, in a very orderly manner, the plethora of wort nutrients. Wort sugars, as will be discussed in greater detail later in this document, are removed in a distinct order as are wort amino acids,[52] different amino acids being removed at various points in the fermentation cycle (Table I). Amino acids, like many sugars, do not permeate freely into the cells by simple diffusion; there is a regulated uptake by a limited number of transport enzymes.[95]

In terms of the development and selection of yeast strains for use in a brewery, there are a number of parameters that are important as scale-up criteria: (i) fermentation rate, (ii) decrease in specific gravity (°Plato of the wort), (iii) taste and flavour match of the final product, (iv) fusel oil, ester and organo-sulphur production, (v) consistent high cell viability, (vi) ethanol tolerance and production and (vii) inoculation (pitching) rate. More specifically, there are a number of factors that will effect fermentation rate *per se*, and these include: (i) inoculation (pitching) rate, (ii) yeast cell viability, (iii) fermentation temperature, (iv) wort dissolved oxygen concentration at pitching, (v) wort soluble nitrogen concentration, (vi) wort fermentable carbohydrate concentrations, and (vii) yeast storage conditions, e.g., the influence of intracellular glycogen levels.

Genetic Manipulation Techniques

As previously discussed, there has been considerable interest during the past decade in the genetic manipulation of brewer's yeast strains.[123,126,137] Although a plethora of papers have been presented at brewing congresses (e.g., IOB, EBC, MBAA and ASBC) the perennial question has been asked 'What is the practical significance of the data?' It is to be hoped that in the remaining paragraphs of this document some answers to this question will be given.

There are a number of methods that are employed in the genetic research and development of brewer's yeast strains.[119] These include hybridisation, mutation and selection, rare mating, spheroplast fusion, and transformation. Transformation can be carried out using native DNA,[102] recombinant DNA[53,104,140] or by liposome-mediated DNA transfer.[98] Hybridisation has not been employed directly as a means until now to 'manipulate' brewer's yeast strains for the reasons already discussed but the prospect of improved methods of spore induction could alter this situation. Nevertheless hybridisation is a technique that has made an invaluable contribution to the field of yeast genetics and is today by no means obsolete. Hybridisation has been employed, in conjunction with more novel genetic techniques, to verify the genetic composition of recombinants.[101] It can also be employed to provide a great deal of relevant genetic information about traits that are germane to brewing fermentation systems.[82,130] Hybridisation has been used to study the genetic control of flocculation,[100] phenolic-off-flavour production[97,119] and the uptake of wort sugars[132] and dextrins.[29,124]

Techniques that have greatest potential and promise as aids in the genetic manipulation of industrial yeast strains are: mutation and selection,[10,22] rare mating,[137] spheroplast or protoplast fusion (also called somatic fusion)[138] and transformation (usually associated with recombinant DNA techniques).[16,40,57] All of these methods have a total disregard for ploidy and mating type and thus have great applicability to brewing yeast strains. Mutation and selection has been used to isolate derepressed mutants of brewing strains such that these strains possess the ability to metabolise maltose in the presence of glucose and thus have increased wort fermentation rates. Mutation and selection has been used to induce auxotrophs and to select easily recognisable characteristics of brewing strains in order that such strains can be employed as spheroplast fusion partners and as recipients for transformation experiments.[10,22] Rare mating has been used in conjunction with the *kar* (karyogamy defective) strains to introduce zymocidal (killer) activity into strains.[35,103,149,150] Spheroplast fusion has been employed to fuse strains constructed by hybridisation with brewing strains in order to introduce the novel capabilities of the hybridised strain into the brewing strain whilst still maintaining all the characteristics of the latter.[103,131] Finally, transformation is being employed to introduce genes from non-*Saccharomyces* yeast strains into brewing strains.

Wort Sugar Uptake

Wort contains the sugars sucrose, fructose, glucose, maltose and maltotriose together with dextrin material. In the normal situation, brewing yeasts are capable of utilising sucrose, glucose, fructose, maltose and maltotriose in this approximate sequence, although some degree of overlap does occur, leaving maltotetraose and the other dextrins unfermented (Fig. 3).

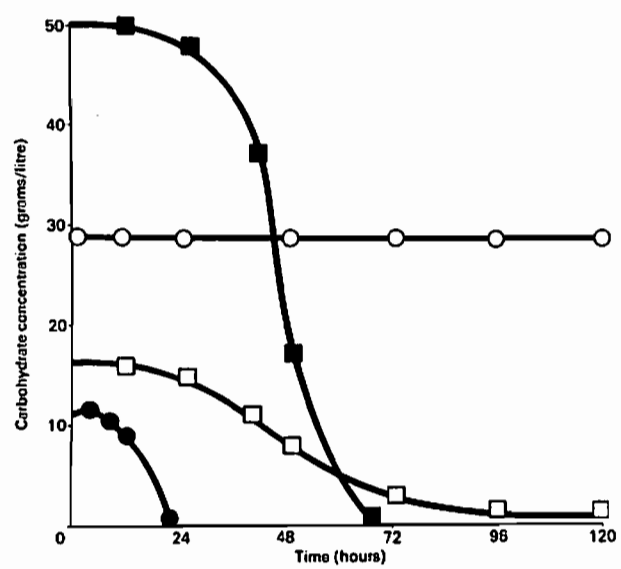


Fig. 3. Uptake of the major wort sugars during fermentation by yeast: maltose (■——■), maltotriose (□——□), glucose (●——●) and dextrin (○——○).

Saccharomyces cerevisiae and *Saccharomyces uvarum (carlsbergensis)* have the ability to take up and ferment a wide range of sugars for example; sucrose, glucose, fructose, galactose, mannose, maltose and maltotriose. *Saccharomyces diastaticus* is able to utilise dextrin material. The initial step in the utilisation of any sugar by yeast is usually 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. Maltose and maltotriose are examples of sugars that pass intact across the cell membrane whereas sucrose and dextrin (with *Saccharomyces diastaticus*) are hydrolyzed by extracellular enzymes [invertase for sucrose and glucoamylase (amyloglucosidase) for dextrins] and the hydrolysis products are taken up into the cell.

Maltose and maltotriose are the major sugars in brewer's wort and, as a consequence, a brewing yeast's ability to use these two sugars is vital and depends upon the correct genetic complement. Brewer's yeast possesses independent uptake mechanisms (maltose and maltotriose permease) to transport the two sugars across the cell membrane into the cell. Once inside the cell, both sugars are hydrolysed to glucose units by the α-glucosidase system. The transport, hydrolysis and fermentation of maltose are particularly important in brewing, distilling and baking, since maltose is the major sugar component of brewing wort, spirit mash and wheat dough. There are (at least) five unlinked polymeric genes that control the ability of yeast to produce α-glucoside permease in response to maltose (*MAL1, MAL2, MAL3, MAL4* and *MAL6*) and all of these loci have been allocated chromosome locations on one of the 17 *Saccharomyces* species chromosomes.[71] Strains carrying any one of these *MAL* genes will be able to take up and metabolise maltose. However, the role of the *MAL* gene is far from fully understood. It has been suggested that the *MAL* loci are either: (i) structural genes for α-glucosidase, (ii) regulatory genes controlling both α-glucosidase and α-glucoside permease, or (iii) complex loci containing both regulatory and structural elements. Indeed, recent evidence would suggest that the *MAL* loci include both the structural genes for α-glucosidase and maltose permease.

A major limiting factor in wort fermentation rates is the repression of maltose and maltotriose uptake by glucose.[112] Only when approximately 50% of the wort glucose has been taken up by the yeast will the uptake of maltose commence. In other words, in most *Saccharomyces cerevisiae* and related strains, maltose utilisation is subject to control by carbon catabolite repression, such that even in the presence of maltose, the maltose-utilising system will be inactivated by high concentrations of glucose. In order to illustrate this phenomenon, two brewing strains of *Saccharomyces cerevisiae* (coded 154 and 3001) have been studied. In both instances, the presence of glucose repressed the uptake of maltose. In an attempt to isolate spontaneous mutants of these strains in which the presence of glucose in the medium did not repress maltose uptake, mutants capable of growth in the non-metabolisable glucose analogue 2-deoxyglucose (2-DOG) were isolated (Fig. 4). 2-DOG was used to simulate conditions of catabolite repression by glucose, thus providing a means to isolate spontaneously arising mutants resistant to the repressive effects of glucose.[121]

A number of stable 2-DOG mutants were found to be capable of utilising maltose in the presence of significant concentrations of glucose. For example, 2-DOG resistant mutants of *Saccharomyces cerevisiae* (strain 154) in a synthetic medium containing both maltose 8% (w/v) and glucose 3% (w/v), were able to completely metabolise the maltose (Fig. 5) whereas in the same medium, maltose uptake was slow with the parental strain and only 60% complete when fermentation ceased. Fermentation and ethanol formation rates in 12°P wort were also increased in the 2-DOG mutants when compared to the parental strain (Fig. 6).

Similar results were obtained with the other strain of

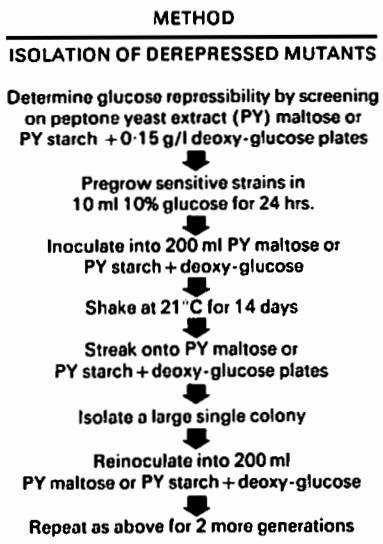


Fig. 4. Methodology for the isolation of derepressed mutants.

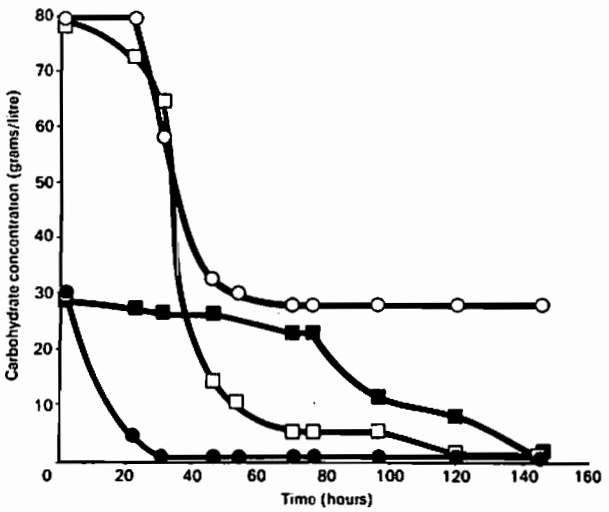


Fig. 5. Carbohydrate uptake in an 8% (w/v) maltose 3% (w/v) glucose peptone-yeast extract medium by polyploid *Saccharomyces cerevisiae* strain 154 and its derepressed mutant.
Glucose uptake: parent (●——●) and mutant (■——■).
Maltose uptake: parent (○——○) and mutant (□——□).

Saccharomyces cerevisiae (strain 3001) studied; maltose uptake in 2-DOG mutants was not repressed by glucose. In 12°P wort, the overall fermentation rate was significantly faster in the 2-DOG mutants (Fig. 7) with complete fermentation being achieved in 45 h compared to 65 h in the parental strain. This increased fermentation rate was due to an increased maltose uptake rate in the 2-DOG mutants compared to the parental strain, with glucose having little influence upon maltose uptake in the mutant. A trained taste panel operating in the triangular mode, determined that the beer produced with 2-DOG mutants of both strains (i.e., 154 and 3001) and was significantly different from that produced using the parental yeast strain; all beers were produced under similar pilot plant brewing conditions.[121] The mechanism by which 2-DOG resistant mutants are derepressed is far from understood, but such mutants have been reported to possess diminished levels of hexokinase.[26] Possibly hexokinase is associated with a general regulatory system in yeast involving overall repression; a theory that is currently under investigation in this laboratory.

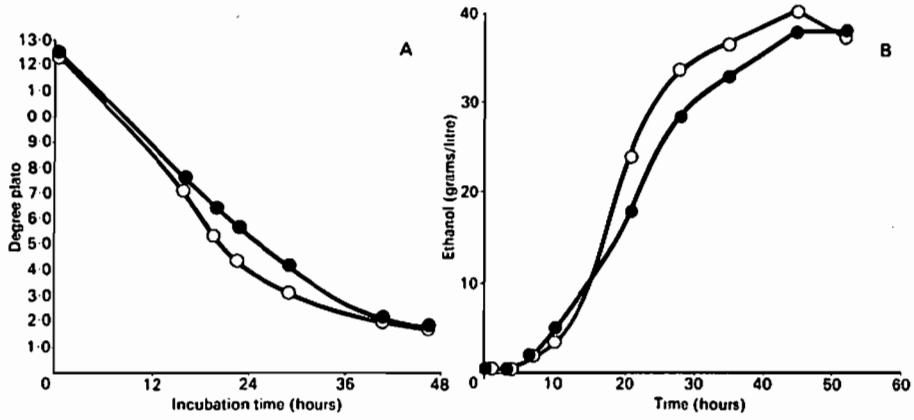


Fig. 6. A. Wort fermentation by polyploid *Saccharomyces cerevisiae* strain 154 (●——●) and its derepressed mutant (○——○).
B. Ethanol production by polyploid *Saccharomyces cerevisiae* strain 154 (●——●) and its derepressed mutant (○——○).

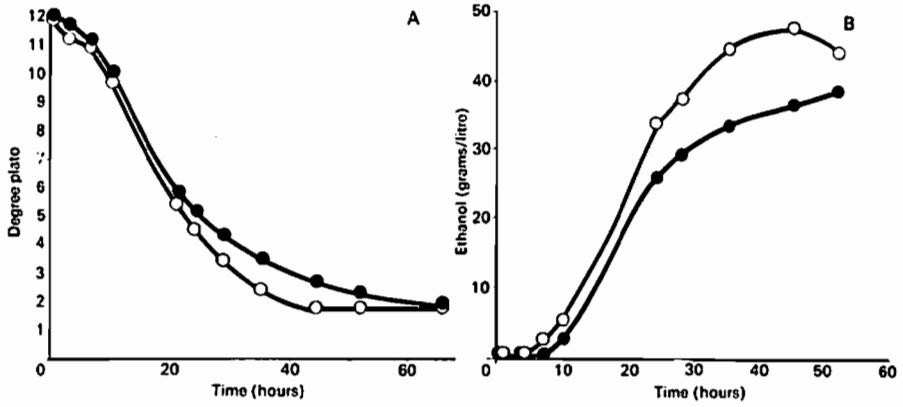


Fig. 7. A. Wort fermentation by ale brewing strain 3001 (●——●) and its derepressed mutant (○——○).
B. Ethanol production by ale brewing strain 3001 (●——●) and its derepressed mutant (○——○).

Bailey and Woodward[4] have described a mutant allele in *Saccharomyces cerevisiae* that is characterised not only by 2-DOG resistance, but insensitivity to glucose repression for invertase, maltose and galactose, as well as the mitochondrial enzyme cytochrome *c* oxidase. The levels of hexokinase activity towards both fructose and glucose were also approximately three-fold higher in mutant clones. The molecular mechanisms by which catabolite repression is affected in yeast is still unknown although many theories have been discussed. Nevertheless, derepressed mutants of industrial yeast strains will be of significant economic importance particularly with respect to increased control of fermentation rate.

Anti-Microbial Activity

Some strains of *Saccharomyces* [and other yeast genera[36,81,149]] secrete a proteinaceous toxin called a 'zymocide' or 'killer' toxin which is lethal to other strains of *Saccharomyces*.[147] Toxin producing strains are termed 'killers' and susceptible strains are termed 'sensitives'. However, there are strains that do not kill and are not themselves killed and these are called 'resistant' (Fig. 8).

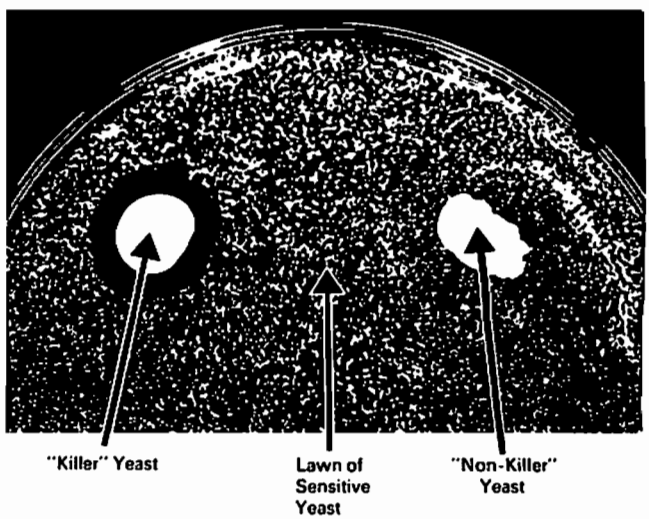


Fig. 8. *Saccharomyces* brewing yeasts with and without zymocidal ('killer') activity.

Although the original terminology for the factor was 'killer',[147] it has been re-named 'zymocide'[149] to indicate that it is *only* lethal towards yeasts and not towards bacteria or cells of any higher organism. Zymocidal yeasts have been recognised to be a serious problem in both batch and continuous brewing fermentation systems[65] since an infection can completely eliminate all the brewing yeast from the fermenter. Thus the brewer can protect the process from this occurrence in one of two ways: (i) maintain a vigorous standard of hygiene to ensure that contamination with a wild yeast possessing zymocidal activity is prevented, or (ii) genetically modify the brewery yeast so that it is not

susceptible to the zymocidal toxin. The first method is the one most brewers to date have been invoking to protect their process; however, genetic manipulation can also be employed to produce a brewing stain that is less vulnerable to destruction by a zymocidal yeast infection.

The killer character of *Saccharomyces* species is characterised by a form of genetic control that is located outside the nucleus—that is termed extra-nuclear or cytoplasmic.[36] Specifically, killer character in *Saccharomyces* species is determined by the presence of two species of cytoplasmically located double-stranded (ds) RNA.[35] M dsRNA (killer plasmid), which is killer strain specific, codes for killer toxin and also for a protein or proteins which render the host immune to the toxin.[37] L dsRNA, which is also present in many non-killer yeast strains, specifies a capsid protein which encapsulates both forms of dsRNA thereby yielding virus-like particles. Although the killer plasmid is contained within these virus-like particles, the killer genome is not naturally transmitted from cell-to-cell by any infection process. The killer plasmid behaves as a true cytoplasmic element and requires at least 29 different chromosomal genes (*mak* for its maintenance in the cell). In addition, three other chromosomal genes (*kex1, kex2* and *rex*) are required for toxin production and resistance to toxin.[147] The zymocidal toxin of *Saccharomyces* species acts as a protonophore, causing ATP leakage and as a K^+ ionophore.[150]

The zymocidal activity has been successfully introduced into a variety of brewing yeast strains by a number of research laboratories.[37,99,150] The technique of rare mating has been employed to produce hybrids with killer activity. When non-mating strains are mixed together at a high cell density, a few true hybrids with fused nuclei form which usually are selectively isolated. An even more useful variation of this technique employs a strain which harbours a specific nuclear gene mutation, designated *kar* for karyogamy defective.[18] When this strain hybridises with another strain, the nuclei will not fuse and this permits the formation of cell lines with mixed cytoplasmic contents (heteroplasmons).[37] Consequently, selected hybrids that contain only the brewing strain nucleus can be isolated from such a rare mating. However, such hybrids contain the cytoplasm of both parental cells, thereby permitting the introduction of cytoplasmically transmitted characteristics, such as killer toxin production, into brewing strains without altering the nucleus of the brewing strain.

A brewing polyploid lager yeast strain has been rare mated with a laboratory 'zymocidal' (killer) haploid strain and a number of rare mating products isolated (Fig. 9). In addition to biochemical tests to characterise the rare mating products, agarose gel electrophoresis demonstrated that some rare mating products contained not only the 2 micron plasmid (from the parental brewing lager strain) but also the L and M ds RNA (from the haploid zymocidal mating partner) which code for zymocidal toxin production. Beers were brewed on a 40 litre scale from both the parental lager and the zymocidal rare mating products.[99] The strains exhibited similar fermentation patterns and a comparison of the profiles of the beer flavour congenors present revealed little difference (Table 2).

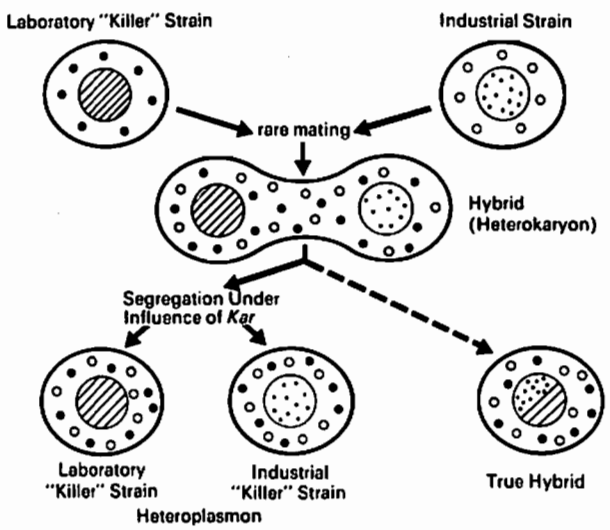


Fig. 9. Rare mating protocol to produce industrial strains with zymocidal activity.

TABLE II. Taste Panel Data (Triangular Mode) of Brewing Strain 3001 and Its Derepressed Maltose Mutant

	Number of panelists detecting difference	Total number of panelists
Trial no. 1	4	12
Trial no. 2	5	10
Total	9	22

No significant difference was detected.

However, an 'expert' taste panel assessment indicated that although the beer produced by the rare mating product was very palatable, it could be distinguished from the parental lager. This difference can be attributed to the cytoplasmic effect of the haploid rare mating partner.

The effect this zymocidal lager strain had on a typical brewery fermentation was studied by mixing this strain at a concentration of 10% (w/v) with an ale brewing strain (Fig. 10A). The control was the ale strain mixed with 10% (w/v) of the 'non-zymocidal' lager. The yeast was sampled throughout the fermentation and viable cells determined by plating onto nutrient agar plates incubated at 37°C; it has already been discussed in this document that this temperature is resistant to the growth of lager yeast but permits the growth of ale yeast. Within 10 h the zymocidal lager strain had almost totally eliminated the ale strain.[104] When the concentration of killer yeast was reduced to 1% (w/v), within 24 h the ale yeast was again eliminated (Fig. 10B). The speed at which zymocidal activity is effective may well make a brewer apprehensive about employing such a yeast in his fermentation cellar particularly where several yeasts are employed for the production of different beers. An error on an operator's part in maintaining lines and yeast tanks separate could result in serious consequences. In a brewery with only one yeast strain this would not be a cause for concern.

An alternative to the 'zymocidal' strain would be to produce a yeast strain that does not 'kill' but is 'killer resistant'. That is to say, it has received the genetic complement that renders it immune to zymocidal activity. The construction of such a yeast would be a logical compromise. Since it does not itself kill, it allays the fear of the brewer that this yeast might kill all other production strains in the plant and at the same time, it is not itself killed by a contaminating yeast with zymocidal activity.

Certain bacteria are well known to impart undesirable qualities to beer. In particular, the metabolic activities of bacterial groups such as the lactic (*Lactobacilli* and *Pediococci*) and acetic acid bacteria are of concern to brewers since they can contribute an array of organic compounds that spoil the aroma and flavour of beer.[45] As there is a constant need for strict microbial control to ensure consistent production of high quality beers, the identification of yeast strains that secrete thermolabile (i.e., compounds inactivated by pasteurisation) antibacterial substances and the introduction of such characteristics into routinely used brewery strains would be of considerable value.

Although it is well known that *Penicillia, Aspergilli, Actinomycetes*, and certain members of other microbial

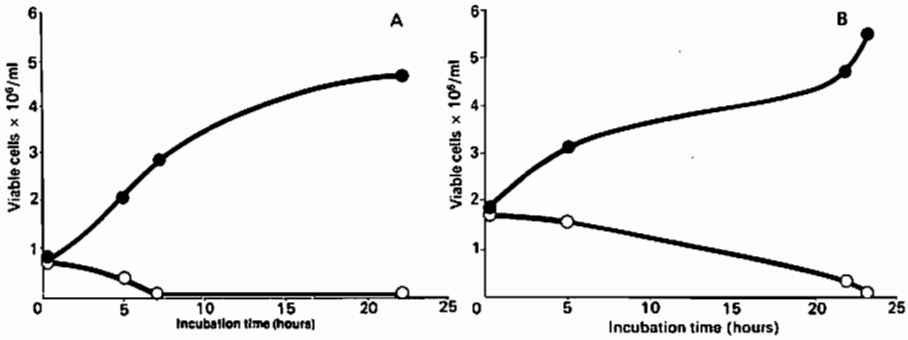


Fig. 10. Effect of a 'killer' lager yeast on growth of an ale yeast during a 12°P wort fermentation. The yeast was sampled throughout the fermentation and viable cells were determined by plating on nutrient agar plates incubated at 37°C to inhibit growth of lager yeast but allow growth of ale yeast.
A. Control—growth of ale yeast from 90% ale yeast and 10% non-killer lager yeast (●——●).
Test—growth of ale yeast from 90% ale yeast and 10% killer lager yeast (○——○).
B. Control—growth of ale yeast from 99% ale yeast and 1% non-killer lager yeast (●——●).
Test—growth of ale yeast from 99% ale yeast and 1% killer lager yeast (○——○).

groups possess an ability to synthesise and secrete secondary metabolites that are pharmacologically active against the growth of other microorganisms, little attention has been given to *Saccharomyces* species as possible producers of substances with similar properties.[23,107] Bilinski *et al.*[8] have employed a general screening test for the expression of antibacterial activity and have studied over 400 yeast species belonging to 31 genera. Of these cultures, only two, *Kluyveromyces thermotolerans* and *Kloeckera apiculata*, were found to produce zones of inhibition of bacterial growth on Diagnostic Sensitivity Test Agar (DSTA) medium supplemented with 0·002% methylene blue. Of nine bacteria employed as test organisms, no antibacterial activity was evident against four Gram-negative bacteria employed and only *Lactobacillus plantarum* and *Bacillus megaterium* were inhibited. In order to determine whether cultivation in the presence of methylene blue added to the agar culture media was a prerequisite for the development of inhibition zones, *Kloeckera apiculata* and *Kluyveromyces thermotolerans* were cultivated with and without the presence of the dye. Zones of inhibition only developed when the dye was included in the culture medium, indicating a role for the dye in the determination of antibacterial activity with the two yeast species in question. It is conceivable that the expression of antibacterial activity by these two yeasts is not due to the secretion of secondary metabolites but instead involves the transformation of methylene blue to a pharmacologically-active form.

Ogden and Tubb[78] have also failed to isolate yeast strains that possess antibacterial activity. Consequently, they have sought a suitable antibacterial agent that is active against a wide range of beer-spoilage bacteria and in particular against lactic acid bacteria. They have studied the effects of Nisin; a 34 amino acid residue polypeptide with a molecular weight of 3510 daltons produced by lactic *Streptococci*.[34] It has been recognised for over 40 years[44] as both an antibiotic and a bacteriocin and is internationally accepted as a food preservative, specifically approved for use in cheese, canned foods, and clotted cream in many countries.[148] One hundred and forty-nine strains of bacteria, mostly brewery contaminants able to spoil wort or beer, and 12 brewing strains of yeast have been screened for their sensitivity to Nisin. Nisin inhibited growth of 92% of the Gram positive strains, predominantly lactic acid bacteria of the genera *Lactobacillus* and *Pediococcus*. In contrast, all 32 Gram negative strains tested, except three *Flavobacter* strains, were Nisin-resistant. In addition, none of the brewing yeast strains tested showed Nisin-sensitivity, suggesting that it could be added to fermentations without altering yeast performance. Rather than adding Nisin to all fermentations, it could be applied as a palliative measure, treating only those fermentations or beers that are found to contain lactic acid bacteria. The use of Nisin in this context would depend largely on the development of sensitive and rapid methods of detecting spoilage microorganisms. Yet another possible use of Nisin is as a cleansing rinse for plant equipment or to wash pitching yeast. Finally Ogden and Tubb[78] consider that it may be possible to clone the structural gene from *Streptococcus lactis* into a brewing yeast strain. However, research on the application of Nisin to the brewing industry is still very much in its infancy. Considerable future research will be required to assess the full potential of Nisin and to define the role in which it might be most usefully employed.

Flocculation

The flocculation properties, or conversely lack of flocculation, of a particular brewing yeast culture is one of the major factors when considering important characteristics during wort fermentation. Flocculation involves the formation of an open agglomeration of cells, the mechanism of which depends upon molecules acting as bridges between the cells; this open structure is implicit in the word flocculation. Unfortunately, a certain degree of confusion has arisen by use of the term 'flocculation' in the scientific literature to describe different phenomena in yeast cell behaviour. The term flocculation, as employed most commonly within the brewing industry in particular, is defined here as '*the reversible aggregation of dispersed yeast cells into flocs, generally toward the end of fermentation, and the subsequent segregation of the flocs from the suspending liquors*'.[127] This definition excludes other forms of aggregation, particularly those of 'clumpy growth' and 'chain formation'. This non-segregation of daughter and mother cells during growth has sometimes erroneously been referred to as flocculation.[128] The term 'non-flocculation' thereby implies the lack of cell aggregation and consequently a much slower separation of (dispersed) yeast cells from the liquid medium. Flocculation occurs in the absence of cell division and only under rather circumscribed environmental conditions and involves the cross-bridging of divalent ions, usually calcium, bridging anionic groups at the cell surface. Although yeast separation often occurs by sedimentation, it may also be by flocculation, because of cell aggregates being entrapped in bubbles of carbon dioxide, as in the case of 'top-fermenting' ale brewing yeasts.

The most important aspect of the flocculation characteristics of a brewing yeast strain is the period during the fermentation cycle that the yeast culture flocculates. Certainly one of the most troublesome problems encountered by the brewer is a 'hung' or 'stuck' fermentation, i.e., incomplete attenuation of the wort. A 'hung' fermentation is invariably caused by one of two factors: (i) premature flocculation of the yeast culture in the fermenting wort, and (ii) failure of the

yeast, although still in suspension, to utilise all of the fermentable sugars; this is usually due to the inability of the yeast to take up and metabolise maltotriose. It is also possible that the yeast may fail to flocculate, thus making its removal from the fermented wort very troublesome if a centrifuge is not available. This in turn will cause difficulty in obtaining a bright sparkling beer and under such circumstances off-flavours, due to yeast autolysis, can often result. However, with the advent of centrifuges in the brewing process, at the end of the fermentation, the yeast is centrifuged out of the fermented wort and the centrifuged fermented wort is cooled and placed in an aging tank. A non-flocculent yeast is now required because prior to centrifugation, the absolute minimum of yeast sedimentation is essential. Indisputably, knowledge of the mechanisms that control flocculation at the biochemical, molecular and genetic levels are of paramount importance to both the brewer and the brewing microbiologist [for reviews see those by Calleja;[15] Johnston and Reader;[50] Rose;[94] Stewart and Russell].[127]

There is little doubt that differences in the flocculation characteristics of various yeast cultures are primarily manifestations of the yeast culture's cell wall structure. Studies in many laboratories have failed to reveal any meaningful differences in gross composition between the walls of the two culture types that could be directly correlated with the phenomenon of flocculation. The aggregates formed when *Saccharomyces cerevisiae* flocculates can be dissociated by sonication or by removal of Ca^{++} ions, as floc formation is brought about by an incompletely understood bridging mechanism between the cell walls of flocculent strains. The bonds connecting cells in a floc are non-covalent and depend on the presence of divalent cations, Ca^{++} being the most effective. Several mechanisms for flocculence have been proposed. One hypothesis is that anionic groups of cell wall components are linked by Ca^{++} ions;[68] these ionic groups, in all likelihood, are cell wall proteins.[7,49] Another hypothesis implicates glycoproteins specific to flocculent strains acting in a lectin-like manner to cross-link the cells;[67] here Ca^{++} ions act as ligands to promote flocculence by conformational changes. Electron microscopy of flocculent and non-flocculent cultures shadowed with tungsten oxide has revealed that flocculent cultures were found to possess a 'hairy' outer surface whereas non-flocculent cells possessed a smooth one (Fig. 11).[21]

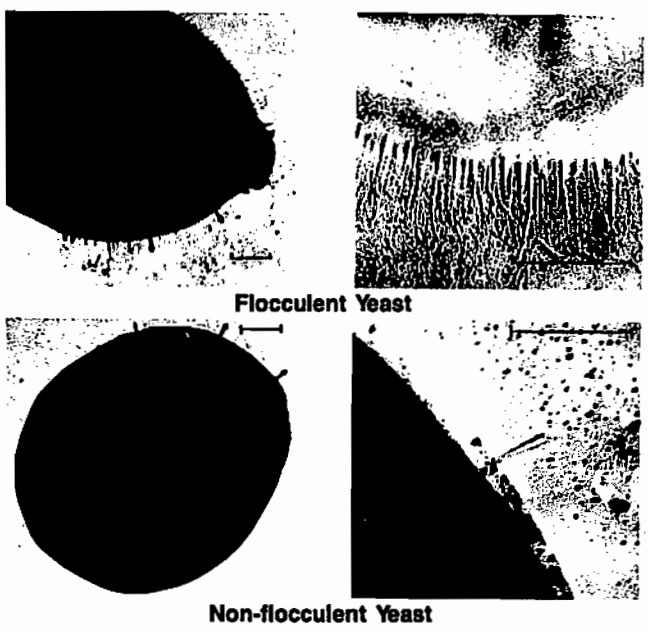


Fig. 11. Electron photomicrographs of *Saccharomyces cerevisiae* shadow cast with tungsten oxide. Bar = 1 μm.

During the course of studies on the flocculation characteristics of a mixed ale yeast culture comprised of two closely related strains of *Saccharomyces cerevisiae*, the phenomenon of *co-flocculation* has come to light.[116] Co-flocculation is defined as the phenomenon where two strains are non-flocculent alone but flocculent when mixed together. Co-flocculation should, however, not be confused with flocculation in pure strains because, although there are many features in common, co-flocculation is an interaction of two discrete strains.

The flocculation characteristics of this complementary pair of *Saccharomyces cerevisiae* strains have been studied.[120] When cultured separately in wort, both strains proved to be non-flocculent, however, when the two strains were mixed together after growth (or grown together), they were found to be flocculent whether in a 50:50 proportioned mixture or in a 90:10 proportion in either strain's favour. A survey of non-flocculent pure strains of *Saccharomyces cerevisiae* revealed a number capable of exhibiting co-flocculence capability when mixed with other strains. There are, however, a number of non-flocculent strains of both ale and lager, which are unable to co-flocculate and these have been designated as neutral types. A survey of non-flocculent strains of *Saccharomyces uvarum (carlsbergensis)* has revealed only neutral types.

Genetic studies on yeast flocculation commenced in the early 1950s.[32,136] However, because of the polyploid/aneuploid nature of brewing strains most, but not all,[51,117] of the research on flocculation genetics has been conducted on haploid/diploid genetically defined laboratory strains. Using a flocculent haploid yeast strain, a single dominant gene for flocculation has been identied, *FLO1*, and found to be allelic with *FLO2* and *FLO4* which had previously been identified.[100,125] Mapping studies have revealed that *FLO1* is linked to *ade1* and thereby located on chromosome I. It is located 45 centimorgans (cM) from the centromere and 40 cM from *ade1*. A further dominant gene (*FLO5*) has been identified but attempts to date to map this gene have been to no avail.[50] A comparison of the flocculation characteristics of yeast strains containing either *FLO1* or *FLO5* in their genotypes has revealed some interesting similarities and differences between the two phenotypes. The flocculent phenotypes conferred by both the *FLO1* and the *FLO5* gene were irreversibly lost upon treatment of cells with pronase, proteinase K, trypsin or 2-mercaptoethanol treatments. However, the floc-forming ability of cells of *FLO1* strains was destroyed by chymotrypsin digestion and was stable to incubation at 70°C whereas the floc-forming ability of cells of the *FLO5* strains was resistant to the action of chymotrypsin and was heat labile.[41] This data would indicate that expression of the *FLO1* and *FLO5* alleles results in the production of different and characteristic cell wall located proteins which are responsible, or at least are required, for their flocculent phenotypes.

Although the technological importance of the flocculation characteristics in yeast has been associated predominantly with brewing, it is becoming increasingly evident that the ability to flocculate is a characteristic to be considered in the choice of yeast strains for other alcoholic fermentations. The ability to flocculate strongly is included amongst criteria for selection of improved rum yeasts, yeasts for the Scotch whisky industry and for the production of fuel ethanol from sugarcane juice in Brazil.[62]

Yeast Storage Conditions—Influence On Intracellular Glycogen Levels

It has previously been mentioned in this review that one of the factors that will affect fermentation rate is the condition under which the yeast culture is stored between fermentations, and of particular importance in this regard is the influence of these yeast storage conditions on the intracellular glycogen level of the yeast cell. Glycogen is the major reserve

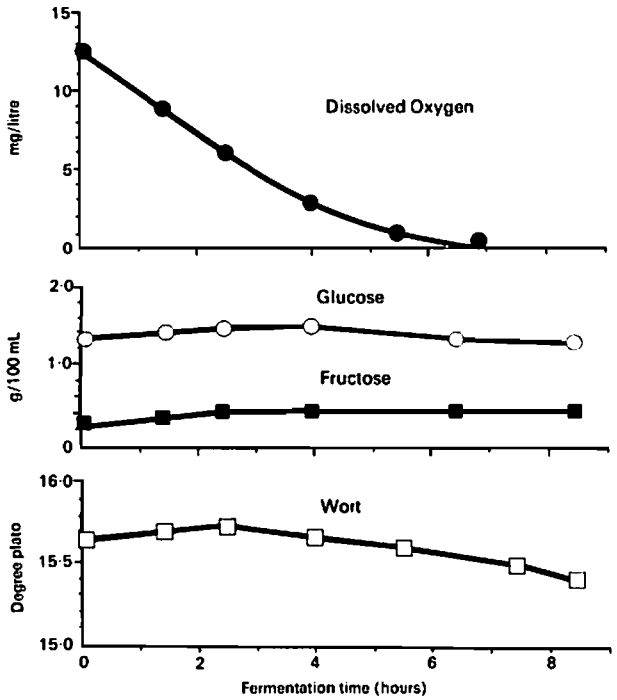


Fig. 12. Dissolved oxygen (●——●) and carbohydrate utilisation [glucose (○——○), fructose (■——■), and wort (□——□) in a lager fermentation.

carbohydrate stored within the yeast cell and is similar in function and structure to plant amylopectin. It serves as a store of biochemical energy and carbon to sustain the yeast cell during periods of starvation and also to provide the cell with an immediate supply of energy during the lag phase of fermentation when the demand for energy is intense for the synthesis of such compounds as sterols and fatty acids (i.e., lipids). Thus an intracellular source of glucose is required to fuel the synthesis of lipid at the same time that oxygen is available to the cell. Brewery fermentations are somewhat unique in this regard in that oxygen is supplied in limited amounts and on a one time basis, usually with the incoming wort. The uptake of oxygen by the yeast cell is very rapid and at the same time, there is a delay in the passive diffusion of wort glucose into the cell. The data presented in Fig. 12 confirm the earlier studies of Quain and Tubb.[91] With a 16°P wort the difference in time between the availability of intracellular wort glucose and wort oxygen during the first 8 h of fermentation is illustrated. There is no appreciable wort glucose uptake until 5 h or even later after pitching whilst the wort dissolved oxygen is almost completely depleted in this same time period.[72]

In order to synthesise lipid the yeast immediately mobilises its reserve of glycogen in order to fulfill the requirement of the cell for glucose. The high levels of ATP resulting from respiration, activate the phosphorylase system which is necessary for the hydrolysis of glycogen to glucose. Yeast phosphorylase activity during wort fermentation has been found to peak coincidentally with glycogen hydrolysis which is within the first 10 h after pitching.[122] Dissimilation of glycogen and the synthesis of lipid are both rapid (Fig. 13). The hydrolysis of glycogen from approximately 27% to 5% and the corresponding production of lipid from 5% to 11·5% of the cell dry weight occurs within the first 6 h after pitching. Towards the later stages of fermentation, the yeast restores its reserve of glycogen. The actual maximum glycogen content is a function of the yeast strain, fermentation temperature, wort gravity and plethora of other factors. However, the concentration of glycogen stored and the degree of depletion at the end of the fermentation will, to a great extent, determine the ability of the yeast culture to

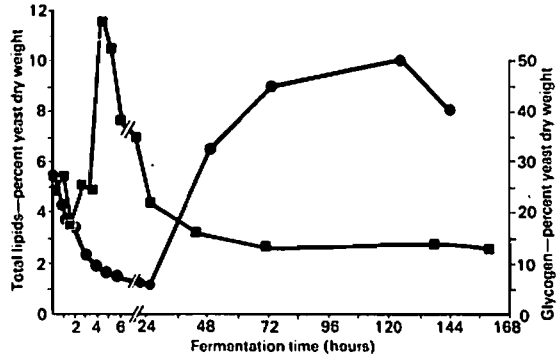


Fig. 13. Yeast glycogen (●——●) and lipids (■——■) during a 16° Plato lager fermentation.

survive extended storage periods and still ferment at an acceptable rate when pitched into wort.

Storage in most brewery yeast handling systems usually involves maintaining the yeast in beer or water, in varying concentrations from collection-to-collection. These conditions are often far from ideal for growth or even maintenance, since limited assimilable carbon and soluble nitrogen are present, together with a relatively high concentration of ethanol. Under these conditions the yeast must survive for an indeterminate period of time and to do it requires a basal level of metabolic energy. To a large extent, glycogen must provide the cell with these requirements. In order to study the change in glycogen content during storage, its concentration has been monitored as a function of time and storage temperature. It can be seen in Fig. 14 that storage temperature has a direct influence on the rate of glycogen dissimilation, as might be expected, considering the effect that temperature has upon metabolic rates in general. Of particular interest is the fact that within 48 h, the yeast which had been stored aerobically at 15°C had approximately 50% of its original glycogen concentration remaining; whereas the yeast stored at 4°C had 80% of its glycogen remaining after 48 h. It is therefore evident that the conditions under which yeast is stored and collected and the time of storage can result in detrimental effects to the yeast which will result in sluggish fermentation rates and modifications to the flavour and stability of the final beer. Good yeast handling practices should include collection and storage procedures which avoid inclusion of oxygen in the slurry, cooling of the yeast slurry to 4–6°C as soon as possible after collection and perhaps most importantly, recognition, prior to pitching, of a yeast that contains a low glycogen concentration in order that an appropriate increase in the pitching rate can be made.

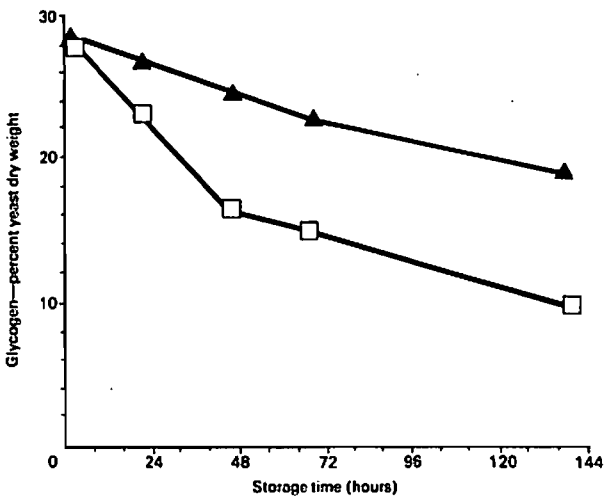


Fig. 14. The effect of aerobic yeast storage on intracellular glycogen at 4°C (▲——▲) and at 15°C (□——□).

Fermentation of High Gravity Worts

In the past two decades an increasing number of breweries have adopted the 'high gravity brewing' (concentrated brewing) procedure and at this time considerably more beer is produced in North America according to this production method than by conventional 'low gravity' means. High gravity brewing is a procedure which employs wort of higher than normal concentration and hence requires dilution with de-oxygenated water at a later stage in processing. By reducing the amount of water employed in the brewhouse, increasing production demands can be met without expanding existing brewing, fermenting and storage facilities. Beers produced according to high gravity brewing procedures (i) have improved colloidal haze and flavour stability, (ii) provide a more efficient use of existing plant facilities, (iii) reduce energy costs, (iv) yield more alcohol per unit of fermentable extract, (v) may contain high adjunct ratios, and (vi) are rated smoother in taste. On the other hand, beers produced according to this procedure suffer from the fact that they (i) decrease brewhouse material efficiency, and (ii) are sometimes difficult to flavour match with an existing normal gravity product.[88]

High gravity worts influence yeast performance with effects normally readily apparent upon both fermentation and flocculation characteristics.[87] Due to differences in the run-off characteristics of varying gravity worts, and that invariably the grain beds of high gravity wort in a lauter tun operation are sparged less intensively than conventional worts, high gravity worts do not contain those substances which are extracted during the final stages of the lautering process of normal gravity worts. The composition of the extract from the first wort differs significantly from the composition of the extract of the last runnings. The latter, for instance, contains more nitrogenous matter, ash and tannins. Further, marked differences in oleic acid and linoleic acid levels are found when comparing the amounts of high gravity worts to the amounts in a normal gravity wort, or a first wort versus a last wort. Most of the above mentioned compounds have been implicated in beer taste stability and ester formation during fermentation[3] and as some of these compounds are modified in high gravity worts, it is not unreasonable to assume that some of the peculiar characteristics of high gravity beers are directly attributable to the reduced levels of these substances.

In a detailed analysis of large-scale brewery trials of three worts, 11·5, 13·7 and 16·5°P, it was readily apparent that the brewhouse material efficiency is reduced as the gravity of the wort is increased. This fact is reflected in the increase in last wort concentration and by the decrease in hop utilisation. However, the portion of the total extract representing the α-amino nitrogen remained constant. This latter observation was somewhat surprising since the fermentability of worts with a high proportion of corn adjuncts has been found to be increased when compared to all malt worts of similarly elevated gravity and also to low gravity/high adjunct worts. The improved fermentability of high gravity/high-adjunct worts despite the constant percentage of α-amino nitrogen in wort solids has been correlated to the observation that a doubling of wort concentration did not double the total yeast crop.[1] Therefore, since high gravity worts do not produce more yeast on a proportional basis, it becomes clear that they would not require the same amount of assimilable nitrogen on a proportional basis. It is also clear that the concentration of assimilable nitrogen in high gravity worts does not become limiting as rapidly as in normal gravity worts.

In many instances it has been found that the higher the wort gravity the more flocculent and sedimentary the yeast will become.[87] Beer produced under high gravity conditions has a marked improvement in both flavour and physical stability. However, beers brewed by the high gravity process are often different from their conventional counterparts. Studies by Anderson and Kirsop[1] have revealed that high gravity fermentations produce significantly increased amounts of acetate esters over their normal gravity counterparts. It has been demonstrated that the interaction of these esters by yeast was amenable to control by oxygenation during fermentation. Such treatment involved the introduction of oxygen, in the form of oxygen saturated water, into the fermenter.[2] The addition of up to 9% of the wort volume as oxygenated water was necessary for the control of acetate esters. The rationale behind this approach was to channel cell energy away from ester production and into cell growth.

Pfisterer and Stewart[88] have taken a different approach. Their research efforts have been directed to finding fermentation conditions which permit control of the acetate esters in high gravity worts by means other than stimulating growth. The investigation into the mechanism of ester formation was based on the following reaction:

$$CH_3COSCoA + C_2H_5OH \rightarrow CH_3CO_2C_2H_5 + CoASH$$

Any means to reduce the pool of acetyl-S-CoA in the yeast cell, either by depletion or by inhibition of its synthesis, might also reduce the level of ethyl acetate. In the presence of high levels of fermentable sugar the wort medium acetyl-S-CoA molecules are directed in large numbers towards the lipid synthesis and away from the formation of acetate esters. It has been observed in an 18°P wort that the amounts of ethyl acetate were reduced when increasing proportions of brewing syrup were employed as adjunct material. In addition, analysis of total lipids revealed a simultaneous accumulation of these compounds in yeast. It would therefore appear that, in the early aerobic stages of fermentation, the yeast cell develops the metabolic mechanisms for the production of fatty acids and lipids which are required for membrane structural purposes. Brewery worts, which contain higher than normal levels of fermentable sugars may prolong this condition and thus enable the yeast to build up a substantial 'lipid credit'. Later in the fermentation under anaerobic conditions, the yeast can use this potential by drawing upon the pool of acetyl-S-CoA for synthesis, thus reducing the availability for ethyl acetate formation.

The use of high gravity worts in brewing has introduced another yeast factor that has relevance to the overall process, namely a yeast strain's ethanol tolerance. For example, an 18°P wort has the potential of being converted into greater than 8% (v/v) ethanol. It has long been appreciated that strains of *Saccharomyces cerevisiae* differ in their ability to remain viable in the presence of ethanol.[12] In general, it is believed that strains used in brewing have only moderate tolerance,[20] whilst those employed in distilleries, predictably, have a greater ethanol tolerance.[38] Unfortunately, there is no recognised method for measuring the ethanol tolerance of yeast strains, and considerable confusion pervades this area of investigation.[93] The simplest method, but not necessarily the most relevant, is to assess the effect of ethanol, incorporated into the media, on the batch growth of *Saccharomyces cerevisiae* and related species. The first data on ethanol tolerance of *Saccharomyces cerevisiae* was obtained in this manner. Other workers have elected to assess the ability of *Saccharomyces cerevisiae* to tolerate ethanol by determining the effect of ethanol on the fermentative activity of cells. For example, Nojivo and Ouchi[77] found that the ethanol tolerance of yeast strains examined fell in the range of 20–30% (w/v) and surprisingly, differences in the tolerance of sake and distilling yeast, as compared with brewer's and baker's yeasts, were difficult to find.

What exactly is 'ethanol tolerance'? For a yeast property of such monumental industrial importance, surprisingly little universal agreement exists on the physiological nature of ethanol tolerance, the excretion of ethanol, the location of ethanol-sensitive sites and the method by which it can be best defined and assayed. This is not totally surprising because

ethanol tolerance is a property of yeast under polygenic control.[48] For this reason, ethanol tolerant mutants cannot easily be isolated by conventional screening and selection techniques. Recent studies by Brown and Oliver[14] have made use of a continuous selection system and this technique does hold promise for isolating spontaneous mutants with enhanced ethanol tolerance properties. Research on the physiological basis of ethanol tolerance and toxicity in *Saccharomyces cerevisiae* and related species was minimal until the upsurge of interest in high gravity brewing and the manufacture of industrial ethanol by fermentation. Despite the centuries of experience in fermentation ethanol production, little is known about important aspects of the biochemical conversion process such as the mechanism of ethanol inhibition of fermentation. The fermentative activity of all ethanol-producing microorganisms declines progressively as ethanol accumulates in the surrounding medium. Product inhibition is one of the factors which decreases the rate at which sugars are converted into ethanol and limits the final concentration of ethanol which can be produced. The accumulation of ethanol in the microbial environment represents a form of chemical stress on the microorganisms living there. High concentrations of ethanol represent an environmental extreme analogous to the extremes of pH or temperature. Organisms which are adapted to live in these environmental extremes have evolved modifications in many enzymes and in their membrane structure which are not found in organisms living in moderate environments.

During the past few years a number of research laboratories have been investigating the ethanol-induced changes in a variety of different microorganisms, including industrial yeast strains,[47,139] as an approach to understanding the mechanisms of ethanol inhibition and the evolution of microbial tolerance to ethanol. There are two basic hypotheses for the mechanism of alcohol inhibition of fermentation: damage to the cell membrane and end-product inhibition of glycolytic enzymes.

Although it is an intuitively attractive hypothesis, end-product inhibition of glycolytic and ethanologenic enzymes does not appear to be important in *Saccharomycces cerevisiae* and related species. *In vitro* studies have shown that these enzymes are relatively resistant to ethanol and are inhibited only at ethanol concentrations which are much higher than those occurring during fermentation.[69,73] Considerable controversy has existed concerning the possibility that the intracellular concentration of ethanol in yeast may be much higher than that of the surrounding medium, thereby providing sufficiently high ethanol concentration to inhibit glycolytic enzymes. Early research in this area indicated that under certain environmental conditions ethanol did accumulate within the cell.[83,135] However, these elevated intracellular ethanol concentration figures may have been due to the idiosyncrasies of the analytical methods employed, particularly with regard to the preparation of the cell-free extracts. Studies from several laboratories in the past few years would appear to indicate that in most circumstances ethanol diffuses freely across cell membranes, rapidly equilibrating intracellular and extracellular concentrations[19,24].

Ingram[46] is of the view that the potency of alcohols as inhibitors in a wide variety of biological systems, including yeast, can be directly correlated with their lipid solubility, implying a hydrophobic site of action such as the membrane. In recent years, a number of research workers have turned to a study of the effect of ethanol on transport processes in the plasma membrane. Rose,[93] as part of a study of the relationships between lipid composition and function in the plasma membrane of a strain of *Saccharomyces cerevisiae*, has reported that cells in which the plasma membrane was enriched with linoleyl ($C_{18:2}$) residues were more resistant to the lethal effects of one molar ethanol compared with cells enriched in oleyl ($C_{18:1}$) residues. Further, exponential growth of linoleyl residue-enriched cells was inhibited to a lesser extent when cultures were supplemented with ethanol than were cells in cultures enriched with oleyl residues suggesting that the fluidity of the membrane plays a major role in ethanol tolerance, perhaps by facilitating excretion of ethanol.

As is hopefully evident from the preceding paragraphs, understanding of ethanol tolerance has significantly increased in the past decade but a clear picture of the mechanisms controlling the process is not yet at hand. Nevertheless, all evidence now indicates that ethanol tolerance in *Saccharomyces cerevisiae* is not simply a result of the ability of various strains to tolerate differing levels of ethanol. Indeed, it has become clear that in brewing, ethanol tolerance is influenced by the nutritional conditions of the wort, as well as by the other environmental conditions employed.[17]

Yeast Excretion Products

It is axiomatic to state that the major excretion product produced during wort fermentation by brewer's yeast strains is ethanol. However it is the type and concentration of the plethora of the other yeast excretion products during wort fermentation that will determine, to a significant extent, the flavour of the product; this formation will depend upon the overall metabolic balance of the yeast culture. There are many factors that can modify the balance and hence the flavour of the product; these include the yeast strain, incubation temperature, adjunct level, wort pH, buffering capacity, wort gravity, etc. Research on beer flavour has been intensively studied in a number of laboratories over the past 25 years.

A great many volatiles, most but not all are fermentation by-products, have been identified in beer, and different substances may influence the aroma and flavour of the product to varying degrees. Some volatiles are of great importance and many contribute significantly to beer flavour, whilst others are of importance merely in building the background flavour of the product. The following groups of substances are found in beer: alcohols, esters, carbonyls, organic acids, sulphur compounds, amines, phenols and a great number of other compounds.

Alcohols

In addition to ethanol a large number of other alcohols are found in beer, and higher alcohols or fusel oils constitute an important part of the by-products formed during wort fermentation. Their formation is linked to yeast protein synthesis and they are formed by β-acids, which in turn may be formed by transamination and deamination of the amino acids in wort, or synthesised from wort carbohydrates. The yeast strain used for fermentation is of great significance in determining the level of higher alcohols in beer. With other conditions constant, some brewery yeasts have been reported to produce five times as much higher alcohols as others. Laboratory and pilot plant fermentations, as well as full scale trials in different breweries, have demonstrated the importance of the yeast strain [for a review see Engan].[25] The formation of higher alcohols is also very dependent upon the fermentation temperature with an increase in temperature resulting in increased concentrations of higher alcohols in beer. It would appear that temperature changes have a more significant influence upon the formation of the aromatic alcohol 2-phenylethanol than on the aliphatic alcohols such as propanol, butanol and hexanol.

Esters

The esters constitute an important group amongst the beer volatiles due to their strong penetrating fruity-flavours. Most of the esters found in beer are formed during fermentation and their formation is linked to the lipid metabolism of the yeast (ester formation is also considered in the section on

high gravity brewing). The direct, enzyme-free formation of esters is an equilibrium reaction between an alcohol and an acid, and this reaction is a possible route to ester formation in beer. As wort and beer contain a large number of alcohols and acids, and they may all react to form esters, the theoretical number of esters in beer is large, however, direct formation of esters would be too slow to account for the concentration of some of the esters found in beer. It is now well documented [for review see MacDonald *et al.*],[63] that the formation of ethyl acetate during fermentation proceeds according to the following reaction:

$$CH_3COSCoA + C_2H_5OH \rightleftharpoons CH_3CO_2C_2H_5 + CoASH$$

(acetyl coenzyme A) + (ethanol) ⇌ (ethyl acetate) + (coenzyme A)

A number of factors have been found to influence the amount of esters formed during fermentation and these include yeast strain, fermentation temperature (where an increase in temperature from 10 to 25°C has been found to increase the concentration of ethyl acetate from 12·5 to 21·5 μg ml^{-1}), fermentation method (continuous fermentation appears to result in higher levels of esters than conventional batch fermentation), pitching rate (higher rates have been found to result in a marked reduction in the formation of ethyl acetate) and wort aeration (which seems to influence ester production in such a manner that low levels of oxygen may enhance ester formation).

Carbonyl compounds

Many carbonyls have a high flavour potential and have a significant influence on the flavour stability of beer thus making them an important group of beer volatiles. The carbonyl found in highest concentration in beer is acetaldehyde. It is formed during fermentation and is a metabolic branch point in the pathway leading from carbohydrate to ethanol. The acetaldehyde formed may either be reduced to ethanol or oxidised to acetic acid, and in the final step of alcoholic fermentation, acetaldehyde is reduced to ethanol by an enzymatic reaction. The concentration of acetaldehyde varies during fermentation and aging/conditioning and reaches a maximum during the main fermentation and then decreases. The question of the influence of yeast strain upon acetaldehyde formation is very much open to question but greater variations in acetaldehyde seem to occur from brewery to brewery than from yeast to yeast. An increase in fermentation temperature does not increase beer acetaldehyde levels but yeast pitching rate does have a significant influence with increasing yeast concentrations resulting in higher acetaldehyde levels.

Excess levels of acetaldehyde in beer can be the result of bacterial spoilage, especially by strains of *Zymomonas anaerobia*. High levels of acetaldehyde can also be caused by high air levels during fermentation, and acetaldehyde levels in bottled beer have been observed to increase during pasteurisation and storage, especially if there is a high air content in the bottle head-space.

Diacetyl and pentane-2,3-dione

Diacetyl and pentane-2,3-dione both impart a characteristic aroma and taste to beer; this is variously described as 'buttery', 'honey or toffee-like' or as 'butterscotch'. The flavour is detectable more readily in lager, where it is almost universally regarded as a defect, than in typical heavily hopped British ales (in the light Canadian ales, diacetyl is regarded as much a defect as in lager). The taste threshold concentration for diacetyl in lager is of the order of 0·1–0·14 μg ml^{-1} and is somewhat higher in ale. In recent years there has been a great deal of interest in the factors that influence the concentration of diacetyl in beer [the topic has been well reviewed by Wainwright].[142]

It is now accepted that diacetyl and pentane-2,3-dione are formed outside the yeast cell, by the oxidative decarboxylation of α-acetolactate and α-acetohydroxybutyrate, respectively. These α-acetohydroxy acids are intermediates in the biosynthesis of leucine and valine (acetolactate) and isoleucine (acetobutyrate) and are leaked into the wort by yeast during fermentation (Fig. 15). Once diacetyl and pentane-2,3-dione have been formed in the fermenting wort, they are normally converted to acetoin or pentane-2,3-diol respectively by the action of yeast reductases. Thus, the final concentration of diacetyl in beer is the net result of three separate steps: (i) synthesis and excretion of α-acetohydroxy

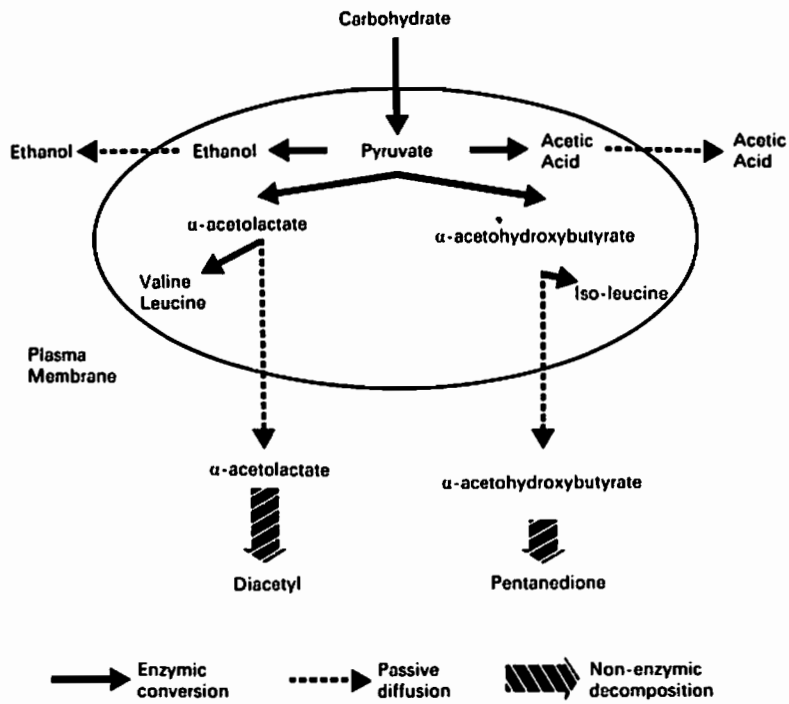


Fig. 15. Mechanisms of diacetyl and pentanedione formation by *Saccharomyces spp.*

acids by yeast, (ii) oxidative decarboxylation of α-acetohydroxy acids to their respective diketones, and (iii) reduction of diacetyl and pentane-2,3-dione by yeast.

The presence of diacetyl in beer at above threshold levels occurs when α-acetolactate has decomposed to give diacetyl at a time when the yeast cells are either absent or have lost their ability to reduce diacetyl to acetoin. Commonly the fault arises because α-acetolactate breakdown has been curtailed by the use of temperatures conducive to yeast settling when the potential to produce diacetyl remains. When the beer becomes warm, which is usually when it is packaged and pasteurised but may not be until the beer is disposed at the point of sale, diacetyl is produced and in the absence of yeast cannot be converted to acetoin and therefore accumulates. Diacetyl levels can thus be controlled by ensuring that there is sufficient active yeast in contact with the beer at the end of fermentation to reduce diacetyl to acetoin. Diacetyl formation from α-acetolactate has been shown to be dependent upon pH, the concentration of α-acetolactate, temperature, the presence of oxygen, the vigour of the fermentation, and certain metal ions. Vigorous fermentations produce more acetohydroxy acids but the decomposition of acetohydroxy acids to vicinal diketones is also more rapid. In addition, since diacetyl is formed earlier in the fermentation, there is more time for diacetyl removal by the yeast. The production of beer containing undesirable concentrations of diacetyl, as a result of yeast metabolism, should today be rare in normal brewing conditions. However, excessive levels of diacetyl can be the result of beer spoilage by certain bacteria such as *Pediococci* and *Lactobacilli*.

Enzymatic procedures have been reported, either for the rapid conversion of acetohydroxy acids to their respective diols or for the reduction of diacetyl. As the rate limiting step in the removal of the diacetyl precursor, α-acetolactate, from beer is its conversion to diacetyl, the use of diacetyl reductases is impractical where α-acetolactate converting enzymes might be employed. It has been found possible to reduce diacetyl to acceptable levels in 24 h at 10°C by 'shunting' α-acetolactate in maturing beer directly to acetoin with the aid of acetolactate decarboxylase obtained from a strain of *Enterobacter aerogenes*:

$$CH_3C(OH)(COCH_3)COSH \rightleftharpoons CH_3CH(OH)COCH_3 + CO_2$$

α-acetolactate⇌acetoin

This finding has important implications in brewing. Provided suitable microbial sources of acetolactate decarboxylase can be identified it may be possible to benefit from such enzymes in commercial brewing.

Attempts have also been made to obtain yeast mutants incapable of producing vicinal diketones. Such mutants have been isolated from haploid laboratory strains of *Saccharomyces cerevisiae* after treatment with ethyl methanesulfonate. The initial isolates produced excessive amounts of propanol but this was circumvented by using recombinants which lacked both acetolactate synthetase as well as threonine deaminase. Such mutants, from one of the haploid strains, have been reported to produce beers which resemble beers obtained with brewer's yeast. However, the industrial use of such mutants will involve improvement of their stability and fermentation characteristics, particularly those relating to the use of maltotriose.

Sulfur Compounds

The biological importance of sulfur has come to be recognised over the past 25 years. The sulfur cycle in nature is just as indispensable for the existence of life as are the carbon and nitrogen cycles. In recent years, considerable progress has been made in understanding the sulfur biochemistry of animals, plants and microorganisms but many facets of the subject are incomplete and obscure. From the standpoint of brewing, sulfur is of additional importance because traces of volatile sulfur compounds such as hydrogen sulfide, dimethyl sulfide, sulfur dioxide and thiols significantly contribute to the flavour of the beer. As early as 1890 it was shown that the stench occurring during primary fermentation of beer was due to the evolution of H_2S caused by the reduction of sulfur compounds in wort. Since that time numerous studies have been published concerning volatile compounds in beer.

Although small amounts of sulfur compounds may be acceptable or even desirable in beer, in excess they give rise to unpleasant off-flavours and special measures such as purging with CO_2 or prolonged maturation times are necessary to remove them. Although volatile organic sulfur compounds are contributed to the wort and beer by hops, adjuncts and malt, a significant proportion of those present in finished beer are formed during or after fermentation. During fermentation, yeasts usually excrete significant amounts of hydrogen sulfide and sulfur dioxide. Indeed, recent analytical studies have suggested that inorganic volatile compounds containing sulfur do not make as important a contribtution to beer flavour as was originally thought. Sulfur dioxide is usually present at a concentration below its taste threshold and normal beer, when free of infection, contains low levels of free H_2S. This latter compound is removed during processing and even beer described as 'sulphury' does not usually contain free H_2S. However, sulfur dioxide is important in beer as an antioxidant and as a complexer of carbonyl compounds; some of these latter compounds in their free state will generate a stale flavour in beer.[5]

Hydrogen sulfide.—Factors that influence the formation and final concentration of hydrogen sulfide in beer have been the subject of a large number of publications over the past 25 years. Nevertheless, the biochemical pathways and the metabolic control are not completely understood. Yeast strains requiring the B vitamin pantothenate for growth form sulfide from sulfite or sulfate if the wort is deficient in this vitamin. It has also been demonstrated that amino acids such as threonine and glycine when added to wort will stimulate H_2S production whereas methionine will retard it. It is therefore important to maintain a high level of methionine in the wort. As previously discussed, methionine is removed from the wort early in the fermentation leaving a relative excess of amino acids which promote H_2S production. Consequently, there is usually a delay in H_2S production early in the fermentation which corresponds to methionine utilisation before the maximum rate of H_2S evolution is observed.

A relationship has been noted between H_2S production during a wort fermentation and the bud index of the yeast cultures (the bud index being defined as: 'the percentage of budded cells of yeast in the suspension', that is 'the ratio of the number of daughter cells to the number of mother cells of yeast in suspension'; 100% budding index means that all yeast cells in suspension have buds). When the budding index was nearly equal to zero, the maximum appearance of H_2S was always observed, which means that the H_2S peaks appeared when almost all of the cells did not possess a bud. Further, when the bud index increased, the H_2S content always decreased, which means that the H_2S content decreased when the cells were budding.[74,133]

Dimethyl sulfide.—Dimethyl sulfide (DMS) is a volatile thioether which makes a significant contribution to the flavour and aroma of lager beers. It is the major organosulfur volatile of most beers and has received much attention in recent years. It is now fairly well established that most of the DMS present in beer originates from *S*-methylmethionine (SMM) which is synthesised during the germination of barley or from small peptides containing SMM. This component (SMM) breaks down on heating, such as occurs during malt

kilning or wort boiling, to give DMS. However, the free DMS is not inert and can be oxidised to dimethyl sulfoxide (DMSO) a heat-stable non-volatile compound, which may in turn be reduced back to DMS by yeast and bacteria. Hence DMS in beer arises from SMM either by chemical decomposition of SMM or via the metabolism of DMSO by microorganisms. In an attempt to establish conclusively that yeast does produce DMS from DMSO during fermentation of wort, experiments have been performed employing [^{14}C] DMSO in order to trace any possible reduction to DMS.[31] It has been found that during fermentation, yeast reduced [^{14}C] DMSO to yield radioactive DMS even under conditions in which a net formation of DMS could not be demonstrated by gas chromatographic analysis.

Low Carbohydrate (Reduced Calorie) Lite Beer

Low carbohydrate (usually low calorie) beer in North America represents a significant share of the beer volume; it constitutes 13% of the market of 22 million hectoliters per annum in the USA and 8% or 1·7 million hectoliters per annum in Canada.

Several procedures for producing low carbohydrate beers are being employed or contemplated and most of them center on one of the following techniques: (i) dilution of regular strength beer with water, (ii) addition of fungal α-amylase or glucoamylase and bacterial pullulanase to the wort during fermentation, (iii) use of glucose, fructose or sucrose as an adjunct, (iv) use of brewing yeast strains with amylolytic activity, and (v) use of a malt enzyme preparation during mashing or fermentation.

As previously discussed, typical brewing yeast strains are capable of utilising sucrose, glucose, fructose, maltose and maltotriose, leaving the maltotetraose and larger dextrins unfermented. However, yeasts of the species *Saccharomyces diastaticus*[6] have been classified as a distinct species from that of *Saccharomyces cerevisiae* due to the fact that the former produces an extracellular enzyme glucoamylase, also called amyloglucosidase (α-1,4 glucan glucohydrolase, E.C.3.2.1.3.). This enzyme possesses the ability to cleave α-1,4 and in some cases α-1,6 linkages, releasing glucose from the non-reducing end of starch chains. On the other hand, α-amylase (α-1,4-glucan 4-glucanohydrolase, E.C.3.2.1.1.) is an enzyme which hydrolyses random α-1,4 glucosidic linkages by-passing α-1,6 bonds. Three genes have been identified that are associated with glucoamylase production of *Saccharomyces diastaticus*, *DEX1*, *DEX2* and *STA3*.[28,134] Recently studies by Erratt and Nasim[27] have ascertained allelism between *DEX1* and *STA2* and *DEX2* and *STA1* and it was proposed that in the future the two nomenclatures be consolidated. Based on the fact that the glucoamylase from *Saccharomyces diastaticus* can hydrolyse both dextrin and starch, it was proposed to retain *STA* as the designation for genes coding for glucoamylase production by this yeast species. Using classical hybridisation techniques a diploid strain, containing the *DEX* and *STA* genes in the homozygous condition, has been constructed and its fermentation rate studied in brewer's wort under static fermentation conditions (Fig. 16). The initial fermentation rate of this strain was slower than a production ale brewing strain, however, the *DEX*-containing strain fermented the wort to a greater extent than the brewing strain due the partial hydrolysis of the dextrins by the action of glucoamylase. Thus, *Saccharomyces diastaticus* strains possess the capacity to produce beer which has been fermented to a high degree and is desirable in the production of low carbohydrate beer; however, it should be noted that beer produced by these strains has a characteristic phenolic-off-flavour. Phenolic-off-flavours in beer are due on may occasions to the presence of 4-vinyl guaiacol (4-VG) which arises by the enzymatic decarboxylation of ferulic acid, a wort constituent.[141] It has been found by Tubb *et al.*[137] that a single dominant nuclear gene designated *POF* (phenolic-off-flavour) codes for the ferulic acid decarboxylation enzyme. Therefore, strains possessing the *POF* gene can produce the enzyme capable of decarboxylating ferulic acid. Whereas, brewing *Saccharomyces* strains normally cannot decarboxylate ferulic acid, all the *Saccharomyces diastaticus* strains initially studied produced 4-VG in the presence of ferulic acid. Assuming that the *POF* and *DEX* genes are independent characteristics, it should be possible to construct a strain containing the *DEX* but not the *POF* gene by means of hybridisation. Thus, a haploid that was *DEX* positive and that carried the *POF* characteristic was mated with a dextrin negative phenolic-off-flavour negative (*pof*) haploid. The resultant diploid fermented dextrin and decarboxylated ferulic acid. When tetrad dissection was carried out a 2:2 segregation for dextrin fermentation and a 2:2 segregation for phenolic-off-flavour was obtained. The *DEX* and *POF* genes segregated independently of each other, allowing the selection of haploids that were *DEX* positive and *POF* negative. Subsequently, a diploid with the genotype *DEX2/DEX2*, *pof/pof* was constructed and an 11·3° Plato wort fermentation was conducted (Fig. 16). Although the initial wort attenuation rate was found to be slower than that of a polyploid ale yeast strain, the yeast was capable of super-attenuating the wort, i.e., it was able to hydrolyse part of the dextrin, into glucose which is readily fermentable, whereas the brewing strain was unable to utilise the dextrins. 'Expert' taste panel assessment has deemed the beer produced from this dextrin positive diploid to be rather winey and to have a slightly sulphury character, however, the characteristic phenolic-off-flavour associated with the *POF* gene (4-VG) could not be detected.

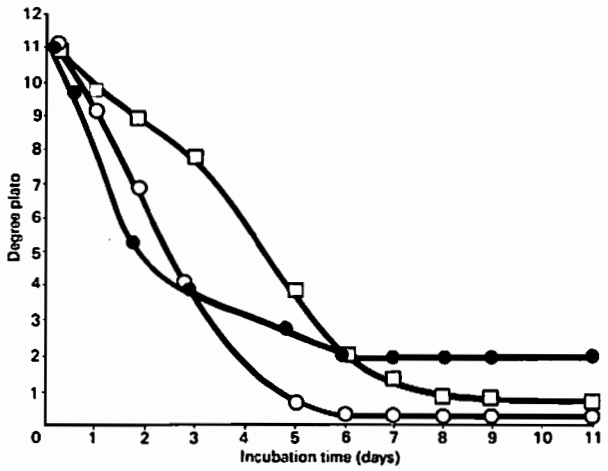


Fig. 16. Fermentation of an 11·3° Plato wort on a 40 litre scale by an ale brewing strain, *pof/pof* (●——●), a *DEX1/DEX1, DEX2/DEX2, STA3/STA3, POF/POF* diploid (○——○), and a *DEX2/DEX2, pof/pof* diploid (□——□).

The production of glucoamylase by strains of *Saccharomyces diastaticus* is subject to carbon catabolite repression by glucose and other sugars.[112] Subjecting two such strains to the 2-DOG selection technique, stable spontaneous mutants derepressed for the formation of glucoamylase and mutants derepressed for maltose utilisation have been isolated. In 12°P wort with a 2-DOG starch mutant of *Saccharomycces diastaticus* (strain 1393), the level of glucoamylase was increased five-fold when compared to the parental strain (Fig. 17). The overall fermentation rates of the starch and maltose mutants were increased when compared to the parental strain (Fig. 18). A second strain of *Saccharomycces diastaticus* (strain 1400) gave similar results to strain 1393. In 12°P wort, the 2-DOG maltose mutants of this strain possessed increased maltose uptake rates together with an overall faster fermentation rate when compared to the parental strain (Fig. 19).

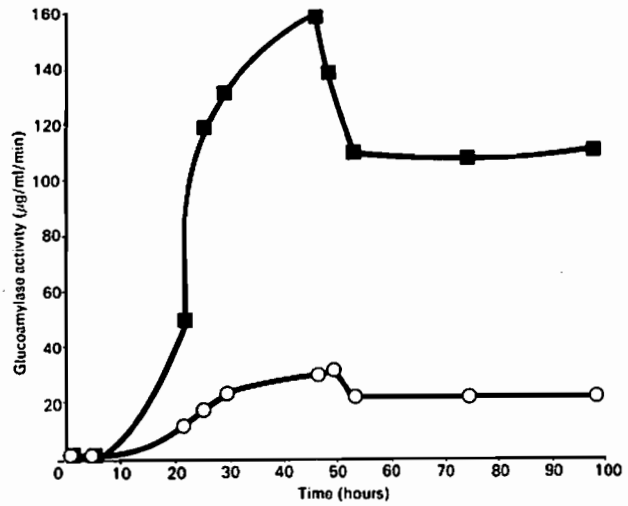


Fig. 17. Glucoamylase production in a 12° Plato wort by parent strain 1393 (○——○) and a derepressed starch mutant of strain 1393 (■——■).

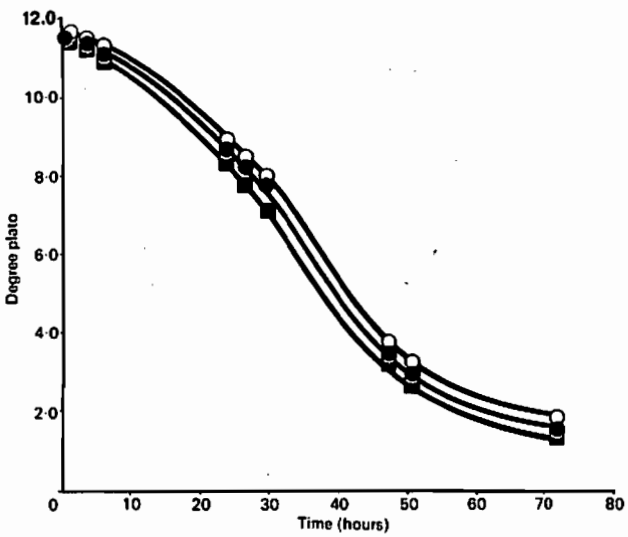


Fig. 18. Fermentation of a 12° Plato wort by parent strain 1393 (○——○), its derepressed maltose mutant (●——●) and its derepressed starch mutant (■——■).

A further problem with the use of strains of *Saccharomyces diastaticus* (*POFor pof*) for the production of beer is the fact that the extracellular glucoamylase is heat stable and remains active in the packaged beer after pasteurisation. As a consequence, upon storage at 21°C, an increasing concentration of glucose can be found in the beer.[29]

A number of laboratories are committed to achieving the objective of obtaining a strain of *Saccharomyces sp.*, that possesses the ability to hydrolyse starch entirely, i.e., able to synthesise and secrete α-amylase and glucoamylase with debranching ability.[111] *Saccharomyces diastaticus* produces a thermostable glucoamylase and no traces of α-amylase or debranching ability can be detected (Table 3). As starch is a polysaccharide composed of two polymers, 20–25% in the form of amylase (liner chains of α-1,4 linked glucose residues) and 75–80% in the form of amylopectin (a highly branched polymer occurring by α-1,6 linkages) debranching activty is essential for complete hydrolysis of the polysaccharide. Two *Endomycopsis fibuligera* strains studied were found to possess α-amylase and glucoamylase activity but no debranching activity (Table 3). A strain of *Pichia burtonii* produced very low levels of debranching activity; however, the yeasts *Schwanniomyces castellii* and *Schwanniomyces occidentalis* produced significant amounts of α-amylase, glucoamylase and debranching activity. These amylolytic systems have been isolated, purified and characterised and it has been found that *Schwanniomyces castellii* possesses a glucoamylase with debranching activity.[113]

TABLE III. Production of Amylolytic Enzymes by Several Yeast Species

Yeast species	Enzymatic activity		
	α-Amylase	Glucoamylase	Debranching
Saccharomyces diastaticus	-	+++	-
Endomycopsis fibuligera	+	+++	-
Endomycopsis fibuligera	++	+++	-
Pichia burtonii	++	++	±
Schwanniomyces castellii	++	+++	+++
Schwanniomyces occidentalis	++	++	++

+++ high activity
++ medium activity
+ low activity
- no activity.

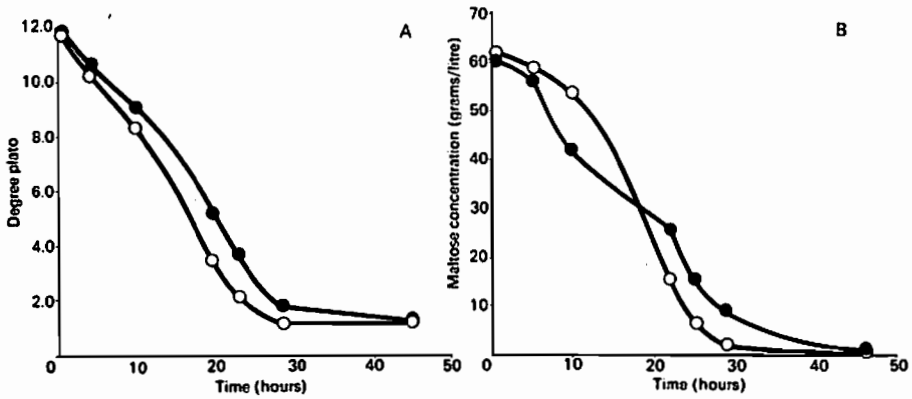


Fig. 19. A. Fermentation of a 12° Plato wort by *Saccharomyces diastaticus* strain 1400 (●——●) and its derepressed mutant (○——○).
B. Maltose uptake in a 12° Plato wort by *Saccharomyces diastaticus* strain 1400 (●——●) and its derepressed mutant (○——○).

It has already been discussed in this paper that one of the techniques for producing low carbohydrate (low calorie) 'Lite' beers is to add fungal amylases to the wort during fermentation. Since 70–75% of the dextrins in wort are of the branched type, a debranching enzyme is essential for total hydrolysis of wort dextrins to fermentable sugars. The fungal glucoamylase used extensively in the production of light beer possesses debranching activity, therefore, it can hydrolise the dextrins. However, normal pasteurisation of the final product employing a conventional temperature/time cycle, does not completely inactivate this enzyme. This is a major impediment as the presence of active glucoamylase in the finished product means that the beer is likely to become sweeter due to the hydrolysis of residual dextrin to glucose, changing the beer's flavour characteristics and introducing a possible microbiological hazard. An important characteristic of amylases from *Schwanniomyces castellii* is their sensitivity to the normal pasteurisation cycle employed in brewing. A representative curve of pasteurisation temperature versus time indicates that there is an eight minute period during which the temperature is maintained at 60–62°C. It has been reported that 15 minutes at 60°C is the time required to inactivate the *Schwanniomyces castellii* glucoamylase, however, this study was conducted at pH 5·5 (this enzyme's optimal pH and in the absence of ethanol.[109] When a commercial fungal glucoamylase preparation derived from *Aspergillus niger* and the glucoamylase from *Schwanniomyces castellii* were compared for their sensitivity to pasteurisation at pH 4·0 (normal beer pH) and pH 6·0 in the presence and absence of ethanol (Fig. 20), the ethanol enhanced the inactivation effect of the pasteurisation. In addition, at pH 4·0, the pasteurisation cycle inactivated glucoamylase (as well as α-amylase—data not shown) from *Schwanniomyces castellii* with or without ethanol, but at pH 6·0 the presence of ethanol was necessary for enzyme inactivation.

A two stage fermentation system has been devised for the production of low carbohydrate beer.[110] Amylases (gluco- and α-amylase) from *Schwanniomyces castellii* are produced in a highly inducing medium containing maltose. Subsequently, the cells are removed, the culture filtrate is concentrated and added to wort previously inoculated with a genetically manipulated strain of *Saccharomyces diastaticus* or with a brewing production strain of *Saccharomyces uvarum (carlsbergensis)*. The *Saccharomyces diastaticus* strain is a diploid containing both the *DEX1* and *DEX2* genes which code for glucoamylase production and, more importantly, this strain lacks the capability to decarboxylate ferulic acid to 4-vinyl guaiacol.

In an attempt to establish the optimal amount of enzyme culture filtrate to be added to the fermenting wort, several concentrations of enzymes were added (Table 4). As expected, increasing concentrations of enzymes had a direct correlation with apparent attenuation, i.e., with the maximum amounts of enzymes added, the degree of fermentation

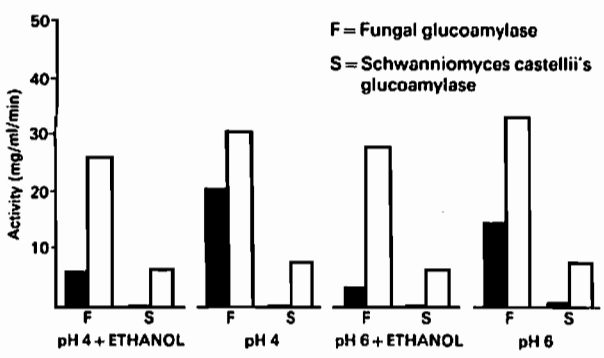


Fig. 20. Effect of pasteurisation on the enzymatic activity of a commercial fungal glucoamylase (F) and a glucoamylase from *Schwanniomyces castellii* (S) with (■——■) and without (□——□) pasteurisation.

TABLE IV. Effect of Increasing Concentrations of Amylolytic Enzymes on Fermentation Characteristics of Worts

Amount of enzyme added (mg/litre)	*Sacch. uvarum (carlsbergensis)*		*Sacch. diastaticus*	
	Final ethanol % (w/v)	Apparent attenuation %	Final ethanol % (w/v)	Apparent attenuation %
0	4·20	84·7	4·30	89·5
4	4·42	88·8	4·37	89·8
8	4·49	93·6	4·40	90·6
12	4·76	95·2	4·55	94·5
16	4·90	99·4	4·69	96·9
20	5·00	100·0	4·95	100·0

16° Plato original wort gravity.

increased to 100% and up to 99·4% with 70% less added enzyme. Fermentations conducted with the diploid *Saccharomyces diastaticus* strain were more sluggish because this particular strain lacked *MAL* genes, therefore, maltose was only hydrolised by the extracellular glucoamylase and a longer time of fermentation was required to achieve 100% of apparent attentuation.

A direct correlation has been found between ethanol production and enzyme concentration employed, i.e., the greater the enzyme concentration, the higher the level of ethanol obtained. Thus, with fermentations employing the lager yeast strain, a maximum 19% increase of ethanol (compared to the control) could be obtained in the final product, whereas with *Saccharomyces diastaticus*, an improvement of 15·1% could be obtained with the maximal enzyme addition (Table 4).

Diastatic Yeasts for Distilled Ethanol Production

The fermentation of starches to ethanol by yeasts conventionally requires pretreatment of the substrate in order to produce fermentable sugars. This pretreatment consists of three steps: gelatinisation, liquefaction and saccharification. Gelatinisation requires heat and free water and must precede liquefaction. Liquefaction, the dispersion of starch molecules into an aqueous solution, is accomplished by the use of heat and amylolytic enzymes. Heat stable bacterial α-amylases or malt enzymes may be employed. During liquefaction starch molecules are only partially hydrolysed producing a form of carbohydrate which cannot be assimilated by ethanol-producing yeasts such as *Saccharomyces cerevisiae*. Therefore, the partially hydrolysed starch molecules must be converted to lower molecular weight sugars such as glucose and maltose by a process known as saccharification. This may be accomplished enzymatically, usually by the addition of fungal glucoamylases to the fermentation vessel at the time of inoculation. The saccharifying glucoamylases represent a significant fraction of the total cost of producing ethanol. Reduction of the amount of added glucoamylase could significantly decrease the cost of the final product.

It has been found possible to decrease glucoamylase addition to starch mash fermentations by employing yeast which actively produce and secrete glucoamylase, e.g., strains of *Saccharomyces diastaticus*.[96,144] A genetically manipulated diploid strain of *Saccharomyces diastaticus* (strain 1393, *DEX1/DEX1, DEX2/DEX2, STA3/STA3, MAL6/mal6*) was studied as the glucoamylase producing strain and compared to a strain of *Saccharomyces cerevisiae* (strain 254) which was unable to produce and secrete glucoamylase. The fermentation performance of the two strains was compared in a corn mash with and without added glucoamylase (AMG). When the *Saccharomyces diastaticus* (strain 1393) culture was employed, added glucoamylase concentrations could be significantly decreased without reducing ethanol production or sugar uptake (Fig.

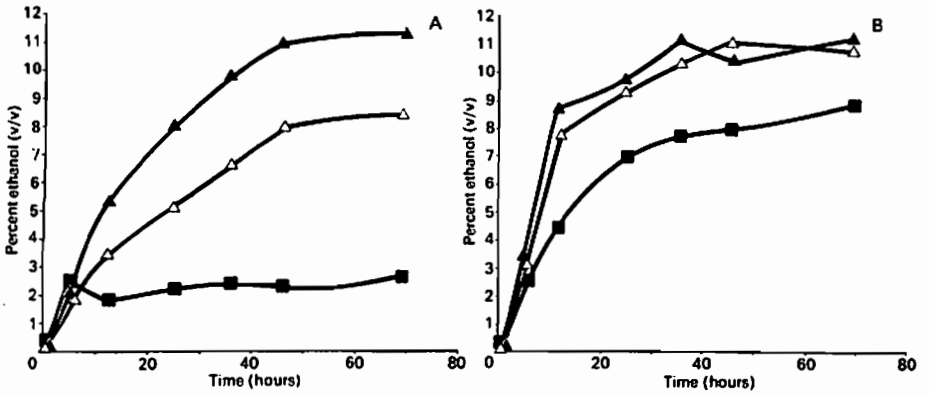


Fig. 21. A. Ethanol production from a 21° Plato corn mash by fuel ethanol strain 254: (■——■) 0·0% (v/w) AMG, (△——△) 0·05% (v/w) AMG, (▲——▲) 0·1% (v/w) AMG.
B. Ethanol production from a 21° Plato corn mash by hybrid strain 1393: (■——■) 0·0% (v/w) AMG (△——△) 0·05% (v/w) AMG, (▲——▲) 0·1% (v/w) AMG.

21). Reduction of the added glucoamylase concentration from 0·1% by volume, based upon the substrate weight, to 0·05% resulted in no significant decrease in ethanol yield or sugar uptake; the reduction of added glucoamylase being possible because the hybrid yeast is able to produce and secrete its own glucoamylase.

Production of glucoamylase by the *Saccharomyces diastaticus* strain and subsequent reduction of added glucoamylase represents a potential financial saving for the ethanol producer. Since the cost of glucoamylase may exceed $3.75 (US) per liter, a 50% reduction of this enzyme can result in a significant decrease in cost. For example, an ethanol manufacturer that is producing 100 million liters of 95% ethanol per year uses 190 million kg of starch (assuming that the fermentation of 20% (w/v) starch yields 10% (v/v) ethanol). For 190 million kg of starch, 0·19 million litres of glucoamylase are required (based upon an addition of 0·1 litres of glucoamylase per 100 kg of starch). This results in an annual enzyme cost of approximately $700,000 US assuming an enzyme cost of $3.75 US per litre. Reduction of the added glucoamylase by 50% represents an annual saving of approximately $350,000 US.

Immobilised Yeast in Brewery Fermentations

In traditional brewery batch fermentations, two metabolic states in which the yeast cell exists can be delineated, namely, the growth phase in which the specific fermentation rate for each of the fermentable wort sugars reaches a maximum and the longer stationary phase where growth is terminated, fermentative power progressively declines, and maintenance activities take precedence.[106] For synthesis of its cellular material, a growing yeast cell employs intermediates of the catabolic glycolytic process as precursors for anabolic synthetic reactions to polysaccharides, proteins, lipids and nucleic acids. Hence, for the production of primary and secondary products of yeast metabolism that define the alcoholic strength and flavour-active quality of beer, comprehensive understanding of growth-regulated activities would be useful. This understanding becomes more important when fermentation systems are considered with the potential for higher productivities than those of the traditional batch system but where yeast growth is limited. It is considered by some[105] that fermentation systems of this nature would be attractive not only as a result of improved volumetric efficiency but also because more substrate is channelled to the product and less to the production of unnecessary biomass.

Immobilised cell technology provides an opportunity to achieve such a fermentation system as that outlined above. Indeed, during recent years, immobilisation of microbial cells by 'active' entrapment within natural polymers or 'passive' adsorption to solid supports has become a rapidly expanding research area.[60,64] Although this is still a novel research area, conclusions to date consider that cell entrapment provides higher reactor productivity and protection to cells when compared to adsorption systems and is thus tending to be a more widely applied immobilisation method. Additional advantages to the employment of immobilised cell systems, rather than free cell systems are: (i) a higher cell mass per unit fermenter volume can be achieved, resulting in potentially faster process times, thus emphasising greater volumetric capacity; (ii) for batch systems, immobilised cells need not be removed at the end of fermentation; the process is reduced to racking the product and refilling the fermenter (reactor) with substrate (wort); thus, complicated microbial handling systems are avoided; (iii) for continuous systems, ease of operational control facilities for process optimisation for yield improvement; (iv) smaller fermenter volumes may be envisaged, hence lower capital costs; and (v) for brewing strains, flocculation properties need not be taken into consideration.

Immobilisation of yeast cells in gels for application in the brewing process have been described.[33,59,75,143] By entrapping brewer's yeast cells in a calcium alginate gel, a continuous wort fermentation in a packed bed column could be achieved[105] yielding a beer with a composition and flavour profile similar to that in a batch fermentation. Onaka *et al.*[79] have reported the development of a novel fermentation system, containing an immobilised yeast reactor, for the rapid production of palatable beer with a low content of diacetyl and diacetyl precursors; the yeast, a strain of *Saccharomyces uvarum (carlsbergensis)* was immobilised in calcium alginate gel beads. When the wort was fed directly into the immobilised yeast reactor, the beer had a higher than acceptable α-amino nitrogen and also possessed an 'off-flavour'. Consequently, in order to produce beer with the requisite α-amino nitrogen content and acceptable flavour characteristics, wort was first fermented for a short period with agitation and aeration before the wort was fed into the immobilised yeast reactor. The initial beer produced in this manner contained an unacceptable level of diacetyl, however, upon reducing the dissolved oxygen content of the fermenting wort from 8·5 mg l^{-1} to 0·04 mg l^{-1} when it was pumped into the base of the reactor containing the immobilised yeast culture, total diacetyl was reduced from 2·25 mg l^{-1} to an acceptable level of 0·05 mg l^{-1}.

Processes employing immobilised yeast cells in brewing are very much in their infancy and still at an experimental pilot stage. Nevertheless, it is a system that augers well for the future and even if a production process does not result, a significant volume of fundamental information regarding brewing fermentation processes will have been obtained. Although immobilised yeast systems can be employed in

batch and continuous fermentation processes, a continuous process would be the most logical for a number of reasons. However, continuous fermentation employing immobilised yeast cells will suffer many, if not all, of the problems and impediments of those outlined for more traditional continuous processes in an earlier section of this document.

Genetic Engineering

The various techniques that are currently available to the geneticist to manipulate brewing yeast strains have already been listed in this manuscript and the potential of these techniques to introduce a variety of novel characteristics into yeast has been discussed. Unlike the other techniques, genetic engineering (recombinant DNA) affords the possibility of cloning a specific genetic character, thereby eliminating the possibility of introducing additional factors of a detrimental nature. In addition, the methods of genetic engineering allow for the transfer of genetic information between completely unrelated organisms so that the recipient organism becomes able to produce heterologous proteins and peptides, that is to say, proteins and peptides which are not produced by their mated genetic constituents, thereby providing considerable scope for transfer of new characteristics into brewing and other industrial yeast strains.

The literature concerning transformation (that is the introduction of DNA into yeast) and recombinant DNA strategies in yeast is extensive and varied. It impinges on every aspect of molecular biology and genetics. Boguslawski[11] provides an excellent introduction to the organised body of research concerned with general principles, methods, vectors, artificial chromosomes, gene isolation and characterisation, as well as, the expression of heterologous genes in yeast. The power of these approaches is highly sophisticated though still at a developmental phase close to infancy.

Genetic transformation is the change in the genetic organisation of an organisms by the introduction of purified DNA. In *Saccharomyces* transformation can be carried out with any one of four different types of DNA.[55] First, circular DNA molecules can be employed that have been constructed *in vitro* by combining a selectable yeast gene with a bacterial plasmid. The second type of DNA is like the first, except that the yeast DNA portion includes a region that confers autonomous replication to the molecule in yeast. In both cases, the transformation will, in addition to the yeast gene(s) introduced, contain genes from foreign species. The third type of DNA also represents circular *in vitro* constructed plasmids, but only yeast DNA has been involved and the DNA has been through no foreign organism.[54,80] The fourth type is total native yeast DNA.[102] The last two types of DNA are more appealing for use in breeding of an organism to be employed for beverage production than the first two. It should be mentioned, however, that it is now possible to remove all foreign DNA sequences from a yeast that has been transfomed with the first type of DNA, leaving behind just the yeast gene that was introduced.[108]

Yeast transformation can be a relatively inefficient process with success depending very largely upon a suitable selection system and suitable recipient strains. Most plasmids currently in use for yeast transformation are selectable because they carry a wild-type gene which complements an auxotrophic mutation (e.g., a requirement for a particular amino acid) in a chosen recipient strain which has usually been a laboratory strain of *Saccharomyces cerevisiae*. However, it has previously been discussed in this review article that Brewer's yeast are prototrophic and have no auxotrophic requirements. Consequently, to select transformants in brewer's yeast strains, it is usually necessary to employ a dominant gene conferring the ability to grow in otherwise adverse conditions. The ability of yeast to resist the metal ion copper is conferred by a dominant yeast gene (*CUP1*), specifying the production of a protein capable of chelating copper *ions*.[30,39] This copper resistance gene has been employed by Meaden and Tubb[66] as a selectable marker for introducing recombinant plasmid DNA into brewing yeast strains. In addition, a *CUP1* plasmid, constructed to include a yeast *DEX* gene, resulted in copper-resistant transformants which produced extracellular glucoamylase and were capable of hydrolising wort dextrins. Hinchliffe and Box[40] and Cantwell *et al.*[16] have employed the copper resistance marker as an aid to the introduction of the *Bacillus subtilis* β-glucanase into yeast. Molzahn[70] has also used the copper resistance marker but to assist in the introduction of genes that code for the production of heterologous proteins of potential commercial value such as β-lactamase and human serum albumin into industrial (including brewing) yeast strains. The genetically modified yeast strain can initially be primarily employed in a process such as the production of ethanol (potable or industrial). The yeast that will inevitably result as a by-product of the process would have improved potential value because of the heterologous protein or peptide which is contains. However, because it is critical that this protein does not occur in the beer or other alcoholic beverage) it must be retained intracellularly, consequently the down-stream processing that would be necessary to isolate and purify such material will be time-consuming and costly. Neverthless, a new dimension has been introduced into the disposal of spent brewer's yeast.

The potential for the genetic engineering of brewer's yeast and other industrial yeast strains is significant and many possibilities could be cited. However, in the short term, the improvement of the substrate specificity of such strains is probably the most commercially viable and attractive development. Some of the recent and ongoing research in this area has already been discussed in this document. A further interesting research project is the construction of strains of *Saccharomyces* that grow on lactose.[115] The lactose in whey, the by-product of cheese-making, represents a potential substrate for the production of ethanol and the growth of microorganisms. However, this potential has not been realised on a large, economically feasible scale. As a result, a great deal of whey must be disposed of through costly effluent treatment systems. Although some yeast species such as *Kluyveromyces fragilis* and *lactis* can metabolise lactose, their ethanol production capability is somewhat limited. Consequently, a strain of *Saccharomyces cerevisiae* capable of metabolising lactose to ethanol would present significant economic opportunities. Attempts to fuse together a strain of *Kluyveromyces lactis* with that of *Saccharomyces cerevisiae* were unsuccessful.[118] However, with the use of recombinant DNA techniques and DNA from a strain of *Kluyveromyces lactis* a *Saccharomyces cerevisiae* transformant, capable of growth on lactose, has been isolated.[115]

Epilogue

It is to be hoped that the preceding sections of this review article have illustrated the significant developments that have occurred in the research and development of brewer's yeast over the past century. These developments have supplied a volume of information that has enriched fundamental information on yeast as a model eucaryote. This research on yeast as a fundamental eucaryote has also greatly assisted research on brewing and other industrial yeast strains. This synergy between fundamental fact-finding research and applied yeast research (of both a basic and developmental nature) will intensify during the next few years and symposia with an objective of bringing together scientists working in these two areas of research will complement and assist in this endeavour. Predicting future developments in this, or any other area of research, is a difficult, if not foolish, pastime. Nevertheless, based upon ongoing trends, it is possible to make an educated guess at what can be expected in this area.

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-glucanase, protease, β-glucosidase production, pentose and lactose utilisation, carbon catabolite derepression (higher productivity) and production of a plethora of intracellular heterologous proteins (value added spent yeast). 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.

Higher gravity worts will be employed, illustrated by the findings of Casey and Ingledew,[17] who have reported satisfactory fermentations employing 28°P worts. However, the ethanol tolerance of yeast recycled in such a medium will be critical and there is no doubt that a greater understanding of both the physiology and molecular biology of this complex phenomenon will be involved. Indeed, the whole question of environmental stress upon brewing, other industrial yeast strains and microorganisms in general, is an area of research currently requiring attention[114] and it is anticipated that this activity will intensify. Another area of yeast research that will receive considerable attention concerns the influence of yeast and fermentation conditions on beer stability. For example, the influence of esters and aldehydes, excreted by yeast, upon overall beer flavour and flavour stability and the role of extracellular lipids upon foam and physical stability.

In concluding this review, one must return to the first reference cited.[92] Rainbow concluded the following '*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 . . .*'. This statement is still true today as it was a decade ago!

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