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# Progress in the Development of New Barley, Hop and Yeast Variants for Malting and Brewing

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## Introduction

This review reports recent genetical developments related to humankind's oldest biotechnology, the brewing of beer. It will, hopefully, become evident to the reader that, despite the longevity of the processes, malting and brewing are anything but stagnant. The review focuses on progress in the development of new and improved barleys, hops and yeasts. In particular, it emphasizes the potential for modern gene technology in these improvements. Readers with no fundamental knowledge of the malting and brewing processes are referred to Hough (1985) for a useful overview. The simplest outlines of the processes involved are given by Bamforth (1993).

## Barley and malt

Barley remains the principal cereal crop, employed worldwide for the production of beer (Bamforth and Barclay, 1993). The vast majority of the barley is prior-malted, in order to mobilize the starch reserves of the endosperm for ready hydrolysis in mashing.

Barley varieties differ in their propensity to 'modify' during the germination phase of malting, viz. to have their cell walls and proteins enzymically hydrolysed, thereby releasing starch. The more readily modified cultivars (so-called 'malting' varieties) are progressively turned over commercially as newer, improved lines emerge through conventional breeding programmes (Sage, 1992). Since the advent of Plant Variety Rights in 1964, there has been a notable surge in the numbers of malting varieties coming to the marketplace. Whereas once varieties might have had a lifetime of 100 years, they may now become outclassed in 4–6 years (Smeaton, 1990).

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Abbreviations: ABA, abscisic acid; ABRE, ABA response element; ALDC, acetolactate decarboxylase; GA, gibberellic acid; GARE, GA-responsive element; mRNA, messenger RNA; rDNA, recombinant DNA; RFLP, restriction fragment length polymorphism.

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Breeding of barleys by traditional techniques is, however, a protracted and imprecise affair, taking perhaps 10–15 years, from the initial cross until a malting variety is fully accepted for commercial use in brewing. Considerable interest exists in identifying rapid methods, applicable to strictly limited quantities of grain, which can be used to select promising malting quality lines very early in a breeding operation (Home and Elamo, 1993). It is clearly advisable to have assessment protocols at all filial stages, which ensure that the barley is not only going to satisfy the farmer, in terms of its agronomic performance, but also the maltster and, ultimately, brewer in terms of the quality (and quantity) of malt which it will yield.

The techniques involved in conventional plant breeding are comprehensively explained elsewhere (Smith, Austin and Haslemore, 1986). Increasing attention, however, is being paid to newer opportunities for more selectively developing barleys with defined and specific characteristics, through modern techniques of genetic modification (Holm *et al.*, 1992). The rate-limiting step in such operations for monocotyledonous plants is the ability to regenerate plants from protoplasts. In the case of barley, this is compounded by an imperfect understanding of the molecular mechanisms which control gene expression. It is useful to address the latter issue first.

## THE CONTROL OF GENE EXPRESSION IN BARLEY

The expression of genes in barley seems largely to involve the hormones gibberellin and abscisic acid (ABA). There is a diversity of gibberellins, but as the majority of studies have involved gibberellic acid (GA), it is convenient to concentrate on this molecule.

The levels of GA peak during the development of seeds (Slominski, Rgowski and Nowak, 1979) whereas ABA peaks later, around the onset of seed desiccation (King, 1976). ABA or osmotic stress induces expression of the so-called *rab* (*r*esponsive to *AB*A) genes (Mundy and Chua, 1988). These genes are valuable models for the investigation of ABA-regulated gene expression (Mundy, Yamaguchi-Shinozaki and Chua, 1990). Such studies, in common with others reported later in this section, involved experiments with so-called transient gene expression in which exogenous DNA remains in a non-integrated form in the host nucleoplasm but can, nonetheless, be expressed for short periods in a manner equivalent to that of integrated genes.

ABA may have a role in regulating dormancy (Fong, Smith and Koehler, 1983). Non-dormant mutants, which lack ABA or are unable to respond to it, do not desiccate properly, and neither do they accumulate *rab* gene products (Koornneef, 1986). The RAB proteins, translated from *rab* genes, peak later in seed formation, but disappear early in germination (Close, Kortt and Chandler, 1989; Mundy and Chua, 1988). The precise function of the RAB proteins is unknown.

Early in germination, the scutellum synthesizes hydrolytic enzymes, although the aleurone soon adopts a priority role in this regard (Mundy and Munck, 1985). Detailed investigations in several laboratories have revealed

something of the influence of ABA and GA on the synthesis of enzymes in germinating barley (*Table 1*).

GA is known to trigger the accumulation of messenger RNA molecules coding for hydrolases critical to endosperm conversion, for example α-amylase (Higgins, Jacobsen and Zwar, 1982), β-glucanase (Mundy and Fincher, 1986) and endo-peptidases (Rogers, Dean and Heck, 1985). GA, or a factor dependent on GA, activates transcription of the principle α-amylase isozyme of barley (Jacobsen and Beach, 1985). Skriver *et al.* (1991) delineated both a *GA*-*r*esponsive *e*lement (GARE) in the α-amylase promoter and an *ABA* *r*esponsive *e*lement (ABRE) in the promoter of a *rab* gene. They showed that ABA represses expression from the GARE. These elements inserted into other promoters directed strong, hormone-responsive expression of genes, showing that they could be used as regulatory cassettes for 'plugging into' any gene. The α-amylase gene promoter is particularly relevant in the context of genes, the expression of which is only desired during malting and not in raw barley.

It appears that sequence motifs in the 3′ untranslated tails of GA-responsive genes have a key role in the stability and processing of mRNA in barley (Khursheed and Rogers, 1989). Secretion of hydrolases in germinating barley is dictated by calcium (Deikman and Jones, 1986).

As can be seen in *Table 1*, ABA antagonizes GA in the expression of genes (Mozer, 1980). ABA, for instance, induces factors capable of either inhibiting the transcription of α-amylase genes or destabilizing the mRNA encoding α-amylase (Nolan and Ho, 1988).

## GENETIC TRANSFORMATION OF BARLEY

The production of transgenic plants, originally tobacco in the early 1980s, was facilitated by the use of the soil pathogen *Agrobacterium tumefaciens*, which is capable of transferring genes into the plant genome. While dicotyledonous plants are readily transformed by vector mediation, such transformation of monocotyledonous plants has proved much more problematic. It seems that differentiated cells of cereals have a poor capacity to dedifferentiate and become embryogenic, viz to eventually reform a plant (Potrykus, 1989). Furthermore, exogenous DNA introduced via *Agrobacterium* may not integrate into the host genome (Grimsley *et al.*, 1987). Accordingly, a range of

**Table 1.** Hormonal regulation of proteins in barley seeds

| Gene | Effect of GA | Effect of ABA | Reference |
|---|---|---|---|
| β(1→3)(1→4)-glucanase | +++ | – | Mundy and Fincher (1986) |
| Thiol protease | +++ | – | Rogers, Dean and Heck (1985) |
| Carboxypeptidase | + | – | Hammerton and Ho (1986) |
| α-amylase 1 | +++ | – | Higgins, Jacobsen and Zwar (1982) |
| α-amylase 2 | +++ | – | Higgins, Jacobsen and Zwar (1982) |
| α-amylase inhibitor | – | + | Mundy *et al.* (1986) |

+, Increased expression; –, decreased expression.

direct gene transfer techniques has been explored as an alternative (*Table 2*). However, there is no evidence for stable transformation from any of these techniques.

The most favoured technique seems to be that of biolistic attack on embryogenic tissues. However, it does appear that there is an associated problem with mutations emerging through the regeneration phase, causing albinism, growth retardation and sterility.

The successful regeneration of barley protoplasts has been reported (Lazzeri and Lorz, 1988) and it has recently been claimed that green barley plants containing an integrated gene for β-glucanase for *Trichoderma reesei* have been produced (Mannonen *et al.*, 1993).

## TARGETS FOR GENETIC TRANSFORMATION OF BARLEY

Successful application of modern gene technology to barley will facilitate a broad range of benefits (*Table 3*). Improvements may lie in manipulation of genes native to barley or by introducing exogenous genes from diverse sources. *Table 3* suggests that progress is limited. This certainly seems to be the case from a survey of the published literature, but there is little doubt that significant progress is being made in various locations, although this activity is shrouded in commercial secrecy. It should be realized that few brewers can have the same direct influence on barley genetic manipulation as they can, for example, on modification of yeast. All brewers have their own yeast strains which, subject to acquiring the necessary legislative permission, they are free to modify as they require. Relatively few brewers produce their own malt, and even fewer are vertically integrated to the extent of growing and, ultimately, breeding their own barleys. Thus, a myriad of commercial decision points divide breeders, with their ability to consider genetical modification of barley, and end-users, that is maltsters and brewers. It is important, then, to have mechanisms to bring the various parties into one place (for example, the Institute of Brewing Barley Committee; see Smeaton, 1990). In the future, one might envisage much more direct control by end-users over raw material supply, for example, contract growing.

**Table 2.** Alternative strategies for introducing exogenous DNA into plants

| Method | Reference |
|---|---|
| Injection of DNA into young floral tillers | de la Pena, Lorz and Schell (1987) |
| Transfer of DNA through the pollen tube into the egg | Luo and Wu (1988) |
| Imbibition of DNA by desiccated embryos | Topfer *et al.* (1989) |
| Microinjection of DNA | Neuhaus *et al.* (1987) |
| Electroporation of DNA | Lindsey and Jones (1987) |
| Bombardment with tungsten/gold particles coated with DNA ('biolistics') | McCabe *et al.* (1988) |

**Table 3.** Goals for genetic modification of malting barley

| Goal | Rationale | Status | Reference |
|---|---|---|---|
| Disease resistance | Avoidance of use of pesticides ('clean labelling') | Current strategy is growth of mixtures of barley varieties, differing in disease resistance. | Wolfe, Bennett and Jenkins (1981) |
| | | Report of transfer of gene for powdery mildew resistance from *H. bulbosum* to *H. vulgare* | Xu and Kasha (1992) |
| Improved yield | Decreased use of fertilizers – reduced nitrogen enabling increased starch | No published reference | |
| Reduced fibre | Fewer processing problems with non-starchy polysaccharides | Low β-glucan barley | Aastrup, Erdal and Munck (1985) Molina-Cano *et al.* (1989) |
| High fibre-conversion potential | Increased enzyme levels to deal with non-starchy polysaccharides | Claim for integration of heat-stable β-glucanase | Mannonen *et al.* (1993) |
| High starch-conversion potential | Increased yield of fermentable sugar per unit weight of barley: enhance limit dextrinase and/or optimize amylose/amylopectin ratios | No published reference | |
| High foam potential | Quality improvements in beer | No published reference | |
| Low haze potential | Quality improvements in beer | Low anthocyanogen barley – improvements needed to agronomic properties | von Wettstein *et al.* (1985) |
| Dormancy and vigour control | Ability to use barley in maltings, as and when required | No published reference – fundamental physiology not fully evaluated | |
| Modifiability | Even and rapid modification of endosperm during germination | No published reference – fundamental physiology not fully evaluated | |
| Low protein accumulation | Low protein, therefore high starch | No published reference | |
| High flavour stability product | Quality improvements in beer | No published reference – fundamental physiology not fully evaluated | |
| Flavour control | Elimination of precursors of possibly undesirable flavours (e.g. dimethyl sulphide) | No published reference | |

**Hops**

Although a relatively small contributor to the cost of beer production, hops have a disproportionate influence on quality, notably in terms of bitterness and aroma. As a consequence, the majority of brewers are conservative with regard to their 'hop bill' and generally accept new varieties with reluctance.

Notwithstanding this, there are several distinct improvements which brewers would like to see in their preferred varieties. Such characteristics cannot be introduced by traditional breeding techniques without severe risk to perceived quality attributes. The only hope has been selection of variants which display the new properties, while retaining all desirable features (so-called 'clonal variation'). There is clearly a substantial chance element to this approach, and for this reason, modern gene technology holds exciting possibilities for hops.

A principal aim is the introduction into hops of resistance to infection and infestation. The cultivated hop (*Humulus lupulus*) is susceptible to powdery mildew (*Pseudoperononspora humuli*) and to the damson hop aphid (*Phorodon humuli*). Its Far Eastern counterpart *H. japonicus* is not. Consequently, there is a considerable interest in transferring the relevant resistance genes. However, while *H. japonicus* can pollinate *H. lupulus*, the resultant embryos abort (Winge, 1914). Nevertheless, there would be some promise if an appropriate culture medium could be identified on which to activate immature 'cross'-embryos, such as was first demonstrated for flax (*Linum*; Laibach, 1925). Having said this, certain varieties of *H. lupulus*, such as Wye Target, contain dominant genes, notably R2, which confer resistance to powdery mildew, so that this may be a useful alternative.

Protoplast formation, fusion and genetic modification by electroporation have all been demonstrated for *H. lupulus*. As yet it has not proved possible to regenerate plants beyond the microcallus stage. However, it is known that hop plants can be regenerated from callus cultures (Heale *et al.*, 1989).

The problem is rather one of which callus lines to select. Evaluation of new varieties is a prolonged affair, which must take into account the considerable impact which growth site and conditions have on expression. Not only this, there is immense undesirability in the methods which need to be used to select for disease resistance, viz growing in strictly quarantined, highly infectious conditions.

As yet there are no suitable selection markers available which can be used as indicators of desired characters. Perhaps restriction fragment length polymorphisms (RFLPs) provide the likeliest opportunity (Gunn, 1989). Certainly, RFLP analysis did detect clear polymorphisms in isolates of *Verticillium albo-atrum*, the organism which causes hop wilt (Heale *et al.*, 1989).

The bitterness components of beer originate from the α-acids, which are normally found only in the cones of female plants. Crossing experiments have confirmed the genetic potential for maximizing levels of α-acids in hops (Gunn, 1988). The genes for α-acid production are found in the male plant as well; the future goal must surely be to identify markers for those genes in

order to facilitate crossing of male and female with high α-acid potential.

## Brewers' yeasts

The basic purpose of fermentation has not changed over the centuries that beer has been produced: it is the conversion of wort, chiefly into an alcoholic, pleasant tasting beer. This is brought about by the activity of brewers' yeast, which converts sugars into ethanol and carbon dioxide, together with small quantities of highly flavoured compounds which give beers their characteristic flavours. The basic biochemistry of these processes is well understood, although the detailed mechanisms underlying the production of many flavour compounds is still a fruitful source of much basic research. Similarly, the factors determining flocculation of yeast from fermenting beer, to facilitate clarification, are only now being elucidated. The current state of knowledge of yeast physiology has been described in some detail in a previous volume in this series (Boulton, 1991), and so the present authors will attempt not to tread the same ground.

Brewers' yeasts all belong to the species *Saccharomyces cerevisiae*. However, they are quite distinct from the well-characterized laboratory strains of the same species. Unlike the latter, which have been bred for their ease of handling in laboratories, brewers' yeasts have been selected for their performance in brewery fermenters. Little effort has been devoted to rendering them amenable to genetic analysis and so laboratory-based breeding programmes have not, until recently, featured in the work schedules of brewing laboratories. This has changed with the advent of 'genetic engineering' and many laboratories have embarked upon work to develop new strains of brewing yeast with defined properties.

### THE SCOPE FOR IMPROVING BREWERS' YEASTS

The properties which are of most importance for a good brewers' yeast strain have been described by Tubb and Hammond (1987). They inclue the ability to ferment wort sugars to ethanol rapidly and efficiently, without excessive yeast growth. They need to be able to withstand the high osmotic pressure and significant levels of carbon dioxide and ethanol encountered in brewery fermenters. They must also produce the required levels of flavour compounds, while being easy to separate from the beer at the end of fermentation. Once separated, they must remain healthy during storage before they are re-used. There is considerable scope for improving the behaviour of traditional brewing yeasts in a number of these areas, either by modifying their physiology or by extending their biochemical capabilities.

### TRADITIONAL BREEDING METHODS

The breeding of laboratory strains of yeast involves sporulation and hybridization of cells. For many years, this was not possible with brewers' yeasts, since they sporulate poorly and many of the spores that are produced are

non-viable (Anderson and Martin, 1975). Recently, techniques have been developed to improve the sporulation characteristics of brewers' yeasts (Gjermansen and Sigsgaard, 1981; Bilinski, Russell and Stewart, 1986, 1987a), but they still sporulate poorly when compared with laboratory strains. Using these techniques, meiotic segregants of brewers' yeasts have been mated and a number of hybrids obtained (Von Wettstein, 1983; Bilinski, Russell and Stewart, 1987b). Although these approaches have proved successful, much work is involved in selecting hybrids with suitable brewing properties and, accordingly, few laboratories are pursuing this approach.

Direct selection of mutants with or without prior mutagenesis has been used to obtain yeasts with altered flocculation and flavour production characteristics (Molzahn, 1977). The selection of mutants resistant to 2-deoxyglucose has yielded derepressed mutants which are able to ferment maltose and glucose simultaneously, increasing the speed and efficiency of fermentation (Jones, Russell and Stewart, 1986).

By mixing large numbers of yeast cells together and by applying a strong positive selection pressure, it is possible to produce hybrids between brewers' yeasts and other yeast strains. The technique is known as rare-mating (Gunge and Nakatomi, 1972) and has been used to produce brewers' yeasts with the ability to ferment dextrins (Tubb *et al.*, 1981) and strains with anti-contaminant properties (Young, 1981, 1983; Hammond and Eckersley, 1984). The technique has been largely superseded by more specific recombinant DNA methods.

Spheroplast fusion provides an alternative means for overcoming the mating difficulties experienced with brewers' yeasts. The cell walls of yeasts are removed enzymatically and the resulting spheroplasts induced to fuse either chemically (van Solingen and van der Plaat, 1977) or electrically (Halfmann *et al.*, 1982). Spheroplast fusion has been used to produce dextrin-fermenting brewers' yeasts (Barney, Jansen and Helbert, 1980; Hansen, Rocken and Emeis, 1990), yeasts with both anti-yeast and anti-bacterial activities (Sasaki *et al.*, 1984), as well as yeasts with changed flocculation character (Urano, Nishikawa and Kamimura, 1990). One problem often experienced with fusion hybrids is that they have poor brewing properties because of the contribution to their genome from the non-brewing strain. Considerable work is required to overcome this and, again, this is a technique largely superseded by more specific methods.

## GENETIC ENGINEERING

The use of DNA molecules to bring about a change in a recipient organism bypasses the mating system and so can be readily applied to brewers' yeasts. With the development of recombinant DNA techniques in the 1970s, such transformation procedures became feasible and have been widely applied to the breeding of new strains of brewers' yeasts. It is normal when carrying out transformation experiments with haploid or diploid yeasts to employ strains carrying auxotrophic markers in order to simplify the identification of transformed cells among the background of parental organisms. However,

since brewers' yeasts are polyploid, it is extremely difficult to produce auxotrophic mutants and so the plasmids used for transformation of brewers' yeasts normally carry dominant selection markers.

For brewing applications, the most widely used selection marker is *CUP1*, which confers copper resistance upon cells harbouring multiple copies of the gene (Henderson, Cox and Tubb, 1985). Almost all brewers' yeasts are sensitive to copper and become resistant when transformed with plasmids containing the *CUP1* gene. Their fermentation behaviour is unaltered and the beer produced is identical to that normally produced (Meaden and Tubb, 1985). Another useful dominant resistance marker is *SMR1*, which encodes resistance to the herbicide sulphometuron-methyl (Casey, Xiao and Rank, 1988a). This is a mutant allele of the yeast *ILV2* gene, which encodes the enzyme acetolactate synthase. Since the *ILV2* gene is a normal part of the genome of a brewers' yeast, the mutant version can not only act as a selectable marker but can also be used as a way of directing integration of DNA into the yeast chromosome (Casey, Xiao and Rank, 1988b). Both the *CUP1* and *SMR1* genes are derived from *Saccharomyces cerevisiae* and so are not 'foreign' to brewers' yeasts, an important consideration when modified yeasts are being considered for commercial production and approval for their use is being sought.

Two other dominant selectable markers widely used are those coding for resistance to geneticin (G418) (Jimenez and Davies, 1980) and to chloramphenicol (Hadfield, Cashmore and Meacock, 1986). Both are derived from bacteria and so, once they have been used for selection purposes, must be jettisoned from a transformed yeast before it can be considered for commercial use. The unaltered bacterial G418 resistance gene is expressed in yeasts but, for efficient expression, it is necessary to place the gene under the control of a yeast promoter. Resistance to chloramphenicol is a mitochondrial function and so can only be used when transformed yeast cells can be grown on a non-fermentable substrate. Despite these complications, both resistance genes have been successfully employed in a number of breeding programmes with brewers' yeasts.

Most early transformation experiments with brewers' yeasts employed multicopy plasmids which led to many copies of the gene of interest being inserted into the host organism. This usually resulted in particularly good expression of the inserted gene. Such over-expression did however, on occasion, have deleterious effects on the fermentation behaviour of the transformed yeast (Perry and Meaden, 1988). The genetic stability of yeasts transformed with multi-copy plasmids is not good, the new property gradually being lost with successive fermentations (Meaden and Tubb, 1985; Cantwell *et al.*, 1985).

More recently, transformation of brewers' yeasts has been carried out by integration of the gene of interest, either into a chromosome (Enari *et al.*, 1987) or into the endogenous 2 μm plasmids (Hinchliffe, Fleming and Vakeria, 1987). Chromosomal integrants are stable but, because of the lower copy number, the level of expression can be less than with multi-copy transformants (Enari *et al.*, 1987). This can be partially overcome by

integrating the gene of interest at several sites within the yeast chromosomes. Alternatively, a highly efficient yeast promoter, typically one involved in controlling a gene from the glycolytic pathway, can be employed to increase the level of expression. This latter approach has now been almost universally adopted.

## DIACETYL-LESS BREWERS' YEASTS

Diacetyl is a major off-flavour in finished beers. It is formed by chemical oxidation of α-acetolactate, which diffuses from yeast cells during fermentation. The need to remove diacetyl is the major reason for the long flavour maturation period at the end of fermentation. In order to eliminate this costly process, various genetic approaches have been proposed to prevent or control diacetyl formation. The rate-limiting step is conversion of α-acetolactate to diacetyl, and so most solutions to the problem have involved either removing α-acetolactate before it is converted to diacetyl or preventing its formation in the first place.

The removal of α-acetolactate can be brought about by converting it to acetoin using the bacterial enzyme acetolactate decarboxylase (ALDC). The gene encoding this enzyme has been cloned from several bacterial species. In initial trials, the gene from *Enterobacter aerogenes* was expressed off the yeast *ADH1* promoter. When transformed brewers' yeasts were used for fermentation, reduced levels of diacetyl were produced compared with control fermentations (Shimizu, Sone and Inoue, 1989). There appeared to be no effect on other aspects of yeast metabolism. In subsequent experiments, the ALDC gene was integrated into the rDNA genes of brewers' yeasts. Because of the many copies of rDNA present in the yeast genome, the ALDC gene was integrated more than 20 times into each cell and consequently all transformants showed a high enzyme activity (Fujii *et al.*, 1990). Again, beers made in laboratory-scale fermentation trials had very low diacetyl contents. The work was extended to investigate the effects of yeast promoters on gene expression and genes from other bacteria were included. Cloned ALDC genes from two strains of *E. aerogenes* and from one *Klebsiella terrigena* strain were placed under the control of both *ADH1* and *PGK* promoters and terminators and were used to transform brewers' yeasts by integration into their *rDNA*, *ADH1* and *PGK* genes. All the transformants fermented well and two, in which the *ALDC* gene had been integrated at the *PGK* site under the control of the *PGK* promoter and terminator, produced beer in which the diacetyl level after primary fermentation was below the taste threshold. The finished beer was of good quality and the transformed yeasts were completely stable (Suihko *et al.*, 1989, 1990; Blomqvist *et al.*, 1991).

Modification of the amino-acid biosynthetic pathway leading to the formation of α-acetolactate (*Figure 1*) is another approach which has been used in the control of diacetyl production. The *ILV* genes coding for the various enzymes have been cloned (Petersen *et al.*, 1983) and attempts have been made to regulate beer diacetyl levels by manipulating these genes in brewing organisms. Mutant yeasts, lacking the enzymes threonine deaminase and

acetolactate synthase, produce no diacetyl but ferment very poorly, presumably because of their inability to synthesize several amino acids (Ryder and Masschelein, 1983). This difficulty can be overcome by only partially reducing the activity of acetolactate synthase. Dominant mutations of the *ILV2* gene are known which prevent sulphometuron methyl inhibiting acetolactate synthase activity. They do this by reducing the binding affinity of the enzyme for the herbicide (Falco and Dumas, 1985). Some of the resistant forms of the enzyme are less active and so should produce less α-acetolactate and hence less diacetyl. Spontaneous dominant mutants of this type have been isolated but reductions in diacetyl levels were disappointingly small (Gjermansen *et al.*, 1988; Galvan *et al.*, 1987). More recently, a much more complex procedure has been developed for the production of recessive mutations in the *ILV2* locus of brewing yeasts (Kielland-Brandt *et al.*, 1989). Strains have been produced having very low acetolactate synthase activities by screening mutagenized, spontaneous herbicide-resistant, meiotic segregants from brewing yeasts for slow growth on media lacking isoleucine and valine. Hybrids, produced by mating these strains, ferment well, produce low levels of diacetyl and yield acceptable beer (P. Sigsgaard, pers. comm.). Another approach to reducing the activity of acetolacetate synthase has involved transforming brewers' yeast with a fragment of the *ILV2* gene in such a way that anti-sense mRNA was made (Vakeria, Box and Hinchliffe, 1991). The level of normal acetolactate synthase mRNA was thereby reduced, and less enzyme was synthesized. Unfortunately, although less diacetyl was produced, the fermentation performance of the transformed yeast was poor.

Another possible way of reducing diacetyl levels is by increasing the flux through the pathway leading to valine production, which would result in any excess α-acetolactate being consumed. This has been attempted by transforming yeasts with multi-copy plasmids containing copies of either *ILV3* or *ILV5* genes. Yeasts transformed with *ILV5* had increased activity of acetolactate reductoisomerase and produced considerably less diacetyl than did parent yeasts (Dillemans *et al.*, 1987; Villanueba, Goossens and Masschelein, 1990; Goossens *et al.*, 1991, 1993). Those transformed with *ILV3* produced just as much diacetyl as did their parents, despite a considerable increase in the corresponding enzyme activity (Goossens *et al.*, 1987).

It is clear that a number of different approaches to producing diacetyl-less brewers' yeasts have been used with different degrees of success. Technically, the ALDC-producing strains are probably the most successful, but their application in breweries must await clarification of the legislative position.

## AMYLOLYTIC BREWERS' YEASTS

Normal brewing yeasts cannot utilize the dextrins, which represent about 25% of wort sugars. Yeasts able to ferment these additional sugars are more efficient than brewing yeasts and can be used to produce both low carbohydrate diabetic beers and lower calorie 'light' beers. At present, these types of beers are often made by the addition of fungal glucoamylases derived from *Aspergillus niger*. Amylolytic brewing yeasts offer a number of advantages,

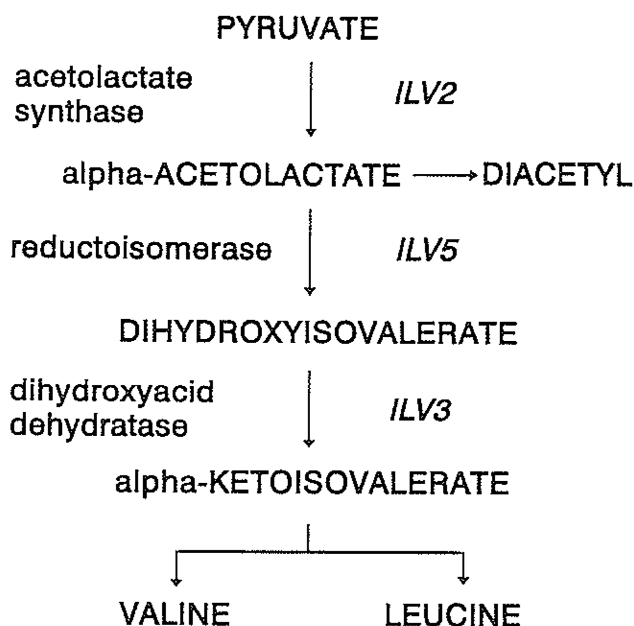


**Figure 1.** Diacetyl formation and amino acid biosynthesis. Diacetyl is formed as a by-product of the biosynthetic pathway for valine and leucine. The *ILV* genes and the enzymes coded by them are indicated on the pathway.

since mistakes in enzyme addition are eliminated and other deleterious activities which are often present in commercial enzyme preparations are avoided.

Various approaches to the production of amylolytic brewers' yeasts have been adopted. A glucoamylase gene (*DEX1* or *STA2*) has been cloned from *Saccharomyces cerevisiae* var. *diastaticus* (Meaden *et al.*, 1985) and inserted into a multi-copy plasmid. Both ale and lager strains have been transformed with this plasmid using *CUP1* as the selectable marker (Meaden and Tubb, 1985). The transformants produced extracellular glucoamylase but, because yeast glucoamylase lacks α-1,6 debranching activity, complete superattenuation was not achieved. The plasmid-based transformants were not stable and both growth and fermentation rates were slower than in the parent organisms (Perry and Meaden, 1988). Plasmid instability was much improved by the development of an all-yeast multi-copy plasmid (Vakeria and Hinchliffe, 1989), but the fermentation rate of the transformant was still slow. The *STA1* glucoamylase gene has also been cloned from *S. cerevisiae* var. *diastaticus* and used to transform brewing yeasts (Sakai *et al.*, 1989; Park *et al.*, 1990). Poor plasmid stabilities and slower fermentations were again observed. Only when the gene was integrated into the yeast chromosome was a stable transformant obtained (Park *et al.*, 1990).

The lack of α-1,6 debranching activity in glucoamylases derived from *S. cerevisiae* var. *diastaticus* is a major problem that can only be overcome by using genes for glucoamylases from other microbial sources. The *GA* gene

from *Aspergillus niger* has been cloned and inserted into an integrating vector (Yocum, 1986). This gene encodes a glucoamylase with both α-1,4 and α-1,6 activities. The vector used for transformation of brewers' yeast was designed to achieve integration of the *GA* gene into the yeast genome while excluding bacterial DNA sequences (*Figure 2*). To avoid instability problems caused by recombination, three copies of the *GA* gene were integrated into three different copies of the yeast *HO* gene. Gene expression and protein secretion were optimized by the use of a yeast glycolytic enzyme promoter and a secretion signal sequence, respectively. Fermentation trials in 100 litre fermenters have been carried out successfully: high levels of extracellular glucoamylase have been obtained and good-quality 'light beer' has been produced (Gopal and Hammond, 1992).

The glucoamylase from *A. niger* is stable at pasteurization temperatures, retaining its activity in packaged beer. Therefore, if any undigested dextrin remains in the beer, this can cause difficulties, the beer gradually becoming sweeter as glucose is produced. The glucoamylase from *Schwanniomyces occidentalis*, unlike the *Saccharomyces* enzyme, possesses debranching activity and is more heat-labile than is the fungal enzyme. It is not, however, commercially available, and so is not currently used for the production of low-carbohydrate beers. The gene for this enzyme has been cloned and placed under the control of the *Saccharomyces ADH1* promoter (Lancashire *et al.*, 1989). Both integrated and multi-copy transformants have been produced, which successfully fermented brewery worts to a lower gravity than normal and produced acceptable beers.

## GLUCANOLYTIC BREWERS' YEASTS

Barley β-glucanase is unstable at the temperatures found during malt kilning and so β-glucans can sometimes be found in beer due to the low level of enzyme activity during wort production. Large molecular weight glucans in beer can cause filtration difficulties and give rise to sediments, gels and hazes. Microbial β-glucanases are often added to mashes or worts to prevent these problems and so there has been much interest in transferring into brewing yeasts the gene for endo-1,3-1,4-β-D-glucanase. This gene has been cloned from *Bacillus subtilis* (Cantwell and McConnell, 1983; Hinchliffe and Box, 1984; Lancashire and Wilde, 1987), *Trichoderma reesei* (Knowles *et al.*, 1985) and barley (Jackson, Ballance and Thomsen, 1986). At first, only low levels of intracellular enzyme activity were obtained from yeasts transformed with the *B. subtilis* gene (Hinchliffe and Box, 1985). When the β-glucanase gene was placed under the control of the yeast *ADH1* promoter (Cantwell *et al.*, 1985), high levels of β-glucanase were produced intracellularly by transformed brewing yeasts but only very low levels were detected in the beer. Significant levels of extracellular β-glucanase were finally obtained when the bacterial gene was placed under the control of the yeast α-mating factor signal sequence (Lancashire and Wilde, 1987). The experimental beers produced using these last transformants had much lower β-glucan contents and viscosities than did control beers and their filterabilities were much improved.

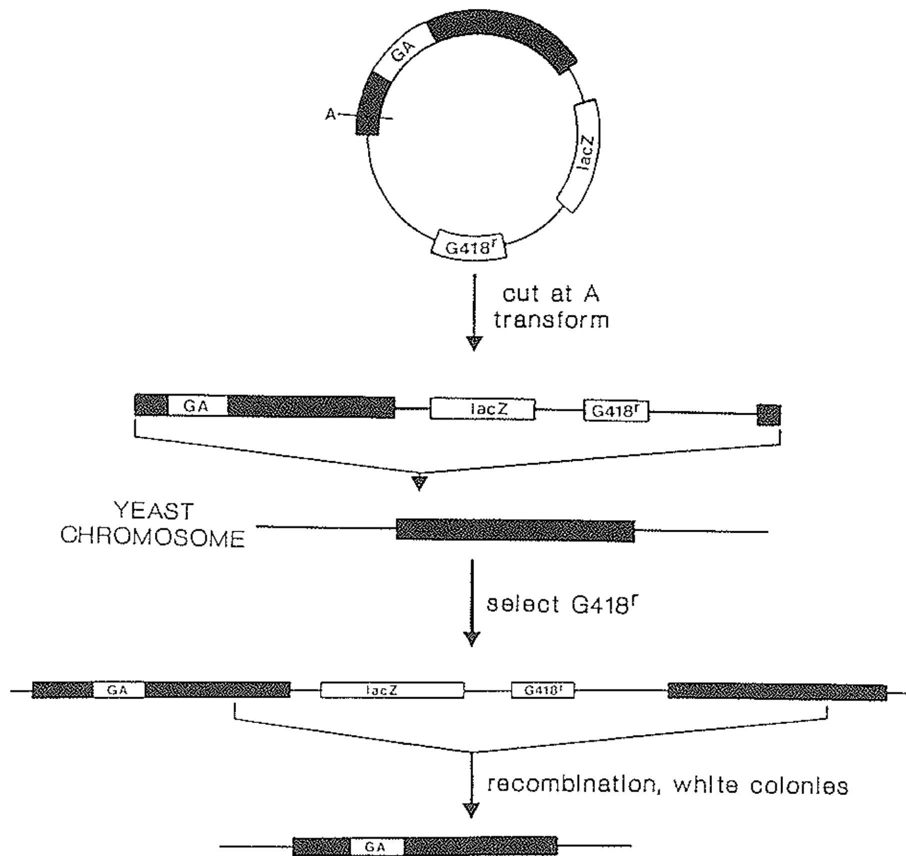


**Figure 2.** Transformation protocol used to achieve stable integration of the *Aspergillus niger* glucoamylase gene into brewers' yeast. The *GA* gene is inserted into a copy of the yeast *HO* gene contained on a plasmid, which also contains copies of the *LacZ* and G418 resistance genes from bacteria. The plasmid is cut at A and used to transform brewers' yeast. Transformants are selected by their ability to grow on plates containing G418. Spontaneous recombination between the two copies of the *HO* gene can lead to loss of the bacterial DNA, while the *GA* gene is retained. This recombination can be detected by virtue of the simultaneous loss of the *LacZ* gene. The desired recombinants can then be detected by screening for glucoamylase activity.

When the β-glucanase gene from *T. reesei* was cloned and used for transformation of brewing yeasts, it was placed under the control of the yeast *PGK* promoter. This was then inserted into both multi-copy and integrating plasmids (Enari *et al.*, 1987). A yeast secretion signal sequence was unnecessary since, unlike their bacterial equivalents, fungal extracellular enzymes are efficiently excreted by yeasts. With both multi-copy and integrated transformants, wort β-glucans were efficiently degraded and beer filterability was much improved, while fermentation and beer characteristics were largely unaltered (Enari *et al.*, 1987; Penttila *et al.*, 1987).

The β-glucanase from *B. subtilis* shows highest enzyme activity at a pH value of 6.7, whereas the barley enzyme is most active at pH 4.7, a value which more closely resembles those encountered during the brewing process.

The barley enzyme would therefore appear attractive for use in transformed yeasts. The barley gene has been cloned by joining two separate DNA sequences isolated from barley aleurone, which together encompassed the barley β-glucanase gene. The gene was then fused to a mouse α-amylase secretion signal sequence and inserted into an expression vector between yeast *ADH1* promoter and terminator sequences (Jackson, Ballance and Thomsen, 1986). Yeast transformants all produced extracellular β-glucanase (Thomsen, Jackson and Brenner, 1988). A similarly transformed brewers' yeast has been used to produce beer in a pilot plant. The β-glucan content of the beer was much lower than usual, flavour and foam stability were unaffected, and filtration rates were markedly increased (Berghof and Stahl, 1991).

As discussed earlier, however, a much more attractive option is the insertion of a heat-stable β-glucan into barley. The earlier the enzyme is introduced, the more benefits it has. β-Glucanase introduced into yeast can have no benefits in wort separation, for instance. Furthermore, there are clear attractions to having foreign enzymes destroyed in the kettle boil.

## FLOCCULENT BREWERS' YEASTS

The factors controlling flocculation of brewing yeasts during fermentation are still far from clear (Boulton, 1991) and the genes involved have not been identified with any certainty. A number of genes (*FLO1*, *FLO5*, *FLO8* and *tup1*) are known to affect flocculation, but their presence in brewers' yeasts is still the subject of much research. Brewers' yeasts appear to flocculate by two different mechanisms (Stratford and Assinder, 1991). The flocculation behaviour of bottom-fermenting lager strains resembles that of laboratory strains containing *FLO* genes and is different from that of top-fermenting ale strains. This suggests that flocculation of ale strains may be caused by something other than the *FLO* genes. The involvement of *FLO1* in the flocculation of bottom-fermenting yeasts has been supported by hybridization studies (Watari *et al.*, 1991a). Recently, the *FLO1* gene product has been tentatively identified as a hydrophobic cell wall protein (van der Aar, Straver and Teunissen, 1993).

The *FLO1* gene has been cloned (Watari *et al.*, 1989) and used to transform both top- and bottom-fermenting non-flocculent yeasts. The transformed yeasts were more flocculent than their parents (Watari *et al.*, 1991b) and fermented satisfactorily (Watari *et al.*, 1991a). As far as lager yeasts are concerned, it would seem that transformation provides a mechanism for manipulating flocculation characteristics.

## THE GENETIC MAKE-UP OF A BREWERS' YEAST

The development of techniques for the modification of brewers' yeasts has taught us much of the structure of the genome of these organisms. The Carlsberg lager yeast strain M244 has been examined in considerable detail and much fascinating information obtained. This has been made possible by

the technique of single chromosome transfer (Nilsson-Tillgren *et al.*, 1980), whereby single chromosomes have been transferred from meiotic segregants of the lager yeast into laboratory strains, thereby allowing detailed genetic analysis of the brewers' yeast chromosomes to be carried out. Chromosomes III, V, X, XII and XIII have been studied in this way.

Within the lager yeast there are two forms of chromosome III: one is functionally and structurally identical to the chromosome III found in laboratory strains, whereas the other is functionally homologous to the laboratory-strain chromosome but is structurally different. Recombination between the two chromosome types only occurs in certain regions (Nilsson-Tillgren *et al.*, 1981; Holmberg, 1982). Similarly, two forms of chromosome V have been identified: one is both structurally and functionally homologous to the chromosome of *Saccharomyces cerevisiae*, while the other is functionally homologous but fails to recombine at all (a homeologous chromosome) (Nilsson-Tillgren *et al.*, 1986).

Three forms of chromosome X have been identified, two of which are not homologous to chromosome X from *S. cerevisiae*, recombination occurring along only part of the chromosome (Casey, 1986a,b). The third chromosome, although the same length as that from laboratory strains, has not been examined in any detail. Chromosome XII occurs in two forms, one homologous and the other homeologous to the equivalent chromosome from laboratory strains (Petersen *et al.*, 1987). In contrast, three different chromosomes XIII exist, one showing homology to that of *S. cerevisiae*, a non-recombining homeologous chromosome and a mosaic-type chromosome exhibiting partial recombination (Petersen *et al.*, 1987).

The chromosome structure of lager yeast M244 is clearly extremely complicated and it appears to have two recombination-incompatible genomes. Direct examination of the number of copies of the *ILV2* gene in the Carlsberg lager yeast suggests that it contains two copies of each of the two verions of the *ILV2* region (Gjermansen *et al.*, 1988). If this is true of all genes, then the brewing yeast would appear to be allotetraploid, although the alloploidy is obviously irregular because of the occurrence of mosaic chromosomes. It also appears that some chromosomes lack certain genes (Nilsson-Tillgren *et al.*, 1986; Casey, 1986b). If all brewing yeasts are allotetraploids with irregular chromosome structures and missing or defective genes, this may explain their poor sporulation behaviour.

## The future

The techniques of modern yeast genetics have been applied to both unravelling the detailed structure of the brewers' yeast genome and to the construction of new yeast strains for the more efficient production of beer. Many of these new strains have been technically very successful and have been used to produce beer in pilot plants. Similar advances have been made in developing new barley and hop varieties. The major barrier to the commercial exploitation of these new plants and yeasts is the classification of the beers produced by them as 'novel foods', together with a requirement for government

approval for their use (Hammond, 1991). There is a pressing need for brewing technologists to devote time and effort to ensuring that this barrier is overcome, and that genetic modification comes to be seen as a normal part of the commercial development of the brewing process. If this does not happen, the application of genetic modification techniques in the brewing industry will be severely curtailed and the benefits available will be lost.

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