# The Genetic Modification of Brewing Yeast with Recombinant DNA[1]

**Edward Hinchliffe and Christine J. Daubney,** *Research Department, Bass Public Limited Company, Burton-on-Trent, England*


## ABSTRACT

The presence of 2-$\mu$m plasmid DNA was demonstrated in each of seven different strains of brewing yeast investigated. These yeasts were used as recipients for 2-$\mu$m-based recombinant DNA plasmids carrying the copper resistance gene of *Saccharomyces cerevisiae* and the $\beta$-glucanase gene of *Bacillus subtilis*. When grown under nonselective brewery conditions, the transformed copper-resistant phenotype of brewing yeast was shown to be relatively stable. The brewing properties of yeast harboring recombinant DNA plasmids were indistinguishable from their unmodified parental counterparts.


The use of commercial enzymes is common practice in modern breweries. Microbial enzymes are used routinely to obtain economic benefits by increasing process efficiency, enhancing product quality, extending product ranges, and resolving intermittant process problems. One enzyme that has received considerable attention in recent years is $\beta$-glucanase, which can be used to obtain process benefits in wort production, fermentation, and conditioning (3). For example, an endo-1,3-1,4-$\beta$-glucanase from the Gram-positive bacterium *Bacillus subtilis* has been shown to produce a quantitative improvement in beer filtration performance (11,12). In view of the cost of adding commercial enzymes, efforts have been made using genetic engineering to construct yeast capable of hydrolyzing $\beta$-glucan (7,11,12). The application of this technology makes it feasible to introduce a specific gene directly into the brewing yeast genome, where it will mediate the production of the appropriate enzyme during the course of fermentation (11).

Here we consider the genetic route by which brewing yeasts are genetically modified. A consideration of paramount importance is the effect which such genetic modification will have upon the brewing performance of the yeast. Previous genetic manipulations of brewing yeast by protoplast fusion and rare mating have resulted in yeast ill-suited to the rigors of modern brewing practice (9,18). If the full potential of gene-cloning technology is to be realized by the brewer, it is important that a similar fate does not befall yeast modified by recombinant DNA.

Here we describe the way in which the copper resistance gene of yeast has been used to introduce the $\beta$-glucanase gene of *B. subtilis* into brewing yeast. The stability of such transformants was investigated, and the brewing performance of the modified yeast was assessed. The results of these studies are discussed in light of the requirements for the future development of brewing yeast genetics.

## EXPERIMENTAL

### Yeast Strains and Plasmids

Brewing strains used were NCYC 74, NCYC 240, and NCYC 1026 (National Collection of Yeast Cultures, Food Research Institute, Norwich) and the Bass brewing production yeasts BB1, BB6, BB9, BB10.2, and BB10.5. The plasmids used were pET13:1 (10) and pEHB10 (12). Each plasmid carries DNA replication functions enabling its extrachromosomal replication in both yeast and *Escherichia coli*, and the gene *CUP*-1, which mediates resistance to copper in yeast (10). Plasmid pEHB10 also harbors the $\beta$-glucanase gene of *B. subtilis* (12).

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### Media and Growth Conditions

Yeasts were grown on YED (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose solidified with 2% w/v agar) or NEP (10) supplemented where appropriate with 0.2 m*M* or 1 m*M* $CuSO_4 \cdot 5H_2O$.

### Extrachromosomal DNA and Double Stranded RNA Analysis

Strains of brewing yeast were screened for the presence of 2-$\mu$m plasmid DNA by the method of Brown et al (6). Double-stranded RNA was extracted by resuspending yeast cells in protoplastic buffer (0.5 ml sodium citrate, 100 m*M*; ethylenediaminetetraacetic acid, 10 m*M*; sorbitol, 1.2*M*; $\beta$-glucuronidase, 1% w/v; pH 5.8) at 30° C for 90 min. Protoplasts were centrifuged and resuspended in 1.2*M* sorbitol before lysis in 0.2 ml of 1% w/v sodium dodecyl sulfate, 8% w/v Ficoll, 25 m*M* ethylenediaminetetraacetic acid at 60° C for 20 min.

### Electrophoresis

Plasmid DNA and double-stranded RNA were analyzed by agarose gel electrophoresis. Samples were applied to 0.8 or 1% (w/v) agarose gels in Tris-acetate buffer, pH 8.1. Gels were run for 8–12 hr at 30 mA and stained with ethidium bromide. Visualization was by fluorescence on an ultraviolet transilluminator (UV Products Inc.).

### Yeast Transformation

Brewing yeast strains were transformed by the protoplast method described by Henderson et al (10), with the exception that Zymolyase (Kirin Brewery Co.) was used for the removal of the cell walls. Copper-resistant transformants were selected by plating protoplasts on NEP regeneration agar (3% w/v agar) containing 0.3 m*M* copper sulfate. Putative copper-resistant transformants were verified by growth on NEP supplemented with 1 m*M* copper sulfate and by the production of $\beta$-lactamase as determined by a plate assay (8).

### Plasmid Stability

The inheritable stability of copper-resistant recombinant plasmids in brewing yeast was determined by growth in nonselective NEP medium. Yeasts were grown to mid-exponential growth phase and then subcultured into fresh growth medium. The presence of plasmids was determined phenotypically by plating cells on NEP followed by replica plating to NEP supplemented with copper sulfate.

### Wort Fermentations, Beer Production, and Analysis

Wort was fermented in stirred mini-fermentation vessels with a nitrogen headspace (15). The specific gravity of the fermenting wort was monitored using an Anton Paar DMA46 density meter. All beers were produced in an experimental brewery such that each batch of wort could be fermented and processed by the various yeast strains in an identical manner. Beers were evaluated by triangular taste testing and by flavor profile analysis (2).

## RESULTS AND DISCUSSION

### Extrachromosomal Genetic Elements in Brewing Yeast

It has been shown that ale and lager brewing yeasts harbor 2-$\mu$m plasmids (1,6,17). This implies that these strains of yeast contain all the necessary genetic information to mediate the successful replication and segregation of the 2-$\mu$m plasmid. Because the

replication of 2-$\mu$m-based recombinant plasmids such as pET13:1 is dependent upon the presence of endogenous 2-$\mu$m DNA in yeast (4), it was considered important to determine whether the different proprietary strains of production yeast also contained endogenous 2-$\mu$m plasmids. The results presented in Figure 1 show that all the strains of yeast tested do possess 2-$\mu$m plasmid with the exception of the control NCYC 74, which is known to be devoid of this extrachromosomal element (13). This confirms the previous observations (1,6,17) and adds credence to the notion that these yeasts should be capable of maintaining recombinant 2-$\mu$m-based plasmid DNA.

Further characterization of the brewing yeast used in this study indicated that these strains do not possess the double-stranded RNA, which is typically associated with the killer phenotype in yeast (19). The absence of the double-stranded RNA was correlated with the sensitivity of all of the yeasts tested to the killer toxin, thereby confirming the observations of Aigle et al (1).

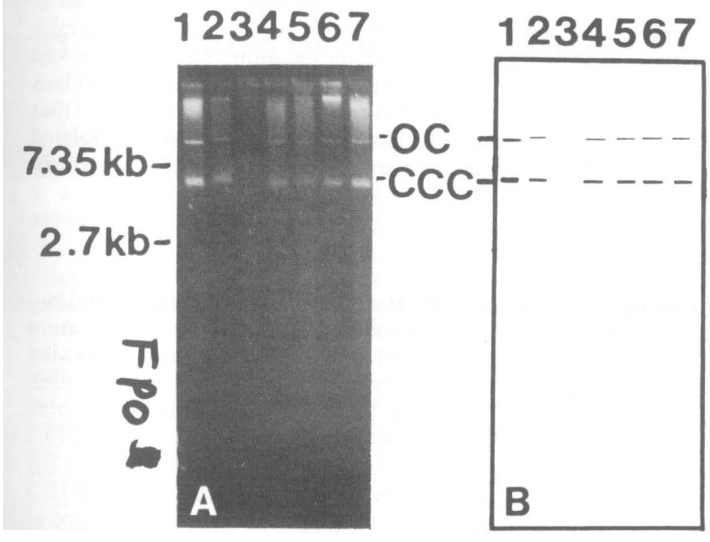


**Fig. 1.** The presence of 2-$\mu$m plasmid DNA in the brewing yeast strains NCYC 1026, 1; NCYC 240, 2; BB6, 4; BB10.5, 5; BB9, 6; and BB1, 7. The negative control strain NCYC 74 (track 3) did not possess 2-$\mu$m plasmid DNA. Open circular (**OC**) and covalently closed circular (**CCC**) forms of 2-$\mu$m are indicated. Molecular size markers are represented in kilo base pairs (**kb**).

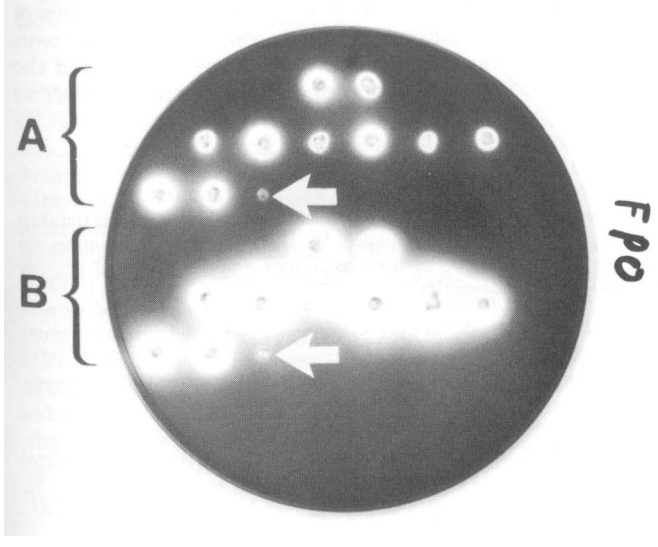


**Fig. 2.** $\beta$-Lactamase producing transformants of BB10.5 harboring plasmid pEHB10 (**A**) and pET13:1 (**B**) showing zones of clearing on starch/iodine supplemented with ampicillin (7). The $\beta$-lactamase negative parental strain BB10.5 is indicated by the arrows.

**Transformation of Brewing Yeast**

Recombinant DNA is ordinarily introduced into yeast as small circular molecules of plasmid DNA that are usually capable of both selection and replication in yeast. Most of the plasmids currently in use for yeast transformation are selectable because they carry a prototrophic gene that complements an auxotrophic allele in a chosen recipient strain. Such plasmids are therefore only of value in effecting the transformation of laboratory auxotrophic yeast. In order to transform brewing yeasts, which are polyploid and do not display auxotrophic requirements, it is necessary to utilize a selection system based upon a dominant selectable gene. In this respect, we utilized the gene *CUP*-1, which confers on the yeast a resistance to copper. The system employed was developed by Henderson et al (10) and has been used to good effect in the transformation of a number of different brewing yeasts (7,10,12,14). Before transformation of the selected strains of brewing yeast, it was necessary to determine the minimal concentration of copper that inhibits yeast growth. Yeasts were streaked for single colonies on NEP and then replica plated to NEP supplemented with increasing concentrations of copper sulfate. All strains tested were sensitive to 0.08 m*M* copper sulfate, with the exception of BB6 and BB10.2, which were sensitive to 0.06 m*M* copper sulfate. However, after transformation with either plasmid pET13:1 or pEHB10, all yeasts were resistant to 2 m*M* copper sulfate. Transformants harboring recombinant plasmids pET13:1 and pEHB10 could also be shown to produce $\beta$-lactamase (Fig. 2).

**Plasmid Stability**

Whenever yeast is transformed with 2-$\mu$m-based recombinant plasmids, the resultant transformed phenotype is unstable (5,16). This is a consequence of either intra- or intermolecular recombination with the endogenous 2-$\mu$m plasmid, or segregational instability through plasmid loss at cell division. In many cases, this latter aspect can be controlled by growth on selective media, thereby exerting a direct selection pressure for either the cloned gene of interest or the plasmid vector. However, such a system is not applicable to an industrial brewing fermentation in which the growth medium is hopped wort. It was therefore considered important to evaluate the inheritable stability of recombinant plasmids pET13:1 and pEHB10 in the brewing yeast host BB10.5 when grown under nonselective conditions. The results indicate that both plasmids are relatively stable in this production lager yeast (Fig. 3). A mean value of 0.18% plasmid loss per cell doubling can be calculated for three independent

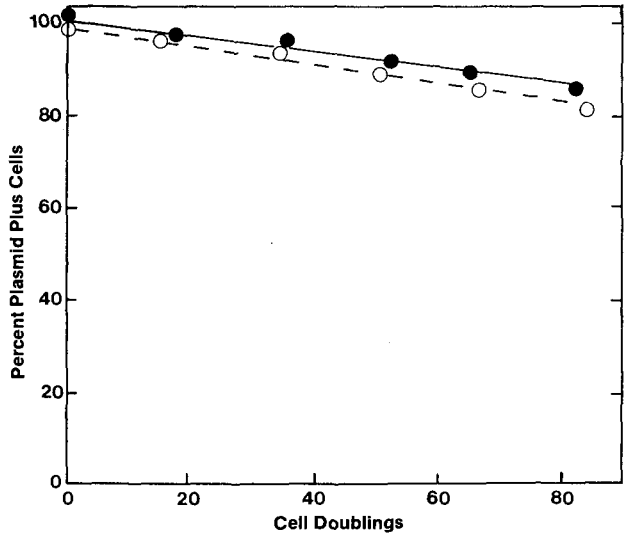


**Fig. 3.** Plasmid stability kinetics in the lager yeast BB10.5: BB10.5 (pET13:1), (O– –O); BB10.5 (pEHB10), (●——●). Each profile is the mean of three independent transformants.

transformants of BB10.5 harboring plasmid pET13:1. As one brewery generation is equivalent to approximately three cell doublings, it would be feasible to operate in excess of 20 brewery fermentations with less than 12% plasmid loss by segregation. This represents a degree of inheritable plasmid stability in excess of that required for good brewing practice, which entails replacement cultures after 10–15 fermentation cycles. However, there appe to be some strain-to-strain variation in the ability to maintain th recombinant plasmids. Figure 4 indicates that plasmids pET1. and pEHB10 are less stable in the ale yeast NCYC 240 than they a in BB10.5. This suggests a requirement for a more stable plasn vector system in strain NCYC 240 compared with BB10.5. T reason for the differing degree of plasmid stability in these t yeasts is not known; whether it is a function of plasmid cc number or intermolecular recombination remains to determined.

**TABLE I**
**The Effect of Washing with Copper Sulfate on the Proportion of Plasmid-Containing Cells in a Mixed Population of Yeast**

| Treatment[a] | Change in % Viability[b] | Change in % Plasmid-Containing Cells[c] |
|---|---|---|
| 0.2 m*M* | −5.9 | +1.9 |
| 1 m*M* | −13.2 | +7.3 |

[a] Yeast was harvested from NEP and resuspended in copper sulfate solution for 10 min.
[b] Percent cell viability was determined by counting cells using a hemocytometer and plating on NEP to determine the percentage of cells giving rise to viable colonies.
[c] The percentage plasmid-containing cells was determined by replica plating viable colonies to NEP supplemented with 1 m*M* copper sulfate.

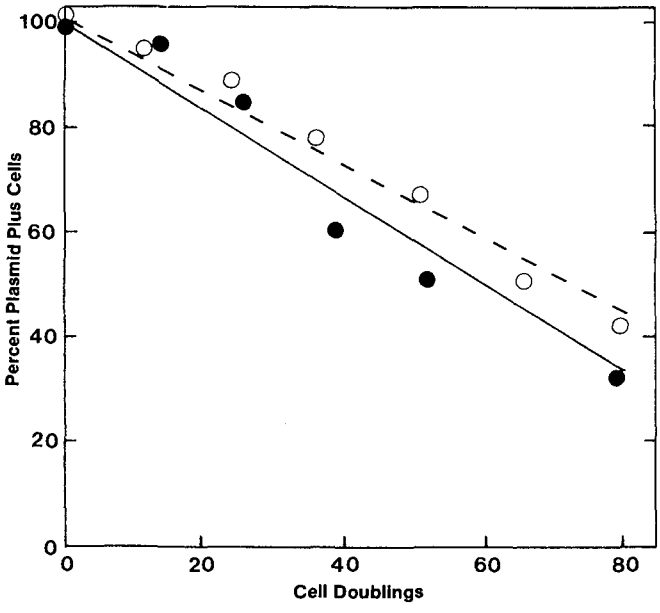


**Fig. 4.** Plasmid stability kinetics in the ale yeast NCYC 240: NCYC 240 (pET13:1), (O−−O); NCYC 240 (pEHB10), (●——●). Each profile is the mean of three independent transformants.

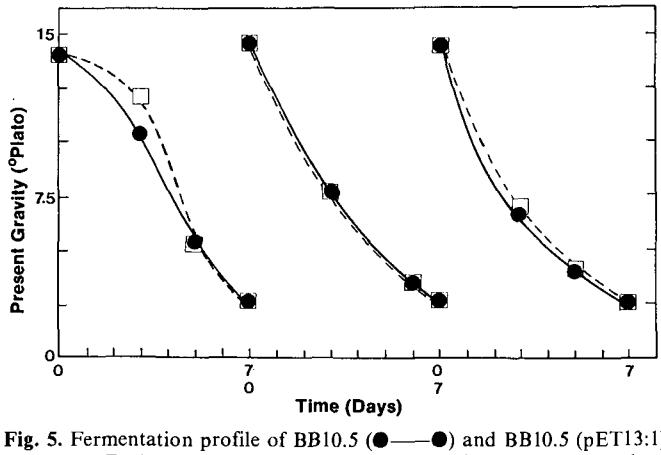


**Fig. 5.** Fermentation profile of BB10.5 (●——●) and BB10.5 (pET13:1) (□- - -□). Each strain was sequentially repitched into the same batch of production lager wort. Fermentations were performed in duplicate as described in the text.

### The Effect of Copper Washing on Plasmid Stability

One way in which the "apparent" stability of recombin plasmids can be influenced is to subject the yeast to direct selecti for the copper resistance phenotype. A mixed population plasmid plus and plasmid minus cells was washed in 0.2 m*M* 1 m*M* copper sulfate solutions with a 10-min rest bef resuspension in NEP. The effect of the highest concentration copper sulfate was to increase the relative proportion of plasm containing cells in the viable cell population. This increase v brought about by a reduction in the viability of those cells that l the copper-resistance plasmid. It can also be seen from Table I t the copper sulfate wash reduced the viability of a small proporti of cells that harbor the copper resistance plasmid. One mig postulate that these copper-resistant cells comprise subpopulation of cells with fewer copies of the copper resistar gene per genome.

### Brewing Properties of Yeast Harboring Recombinant Plasmid

It is important to consider the effect of genetic modificati upon the brewing properties of yeast: the characteristic brewi properties of the yeast must be maintained if the yeast is to be us in normal commercial practice. Extensive trial fermentations w carried out with various yeast strains harboring plasmids pET1 and pEHB10. The results presented in Figure 5 show that fermentation characteristics of strain BB10.5 and BB1 (pET13:1) were similar. When the copper resistance phenotype BB10.5 (pET13:1) was determined during the course of th fermentations, it was found that growth upon nonselective brew wort did not adversely influence plasmid stability.

The brewing properties of BB10.5 and BB10.5 (pET13:1) w further compared in five barrel fermentations in an experimen brewery. Each yeast strain was passed through two success brewery fermentations before evaluation of the beer produced. all cases, the beers produced by yeast harboring the recombin plasmid were indistinguishable from the control beers on the ba of organoleptic analysis. Furthermore, beers matched t production and flavor specifications of the commercial producti beers.

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### LITERATURE CITED

1. Aigle, M., Erbs, D., and Moll, M. *J. Am. Soc. Brew. Chem.*, 42:1, 19
2. Anderson, R. J. *Brew. Guardian* Nov:25, 1983.
3. Bamforth, C. W. *Brew. Dig.* June:22, 1982.
4. Broach, J. R. Page 445 in: The Molecular Biology of the Ye Saccharomyces: Life Cycle and Inheritance. J. S. Strathern, E. Jones, and J. R. Broach, eds. Cold Spring Harbor: New York, 198
5. Broach, J. R., and Hicks, J. B. *Cell* 21:501, 1980.
6. Brown, A. J. P., Goodey, A. R., and Tubb, R. S. *J. Inst. Brew.* 87:2 1982.
7. Cantwell, B. A., Brazill, G., Hurley, J. C., and McConnell, D. J. *E Brew. Conv. Proc. Cong. 20th, Helsinki, 1985*, p. 259.

8. Chevallier, M. R., and Aigle, M. *FEBS Lett.* 108:179, 1979.
9. Freeman, R. F. *Eur. Brew. Conv. Proc. Cong. 18th, Copenhagen, 1981*, p. 497.
10. Henderson, R. C. A., Cox, B. S., and Tubb, R. *Curr. Genet.* 9:133, 1985.
11. Hinchliffe, E. *J. Inst. Brew.* 91:384, 1985.
12. Hinchliffe, E., and Box, W. G. *Eur. Brew. Conv. Proc. Cong. 20th, Helsinki, 1985*, p. 267.
13. Livingston, D. M., and Kupfer, D. M. *J. Mol. Biol.* 116:249, 1977.
14. Meaden, P. G. T., and Tubb, R. S. *Eur. Brew. Conv. Proc. Cong. 20th, Helsinki, 1985*, p. 219.
15. Quain, D. E., Box, W. G., and Walton, E. F. *Lab. Practice* 34:84, 1985.
16. Struhl, K. D. T., Stinchcomb, S., Scherer, S., and Davis, R. W. *Proc. Natl. Acad. Sci. USA* 76:1035, 1979.
17. Tubb, R. S. *J. Inst. Brew.* 86:78, 1979.
18. Tubb, R. S., Brown, A. J. P., Searle, B. A., and Goodey, A. R. Page 75 in: Current Developments in Yeast Research. G. G. Stewart and I. Russell, eds. Pergamon Press. Toronto, 1981.
19. Wickner, R. B. *Genetics* 88:419, 1978.

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