A New Approach to the Application of Genetics to Brewing Yeast[1]

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ABSTRACT

The effect of different mutagens on brewing yeast is described together with the results of selection for strains possessing improved commercial properties. The mutagens n-methyl-n-nitroso nitroguanidine (NTG), ultraviolet light (UV), ethyl methane sulfonic acid ester (EMS), and nitrous acid were assessed by the rate of induction of genetically stable auxotrophic mutants. Commercial brewing strains of *Saccharomyces cerevisiae* and *S. carlsbergensis* were particularly sensitive to NTG and UV. Specific selective techniques were designed to isolate mutants with changed ability to synthesize diacetyl and hydrogen sulfide, and with altered degrees of flocculence. Mutants were isolated successfully for these parameters from three different strains of brewing yeast after NTG or UV treatment. The significance of these results is discussed in relation to the ploidy of the strains employed as determined by estimation of DNA content. The properties of some of these mutants compared with the parental strains show that induced mutation is an effective method for production of improved brewing yeast.

Key words: *Diacetyl, Flocculence, Hydrogen sulfide, Mutation, Selection.*

The genetic characteristics of microorganisms employed in the fermentation industries are of major significance to the efficiency of the process and to the quality, composition, and consistency of the product. Environmental controls in the fermentor are of considerable technological importance, but are secondary to the innate properties of the microorganisms themselves.

Brewers are by tradition careful guardians of the genetic characteristics of their yeasts. Much of the skill of brewing has developed from the need to modify and control the environment of the fermenting yeast in order to encourage expression of desirable properties and to suppress those which are less acceptable. All the biochemical and physical properties of brewing yeast are under genetic control to a greater or lesser extent. This means that wort production, design of fermentation vessels, fermentation procedures, yeast cropping, and conditioning of beer are all influenced by the genetic characteristics of brewing yeast. Therefore, if these inherited properties can be altered to meet specific requirements, there are opportunities for making the brewing process more efficient, easier to control, and more responsive to changing technology.

The techniques available to change the genetic characteristics of yeast are hydridization, transduction, mitotic recombination, transformation, and mutation.

Hybridization is a frequently used method with brewing strains (2,6,7,16), but it appears to have met with little commercial success. It has the disadvantages that many strains do not sporulate to give haploid progeny suitable for mating (2,16), and those that do may not produce stable diploid hybrids under brewery conditions.

Transduction is a poorly documented phenomenon in yeast (10) and, like mitotic recombination, appears to offer few advantages over simpler methods. Reports of transformation in yeast have been contradictory (4,13,14), but it may have future potential (8,13). The outstanding alternative to all of these methods is induced mutation, which is a proven technique for introducing genetic changes into yeast and other microorganisms.

Mutagenesis is widely used in the pharmaceutical industries for increasing yield of antibiotics and improving the technological properties of the strains employed (1). Compared with other techniques, the potential for positive change with mutagenesis is restricted. It is essentially a destructive procedure and abolishes or reduces metabolic activity, which imposes limits on the type of genetic change that can be achieved. However, it is a relatively simple technique compared with hybridization and highly efficient when combined with methods for direct selection of mutants with the desired properties.

This paper describes results of investigations into the application of mutagens to brewing yeast and the selection of improved mutant strains.

MATERIALS AND METHODS

Media

Three basic media were employed: a yeast complete medium (ZYCM), a yeast minimal medium (ZYMM), and hopped wort medium.

The ZYCM contained 0.3% Difco peptone, 0.5% Difco Yeast Extract, 0.5% casein hydrolysate, 4.0% glucose, and 0.4% zinc sulfate w/v. The ZYMM contained 0.67% Difco yeast nitrogen base (without amino acids), 4% glucose, and 0.4% zinc sulfate w/v. Both media were adjusted to pH 5.8. Hopped wort medium was prepared from hopped ale wort of 1.040° specific gravity with addition of 0.4% zinc sulfate. Oxoid No. 3 agar (1.5%) was added to

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these media when required.

All media were sterilized in an autoclave at ten lb/sq. in. for 10 min.

When amino acids were required they were added to media prior to sterilization. Diacetyl additions were made to cooled (49°C) medium immediately before plates were poured.

Organisms

Two commercial brewing strains of *Saccharomyces cerevisiae* (strain D.I. and strain C.A.) and one of *S. carlsbergensis* (strain C.F.) were used. A nonbrewing, haploid strain of *S. cerevisiae* (A184D)[2] was included in experiments to measure ploidy. Yeasts were maintained on ZYCM agar slopes at 4°C and subcultures onto ZYCM for 24 hr at 28°C prior to mutagenesis.

Mutagenesis

Overnight cultures on ZYCM were used for all mutagenesis procedures. Cultures were suspended in 10 ml isotonic saline at a concentration of 2×10^6 cells/ml and subsequently treated as follows. Cells were counted using an electronic cell counter (Coulter Electronics). Control cultures were prepared in an identical manner but not treated with mutagen.

n-Methyl-n-Nitroso Nitroguanidine (NTG). NTG was prepared as a stock solution (1 mg/ml) and added to yeast suspensions to give a final concentration of 25 μg/ml. Suspensions were incubated with agitation for 70 min at 30°C. Mutagenesis was stopped by washing four times in saline (10 ml).

Ethyl Methane Sulfonic Acid Ester (EMS). Cell suspensions were treated with 2% v/v EMS at 30°C with shaking for 70 min. Mutagenesis was stopped by washing once in 2% sodium thiasulfate (10 ml) and three times in saline (10 ml).

Nitrous Acid. Cultures were suspended at 10^6 cells/ml in 9 ml 0.1 M sodium acetate buffer pH 4.5. Sodium nitrite (1 ml 0.2*M*) was added and the cultures incubated with shaking for 10 min at 22°C. The suspensions were washed four times in saline (10 ml) and resuspended to 10 ml in saline.

Ultraviolet Irradiation (UV). Yeast suspensions (20 ml at 10^6 cells/ml) were induced to mutate in a 9-cm diameter glass petri dish by 10-sec exposure to a high-energy UV light source (Anderman G8, T5 UFA). The petri dish and UV light source were 12 cm apart. Cells were kept in suspension during mutagenesis with a magnetic stirrer.

Estimation of Viability

The viability of mutated cultures was estimated by viable counts on ZYCM or by viable staining with fluorescein diacetate and rhodamine B. The mutated culture was stored in saline at 4°C for 3 days during growth of colonies on the ZYCM. Estimation of viability using fluorescein diacetate (0.01%) and rhodamine B (0.025%) was a modification of the technique described by Molzahn and Portno (11) for rapid detection of viable microorganisms. A loopful of cell suspension was mixed on a microscope slide with a drop of a mixture of fluorescein diacetate and rhodamine B and examined under blue illumination (420 nm). With this technique, viable cells fluoresce bright green and dead cells appear pink.

Procedure for Detection of Mutagenic Activity

Cultures treated with mutagen were spread on ZYCM to give 40–50 colonies per plate. After 48 – 72 hr incubation at 30°C, the colonies were replicated onto ZYMM using a technique based on the replica-plating method for bacteria (9). An 11-cm diameter Whatman No. 1 filter paper was used as the replicating material held over a circular block with a narrow ring. The filter paper was sterilized by flaming lightly with a Bunsen burner while in position on the replicating block.

The ZYMM plates were incubated for 72 hr at 30°C and colony growth was compared with the corresponding ZYCM plates. Colonies growing poorly or not at all on ZYMM were scored as presumptive auxotrophs and subcultured to ZYCM for confirmatory tests.

Comparison of the number of auxotrophs from mutated and control cultures was used as an indication of the effects of mutagenesis.

Selection of Mutants with Changed Ability to Synthesize Diacetyl

Mutants of brewing yeast with changed ability to synthesize diacetyl were selected by two techniques.

Selection as Valine Auxotrophs. Mutated cultures were incubated on ZYMM containing valine, isoleucine, and leucine at 100 μg/ml, respectively. Colonies auxotrophic for valine were selected by replicating onto ZYMM, and purified by single-colony isolation before retesting on minimal medium.

Selection as Diacetyl-Resistant Mutants. Mutants resistant to the toxic effects of high concentrations of diacetyl were selected from mutated cultures on diacetyl gradient plates. These plates were prepared by pouring ZYCM (about 10 ml) containing 250 μg/ml diacetyl into a petri dish maintained at approximately 30° from the horizontal. As soon as the diacetyl medium solidified, it was overlayered with about 10 ml ZYCM, but with the petri dish in the horizontal position. The resulting wedge of diacetyl medium gave a gradient of concentrations across the surface of the medium from below 50 μg/ml to 250 μg/ml.

Mutated suspensions were spread at approximately 10^5 cells per gradient plate and incubated at 18°C for 3–5 days. Single colonies growing in front of the zone of confluent growth were subcultured for further testing.

Selection of Mutants with Reduced Ability to Synthesize Hydrogen Sulfide

Mutants with a reduced ability to produce hydrogen sulfide were selected by two methods.

Selection as Cysteine or Methionine Auxotrophs. Cysteine or methionine auxotrophs were selected by incubating mutated cultures on ZYCM containing cysteine (100 μg/ml) or methionine (100 μg/ml) for 2–3 days at 30°C and replicating onto ZYMM without amino acid additions. Selection and purification were the same as those described for valine auxotrophs.

Selection on Lead Acetate Medium. Mutated cultures were grown at 40–50 cells/plate, on ZYCM containing 0.05% lead acetate for 4 days at 30°C. Colonies which gave rise to H_2S turned black, due to formation of lead sulfide. White colonies were selected as non-H_2S producers and were subcultured on lead acetate ZYCM for purification and further testing.

Selection of Flocculation Mutants

A mutated yeast suspension (10 ml) was inoculated into hopped brewery wort (1.040° Specific Gravity) in a laboratory continuous fermentor and grown as a batch culture with continuous aeration. After 2 days, wort flow to the fermentor was started at $0.05 \times hr^{-1}$ dilution rate and increased to $0.10 \times hr^{-1}$ and $0.15 \times hr^{-1}$ after 6 and 10 days, respectively. Isolates were taken from the fermentor after 2 and 4 weeks' continuous operation and tested for flocculence using the method described by Helm *et al.* (5).

Two designs of fermentor were used in these experiments, both relying on yeast flocculence to retain a high concentration of cells. One was a simple vertical tube (8 × 600-mm) modeled on a conventional Tower fermentation system. The second was a stirred chemostat of 1-liter capacity with a small still zone on the outlet to prevent excessive yeast loss in the effluent beer. In both cases, increasing flow rates resulted in preferential washout of less flocculent strains.

Determination of Yeast Ploidy

Yeast ploidy was estimated by measuring the deoxyribonucleic acid (DNA) content per cell and comparing that with values obtained for a known haploid strain of *S. cerevisiae*.

Overnight cultures from ZYCM were inoculated into 250 ml

[2]A184D was provided by R. A. Wood, Department of Genetics, Sheffield University.

ZYCM at 10^6 cells/ml and incubated for 48 hr at 30°C with shaking. Cultures were harvested by centrifugation, washed twice in cold distilled water, and resuspended to 50 ml. Flocs of yeast were disrupted by shaking for 5 min on a rotary shaker, and the cell count was measured with an electronic counter (Coulter Electronics). The dry weight of yeast in suspension was measured by filtering through tared membrane filters (0.45 μ) and drying at 85°C to constant weight. Twenty-five milliliters of cell suspension was disrupted with an X-press (LKB Instruments Ltd), and DNA was extracted according to the technique described by Ogur *et al.* (12). DNA was estimated by a diphenylamine assay for deoxypentose using salmon sperm DNA as a standard.

Trial Fermentations

Mutants were screened in various fermentation systems using hopped brewery wort of 1.040° specific gravity. Fermentations of large numbers of mutants were performed in test tubes (22 × 100-mm) at 20°C inoculated with a standard loop from an overnight culture on ZYCM. A smaller number of strains was tested in EBC 2-liter tall tube fermentors or in 700-ml fermentations in 2-liter flat-bottomed flasks. A few strains were selected for trials in a model brewery using 30-hl fermentations.

RESULTS

Effect of Mutagens on Brewing Yeast

The frequency of auxotrophic mutants induced by treatment of brewing yeast with different chemical and physical mutagens, together with the spontaneous mutation rate of cultures not treated with mutagens, is shown in Table I. Mutants were classified as tight or leaky depending on the severity of their nutritional requirements. A third group, classified as revertant, was characterized initially as auxotrophs, but proved unstable when subcultured and quickly reverted to a prototrophic phenotype.

TABLE I
Induction of Auxotrophic Mutants from Brewing Yeast with Chemical and Physical Mutagens

Strain	Mutagen	% Auxotrophs Recovered		
		Tight	Leaky	Revertant
S. cerevisiae (strain D.I.)	NTG	3.06	16.11	5.25
	EMS	0	0	0.43
	UV	0.93	0.04	1.25
	Nitrous Acid	0	0	3.68
	Control	0	0.06	0.12
S. carlsbergensis	NTG	0.41	5.61	1.10
	EMS	0	0	3.92
	UV	0.14	0	3.33
	Nitrous Acid	0	0	1.93
	Control	0	0	0.13

TABLE II
Effect of Mutagens on the Survival of Brewing Yeast

Mutagen	% Survival	
	S. cerevisiae (strain D.I.)	*S. carlsbergensis* (strain C.F.)
NTG	6.0	4.0
EMS	20.0	1.0
UV	1.45	0.11
Nitrous Acid	70.0	70.0

The spontaneous mutation rate of the brewing strains used in these investigations was low, as indicated by the frequency of auxotroph recovery. Strain D.I. (*S. cerevisiae*) was more unstable than strain C.F. (*S. carlsbergensis*), giving two leaky (0.06%) and four revertant colonies (0.12%) from the 339 colonies screened. Strain C.F. produced no stable spontaneous auxotrophs, but gave four unstable (0.13%) revertants from 3298 colonies tested. Exposure of either strain to any of the four mutagens listed in Table I substantially increased the rate of mutation and gave a higher frequency of stable and unstable auxotroph production.

The mutagens varied in their effectiveness, particularly when judged by the frequency of induction of stable auxotrophs. NTG was the most potent mutagen, with 19.2% of the survivors of strain D.I. after treatment classified as tight or leaky auxotrophs. Strain C.F. was slightly less sensitive to the effects of NTG and yielded 6.02% stable auxotrophs. High-energy UV irradiation gave a lower frequency of mutant production than NTG. Strain D.I. again proved more sensitive than strain C.F. with 0.97% and 0.14% auxotrophs, respectively.

EMS and nitrous acid were substantially less effective mutagens than NTG or UV. Neither mutagen induced stable auxotrophs in either strain but both successfully increased the frequency of unstable revertant colonies. For selection of improved brewing strains, it was essential to have stable mutants, and therefore NTG and UV were used for subsequent mutagenis.

There was a significant difference in the proportion of tight and leaky auxotrophs induced by NTG or UV. When strain D.I. or C.F. was treated with NTG, the majority of stable mutants were classified as leaky. Conversely, exposure to UV produced only one leaky mutant from strain D.I., and none from strain C.F.

The survival rates of strain D.I. and strain C.F. after mutation varied according to the mutagen employed, and these are summarized in Table II. UV and NTG, the mutagens with the greatest capacity to induce auxotrophs, gave the lowest survival rates. There was no direct relation between mutagen efficiency and survival rate. In general, strain C.F. was more susceptible to the lethal effects of mutagens than strain D.I.

Isolation of Mutants with Changed Ability to Synthesize Diacetyl

The most direct method for recognition of diacetyl mutants would be by detection of diacetyl produced by single colonies growing on solid medium. Attempts to develop such a method were not successful and therefore alternative techniques were applied.

Selection of Valine Auxotrophs. Diacetyl is formed by the spontaneous oxidative decarboxylation of acetolactate, an early intermediate in the biosynthesis of valine. The relation between diacetyl and valine synthesis suggested that examination of valine auxotrophs of brewing yeasts might allow changed levels of diacetyl production to be achieved.

The results of selection for valine auxotrophs from brewing strains of *S. cerevisiae* (strain D.I.) and *S. carlsbergensis* (strain C.F.) are shown in Table III. Valine auxotrophs were induced from both yeasts with NTG, but only from strain D.I. with UV treatment. The frequency of selection shown in Table III illustrates

TABLE III
Diacetyl Mutants—Mutation and Selection of Valine Auxotrophs

Strain	Mutagen	% Valine Auxotrophs Recovered
Strain D.I.	NTG	0.75
Strain D.I.	UV	0.04
Strain C.F.	NTG	0.72
Strain C.F.	UV	0

the relative efficiency of each mutagen. The true mutation frequency for valine auxotrophs was lower because mutated cultures were first screened against all other auxotrophic mutants by growth on minimal medium plus valine, leucine, and isoleucine. NTG was considerably more effective than UV at inducing valine auxotrophs.

Selection of Diacetyl-Resistant Mutants. Many brewing yeasts are able to reduce diacetyl (produced during fermentation) to acetoin, but in high concentrations diacetyl will inhibit yeast growth. Therefore, it was proposed that a mutant resistant to high diacetyl concentrations might exhibit increased rates of diacetyl removal and so produce a beer with lower levels than the original strain.

The growth of strains D.I. and C.F. was inhibited by concentrations of diacetyl above 50 μg/ml. Attempts to isolate diacetyl-resistant mutants on solid medium containing diacetyl were unsatisfactory because of the volatile nature of the inhibitor. Adoption of diacetyl gradient plates comprising a wedge of diacetyl medium overlayered with ZYCM gave more reliable results.

Cultures of strain D.I. or C.F. treated with NTG or UV and spread on gradient plates at approximately 10^6 cells/plate gave confluent growth in a well-defined section of the plate. Colonies growing well ahead of the zone of inhibition were isolated from both strains and classified as diacetyl-resistant mutants. One or more mutants were isolated from each gradient plate.

Fermentation Characteristics of Valine Auxotrophs and Diacetyl-Resistant Mutants. A summary of the fermentation properties and diacetyl production characteristics of valine auxotrophs and diacetyl-resistant mutants is shown in Table IV. Fermentations were carried out in small-scale test tube fermentors.

Several mutants produced less diacetyl after a 4-day fermentation than the original culture, but this was often accompanied by poor fermentation performance. A few strains gave beers with very high levels of diacetyl, in excess of four times that produced by controls. There were also significant differences in the size and character of the yeast crop with some mutants.

Some of the diacetyl-resistant strains of *S. carlsbergensis* (C.F.) gave very low levels of diacetyl combined with good fermentation performance. The results of two fermentations with six of these strains are summarized in Table V. Each mutant fermented as well or better than the original yeast and, despite some variation in the overall levels of diacetyl, the mutants generally produced less. The rate at which diacetyl was reduced by the six mutants in Table V was investigated by adding 3 μg/ml diacetyl 21 hr after the start of fermentation in tall tubes. Samples taken over the following 72 hr failed to show any significant difference in the rate of disappearance of diacetyl between mutants and wild type.

TABLE IV
Fermentation Properties and Diacetyl Production of Valine Auxotrophs and Diacetyl-Resistant Mutants

	Valine Auxotrophs		Diacetyl-Resistant	
Gravity °Plato after 4 days	No. of mutants	Diacetyl range mg/l.	No. of mutants	Diacetyl range mg/l.
S. cerevisiae, strain D.I.				
Controls				
2.2 – 2.7	...	0.6 – 0.7	...	0.6 – 0.7
2.5 – 3.7	1	0.6	2	0.8 – 1.0
3.7 – 5.0	14	0.4 – 1.9	2	0.7 – 0.75
5.0 – 7.5	31	0.35 – >2.0	1	0.7
7.5 – 10.0	7	0.15 – 1.30	0	...
S. carlsbergensis, strain C.F.				
Controls				
2.0 – 2.5	...	0.2 – 0.5	...	0.2 – 0.5
1.2 – 2.5	3	0.1 – 0.7	3	0.1 – 0.5
2.5 – 3.7	18	0.1 – 0.8	14	0 – 1.10
3.7 – 5.0	8	0.26 – 0.9	2	0.3 – 1.10
5.0 – 7.5	6	0.2 – 1.3	0	...
7.5 – 10.0	0	...	0	...

Isolation of Flocculation Mutants

Mutants of *S. carlsbergensis* (C.F.) with altered potential for flocculence were selected by inoculating a culture treated by exposure to UV light into a stirred laboratory-scale continuous fermentor. After an initial period of growth and stabilization under batch fermentation conditions, followed by progressive increase in wort flow, 20 single-colony isolates were taken from the fermentor. These strains were tested for flocculence using the method described by Helm *et al.* (5), and compared with isolates taken from a control fermentor inoculated with a nonmutated culture.

The results showed that all 20 isolates failed to form a defined clear zone in the buffer solution and were clearly less flocculent than the controls.

Selection of flocculation mutants in a small-scale Tower fermentation system gave different results from the chemostat. An NTG mutated culture of *S. carlsbergensis* was used as the inoculum and, after 4 weeks' continuous operation, 50 single-colony isolates were taken from the yeast plug formed in the fermentor. A control inoculated with a nonmutated culture was operated alongside the trial fermentor. During continuous operation, the wort flow was progressively increased to promote loss of less flocculent cells in the effluent beer.

Helm test analysis showed that three isolates from the trial fermentor were more flocculent than the controls. They flocculated faster and produced smaller, more compact sediments. The remaining 47 isolates exhibited similar flocculation properties to the controls.

Isolation of Mutants with Reduced Ability to Synthesize Hydrogen Sulfide

Methionine and cysteine are implicated in the formation of hydrogen sulfide by brewing yeast. Accordingly, auxotrophs for

TABLE V
Diacetyl-Resistant Mutants of *S. carlsbergensis*—Fermentation Performance and Production of Diacetyl

	Fermentation Number			
	1		2	
	Gravity[a]	Diacetyl[b]	Gravity[a]	Diacetyl[b]
Wild Type Control	3.6	0.6	3.2	0.3
M139	3.1	0.3	2.0	0.1
M143	3.4	0.3	3.4	0.1
M147	3.2	0.5	...	...
M148	3.5	0.3	2.4	0.1
M149	2.5	0.4	2.7	0
M150	2.6	0.5	...	...

[a]Gravity, °Plato.
[b]Diacetyl, mg/l.

TABLE VI
Selection of Hydrogen Sulfide Mutants of *S. cerevisiae*, Strain C.A.

Selective Technique	% Recovery
Methionine auxotrophs	0.06
Cysteine auxotrophs	0.29
Lead acetate medium	2.27

these amino acids were selected and tested for H_2S production. Mutants were also isolated from ZYCM containing lead acetate, which distinguished between black colonies producing H_2S and white non-H_2S producers.

Methionine and cysteine auxotrophs were selected from NTG mutated cultures of strain C.A. by conventional replica plating techniques at the frequency shown in Table VI. Cultures were screened first on minimal medium plus either cysteine or methionine, and then replicated to minimal medium without additions. Only one methionine auxotroph was isolated; this was leaky and had no stringent requirement for the amino acid. Selection for cysteine auxotrophs yielded 20 mutants, of which 7 were tight and 13 were leaky.

Mutants unable to synthesize H_2S were selected from strain C.A., treated with NTG, and grown on ZYCM containing 0.05% lead acetate. Most of the colonies were dark brown in color, but a small number were white, and these colonies were selected as hydrogen sulfide negative mutants (Table VI). Repeated subculture on lead acetate medium showed that the white phenotype was stable.

Growth of the methionine and cysteine auxotrophs on lead acetate medium was used as a screen for H_2S production. Colonies of the leaky methionine auxotroph were light gray compared with the much darker brown/black color of controls. This was interpreted as reduced H_2S production in the mutant. Among the 20 cysteine auxotrophs, 12 gave white colonies on lead acetate, while the remaining 7 were the same color as wild type.

Fermentation Properties of H_2S Mutants

Results from a series of 30-hl draught beer fermentations with an H_2S mutant isolated from lead acetate medium showed that the mutant fermented at an equivalent rate to the control and gave a beer which fined and conditioned normally.

The GLC headspace analysis of control and trial beers was very similar, and typical results are shown in Table VII. Flavor and aroma assessment of the two beers showed little difference except for the presence of H_2S. The control beer had a distinctive sulfury aroma, while the trial beer fermented with the mutant had a clean aroma and flavor without the presence of sulfur.

TABLE VII
GLC Headspace Analysis of Beer Volatiles from H_2S Mutant of *S. cerevisiae*

	Wild Type Control mg/l	Mutant mg/l.
n-Propanol	13	15
Isobutanol	18	21
Isoamyl alcohol	71	74
Ethyl acetate	16	17
Isoamyl acetate	2.04	2.35
Ethyl caproate	0.20	0.18
Isobutyl acetate	0.05	0.12
Ethyl n-butyrate	0.03	0.03

TABLE VIII
Determination of the Ploidy of Brewing Yeast—DNA Content per Yeast Cell

Strain	μg DNA/cell[a]	Ploidy[b]
Haploid control A184D	0.413×10^{-7} (± 0.051)	1.0
S. cerevisiae, strain D.I.	1.804×10^{-7} (± 0.153)	4.4
S. carlsbergensis, strain C.F.	1.741×10^{-7} (± 0.459)	4.2
Mutant 128 (strain C.F. valine auxotroph)	1.569×10^{-7}	3.8

[a]Average values based on five independent analyses. Results for Mutant 128 are from one analysis only and derived from a direct comparison with the parent strain.

[b]$\text{Ploidy} = \frac{\mu\text{g DNA/cell}}{\mu\text{g DNA/cell A184D}}$

Genetic Stability of Mutants of Brewing Yeast

Six auxotrophic mutants from strain D.I. and four from strain C.F. were serially subcultured on ZYCM with 3–5 days' growth between each stage. At each transfer, growth on ZYMM was tested by replication. One mutant from each strain showed progressive reversion to prototrophy. The remaining mutants retained their auxotrophic phenotype and failed to grow on ZYMM after ten serial transfers.

H_2S mutants of strain C.A. have been shown to retain their mutant phenotype after serial repitching in 30-hl brewery fermentations. Similarly, no revertant cells were detected in a laboratory continuous fermentor inoculated with an H_2S mutant and operated continuously for 4 weeks.

Determination of the Ploidy of Brewing Yeast

The results for brewing strains of *S. cerevisiae* (D.I.), *S. carlsbergensis* (C.F.), and a valine auxotroph of strain C.F. are shown in Table VIII. Results are expressed as μg DNA per cell and compared with the result for a haploid strain of *S. cerevisiae* (A184D) to give an indication of ploidy.

Both brewing strains were shown to contain four times as much DNA per cell as the haploid strain. Similarly, the valine auxotroph derived from strain C.F. by NTG treatment had at least three times as much DNA as cells of A184D. Therefore it can be assumed that both parental strains and an auxotroph derived from one of them have a triploid or tetraploid chromosome complement.

DISCUSSION

The results described show that it is possible to induce stable genetic mutations into commercial strains of brewing yeast. Furthermore, with suitable selective techniques it is possible to isolate mutant strains with characteristics of potential commercial significance.

The high frequency at which mutations were expressed in brewing yeast was unexpected. Previous reports (3,15) have suggested that induced mutation might be possible, but theory dictates that polyploid strains of yeast should appear resistant to the effects of mutagens. The majority of mutations are expected to be recessive and therefore not expressed against the background of wild type genes.

The results show that the strains used in these investigations were possibly tetraploid but the effect of NTG or UV treatment was clearly to increase the rate of mutation. A few mutations could be dominant and this may account for some results, but it is particularly unlikely in the case of auxotrophic mutants. Alternatively, the parental strains could be aneuploid, possessing a single chromosome in which recessive mutations would be expressed. Again, this is unlikely to be the complete explanation because of the variety of mutants isolated from different species of yeast with diverse genetic backgrounds. An alternative explanation ascribes the success of mutagenesis to an increased rate of mitotic crossing-over, which would lead to homozygosity for a proportion of recessive alleles.

Two different sets of diacetyl mutants were isolated. The valine auxotrophs generally fermented less well and produced more diacetyl than the diacetyl-resistant mutants. The high levels of diacetyl from valine auxotrophs are probably caused by metabolic blocks in the valine pathway after acetolactate. Accumulation of acetolactate would therefore lead to increased decarboxylation to diacetyl. The diacetyl-resistant mutants were isolated in order to

achieve increased rates of diacetyl reduction. Despite lower final levels of diacetyl in some beers, there was no significant difference between mutant and wild type in the rate of removal of added diacetyl. The mechanism for the improved performance of these strains is not known.

The relatively poor fermentation performance of valine auxotrophs suggests that auxotrophs are not a suitable class of mutant for brewery application. There is a high probability that any auxotroph would be at a selective disadvantage under brewery conditions. Revertant strains or prototrophic contaminants would have a substantial advantage over a mutant limited by supplies of a wort component. Consequently, selection for H_2S mutants on lead acetate medium is preferable to selection of methionine or cysteine auxotrophs. This does not preclude the use of such mutants for investigating the role played by sulfur-containing amino acids in beer flavor and aroma. Similarly, other classes of auxotroph may assist our understanding of the various roles played by brewers' yeast in affecting beer quality.

The selection of mutants with both increased and decreased powers of flocculence illustrates an important aspect of this work for the future. Mutation generally acts in a destructive manner and retards or prevents a metabolic process from occurring. Therefore, the selection of less flocculent strains was expected, but the appearance of cells with greater flocculence demonstrates the positive potential of mutation. In this case, mutation has probably removed some regulating or controlling element of flocculence and promoted an increased affinity between cells.

In summary, mutation has been shown to be a valuable tool for eliminating unwanted characteristics and, in certain circumstances, for introducing positive and desirable properties into yeast. Mutation can therefore be regarded as a relatively simple and certainly attractive alternative to the technique of hybridization for improving and modifying the behavior of commercial brewing yeasts.

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