Can a Genetically Manipulated Yeast Strain Produce Palatable Beer?[1]

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ABSTRACT

Diploid yeast strains have been produced containing a single gene for flocculation (FLO 4) and the intensity of the phenotypic expression of such strains has been found to vary, the flocculation intensity being modified or suppressed by an unknown factor(s). Although ale strains belong to the species *Saccharomyces cerevisiae* (melibiose negative) and lager strains to the species *S. uvarum (carlsbergensis)* (melibiose positive), hybridized diploids melibiose positive (MEL) and negative (mel) do not produce "typical" lagers and ales, respectively. It is concluded that ale and lager strains are polyploid cultures forming a small grouping within their species. The ability to ferment maltose and maltotriose is controlled by at least six polymeric gene systems (MAL genes). Each gene system's ability to outferment wort varies; MAL 6 is the most capable, whereas MAL 1 is incapable of using maltotriose. In the near future, it will be possible to construct yeast strains—probably polyploid in nature—capable of completely fermenting a wort in static culture and sedimenting out of suspension at the appropriate time. Although the problem of producing a palatable beer still remains, the use of polyploid yeasts may overcome these difficulties.

Key words: *Fermentation, Flocculation, Genetics, Maltose/maltotriose, Yeast.*

The title of this paper is a paraphrase of a question that was asked at the 1976 MBAA Convention (34). Since the reply was less than adequate, it failed to do justice to a very important topic; this paper attempts to consider, in greater depth, the progress made in constructing, via genetic manipulation, novel brewing yeast strains. Although studies in this field have been ongoing for 40 years, developments have been slow and there are still more questions than answers; posing the title of this paper as a question would therefore seem appropriate.

It was in 1935 that Winge (44), working at the Research Laboratories of Carlsberg, in Copenhagen, demonstrated that, in the normal situation, strains of *Saccharomyces* are diploid in the vegetative phase; meiosis precedes sporulation and four haploid spores are formed in the ascus (Fig. 1). Parenthetically, during these studies, Winge commented on the difficulties of genetically manipulating yeast strains to produce palatable beer, "This difficulty is particularly pronounced with regard to beer and wine yeasts, as no objective method for analyzing the quality of the yeast is available...undefined demands cannot be answered to order."

Although the life cycle of *Saccharomyces* normally alternates between haploid and diploid states, yeast cells of higher ploidy have been derived. Some of the possible sequences of events leading to polyploid yeasts are summarized in Fig. 2. Brewer's yeast strains are often polyploid in nature (36) and, as a consequence, have a low degree of sporulation and poor spore viability, rendering genetic analysis of such strains extremely difficult. However, as will become clear in the **DISCUSSION**, polyploidy tends to confer a high degree of genetic stability on such strains.

The virtues of yeast as a research tool for detailed genetic analysis are widely recognized (25,26,32), and it has become an ideal eucaryote (*i.e.*, it is composed of cells possessing a well-defined nucleus surrounded by a definite membrane) for biochemical and genetic studies. While yeasts have greater complexity than bacteria, they still share many of the biochemical advantages which have permitted the rapid progress in molecular genetics of procaryotes (*i.e.*, they possess cells where the nucleus is usually poorly defined and lacking in a clearly distinct nuclear membrane) and their

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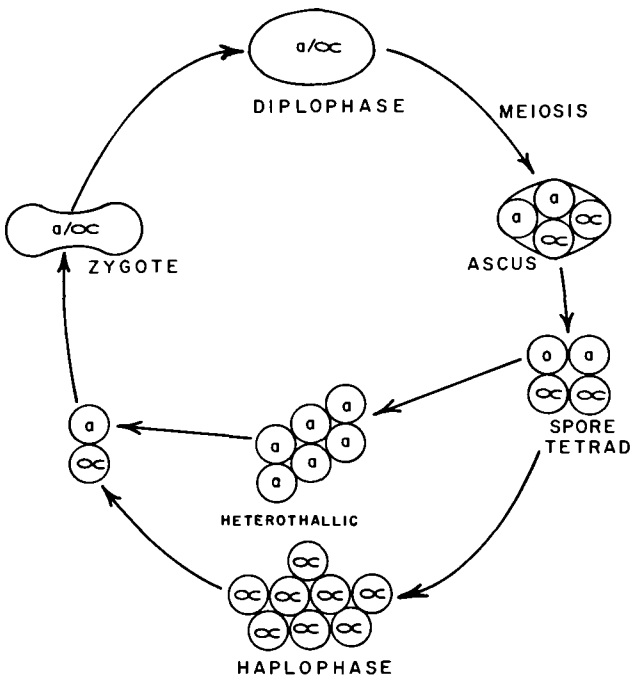

Fig. 1. Haploid/diploid life cycle of *Saccharomyces* sp.

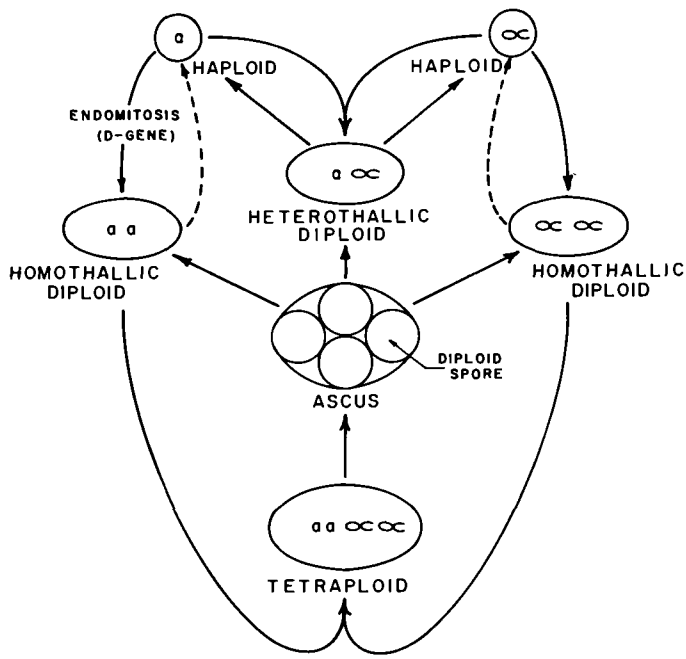

Fig. 2. Development of polyploid yeast cultures.

viruses. Some of the properties which make yeast particularly suitable for genetic studies include the existence of both stable haploids and diploids, rapid growth, ability to form clones, ease of replica plating, and mutant isolation. Yeast has been successfully employed in all phases of genetics, such as mutagenesis, mitotic recombination, regulation via gene action (especially mitochondrial genetics), and other aspects peculiar to eucaryotic systems.

In 1956, the first paper on yeast genetics was presented to the American Society of Brewing Chemists by Lindegren (19) when he considered "The Possible Application of Yeast Genetics to Brewing" and concluded that, "If the brewer can prepare the specification, the geneticist can tell him what characteristics are hereditary and what kinds of yeast he can produce." In 1971, Emeis (5) considered "A New Hybrid Yeast for the Fermentation of Wort Dextrins," and reported on the development of a hybrid strain capable of fermenting wort dextrins containing in excess of 20 glucose units; the resulting beer was unfortunately unpalatable. Emeis does report (5) however that "acceptable beer" was produced from a hybrid capable of fermenting wort dextrins that resulted from repeated crossings with a bottom-fermenting brewer's yeast and dextrin-using haploid. The following year, Anderson (*née* Clayton) *et al.* (4), in a paper entitled "Yeast Hybridization," discussed hybridization as a possible technique for improving traditional brewing strains and introducing properties required by modern technology while retaining the characteristic product flavors. Although a few hybrids of interest were isolated, the study suffered from the fact that a mass-mating technique was routinely used and, consequently, little of the genotypic makeup of the resulting hybrids could be deduced. In 1975, Stewart *et al.* (36) assessed the relative contributions of phenotype (*i.e.*, the nurture effect) and genotype (*i.e.*, the nature effect) to flocculation in a number of yeast strains. It was concluded that both factors play a vital role; however, genetic factors are of paramount importance. Finally, Molzahn (24), in "A New Approach to the Application of Genetics to Brewing Yeast," reported on an investigation into the application of mutagenics to brewing yeast and the selection of improved mutant strains.

In this paper, three examples of the genetic studies currently ongoing in the authors' laboratory will be discussed; these will serve to illustrate some of the current possibilities available to brewing microbiologists that they may gain a better understanding of the overall physiology and genetics of brewing-yeast strains. Evidence is also presented to illustrate that the breeding of yeast strains for use in brewing is no longer a utopian or unattainable goal but a reality simply requiring some further development.

EXPERIMENTAL

Yeast Strains

A number of *Saccharomyces cerevisiae* (ale) and *S. uvarum (carlsbergensis)* (lager) were studied; they are referred to in the text by their number in the Labatt Culture Collection. A number of haploid strains with known genetic markers have been used in this work and are listed in Table I.

Genetic Manipulations

The induction of sporulation, isolation of spores, determination of mating type and hybridization were as previously described (38). Genetic linkage and chromosome location were determined using the methodology described by Mortimer and Hawthorne (25,26) and by Sherman *et al.* (32). The auxotrophic strains listed in Table I were used as marked cultures for this purpose.

Flocculation Tests

The two *in vitro* tests and the *in vivo* test described previously (37) were used as the assay methods for flocculence.

Sugar-Fermentation Tests

The ability of yeast strains to ferment a number of sugars was studied using 2% (w/v) sugar in 0.67% (w/v) yeast nitrogen base media (Difco).

Fermentation Tests

The yeast strains were precultured in hopped wort (11.8° P, 30% adjunct) at the same temperature required in the static fermentation. The yeast was subsequently inoculated into 36 l. of 11.8° P wort in a 40-l. unstirred glass fermentor (inoculation level of 0.25%, w/v, wet weight of cells). Sequential wort samples were taken throughout the fermentation and the specific gravity and concentration of yeast in suspension were determined on each sample. The fermentability of the wort with each yeast culture was determined by incubating 200 ml of the 11.8° P wort with 4 g of yeast in a 300-ml flask on a New Brunswick Gyrotory shaker at 160 rpm for 72 hr. The difference between the original gravity and the gravity after shaken fermentation represents 100% fermentability for the wort and the yeast culture in question at a particular incubation temperature.

Post-Fermentation Processing

After 7 days' fermentation, the fermented "beers" were stored at 0° C for another 7-day period under CO_2 pressure (approximately 30 atmospheres). The beers were subsequently filtered, sodium metabisulfite and chillproofing enzymes added, and they were then stored for an additional 7 days at 0° C under CO_2 pressure, at which time they were refiltered. The bright beer was hand-bottled, crowned, and pasteurized.

Beer Analysis

SO_2 and diacetyl levels were measured in the dropped beer samples by the methods of Stone and Laschiver (39) and Haukali

TABLE I

HAPLOID STRAINS OF SACCHAROMYCES Sp.

STRAIN	Degree of Flocculation	GENOTYPE
1176	F – 0	*a, MAL, mel, SUC .*
1190	F – 0	*a, MAL 2, MEL1, suc.*
1191	F – 0	*a, MAL 3, MEL1, SUC 3.*
1209	F – 5	*α, FLO4, MAL.*
1210	F – 2	*a, FLO4, MAL.*
1239	F – 5	*α, FLO4, MAL, mel.*
1240	F – 0	*α, MAL, MEL.*
1241	F – 0	*α, MAL, mel.*
1242	F – 0	*α, MAL, MEL.*

and Lie (13), respectively. The bottled beers were analyzed for H_2S (1), DMS, aliphatic higher alcohols (43), and aromatic higher alcohols (41).

Taste Assessment

Bottled beers were subjected to taste panel assessment using the profile techniques of Clapperton (2).

α-Glucosidase Activity

α-Glucosidase activity of cell-free extracts of yeast was determined by the method of Halvorson (9) using the techniques described by Stewart (33).

Wort Sugar Analysis

Wort sugars were quantitatively determined by the gas-chromatography method of Clapperton and Holliday (3) as described by Pfisterer (28).

RESULTS

1. Manipulation of the Flocculation Characteristics of Hybrids

Previous studies in this laboratory (38) have identified and mapped a dominant gene for flocculation (FLO 4). The gene is located on the left arm of Chromosome I, 32 cM from the centromere (Fig. 3). The FLO 4 gene has been identified as the sole-gene coding for flocculation in a number of haploids. However, the degree of flocculation intensity has been found to vary (Table I: cf. 1209, F-5, and 1210, F-2), indicating some form of gene modification or suppression. A haploid strain (1209) displaying intense flocculation (F-5) was crossed with a nonflocculent haploid (1191) that possessed the ability to use the five fermentable-wort sugars and the fermentation characteristics of the resulting diploid were then studied under static-fermentation conditions in an 11.8° P wort (Fig. 4). The diploid proved to be too flocculent and consequently only 55% of the wort was attenuated.

A second diploid was constructed possessing a less intense flocculation (F-3), crossing a FLO-4-containing haploid (1239) (F-5) with a nonflocculent wort-sugar-fermenting haploid (1176). This diploid was considerably less flocculent in static fermentation (Fig. 4) than the first diploid; nevertheless, only 90% of the wort attenuated due to premature flocculation of the yeast. A further diploid was developed, crossing a strain (1240) lacking the FLO 4 gene (*i.e.*, recessive flo 4) (F-0) with the nonflocculent haploid 1176. The resulting strain, as well as being nonflocculent (F-0), exhibited good fermentation ability and fully attenuated the wort (Fig. 4). However, after 7 days' fermentation, a considerable quantity of yeast remained in suspension (Fig. 5) and a further 2–3 days' incubation was required to reduce it to less than 0.1% (w/v); nonflocculent diploids, therefore, would appear to be unsuitable for brewing purposes unless the yeast is to be centrifuged out of suspension. In order to achieve acceptable yeast sedimentation within normal production schedules and obtain complete wort attenuation, a strain is required which contains a gene for flocculation wherein the intensity of the phenotypic expression is reduced, as compared to flocculent diploids constructed to date.

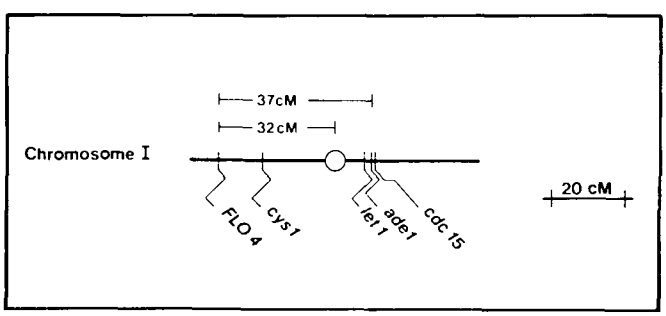

Fig. 3. Location of FLO 4 gene.

2. Genetic Differences between Ale and Lager Yeast Strains

The taxonomic differentiation (20) between ale (*i.e.*, *S. cerevisiae*) and lager [*i.e.*, *S. uvarum (carlsbergensis)*] strains is made on their sugar fermentation capability. Lager strains are able to ferment the disaccharide melibiose, whereas ale strains are unable to ferment this sugar (Fig. 6). *S. uvarum (carlsbergensis)*

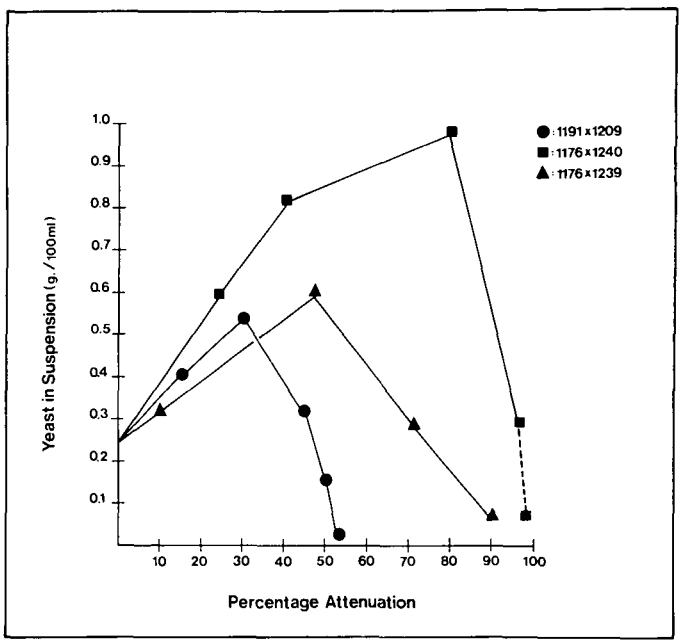

Fig. 4. Static fermentation characteristics of flocculent and nonflocculent hybrids.

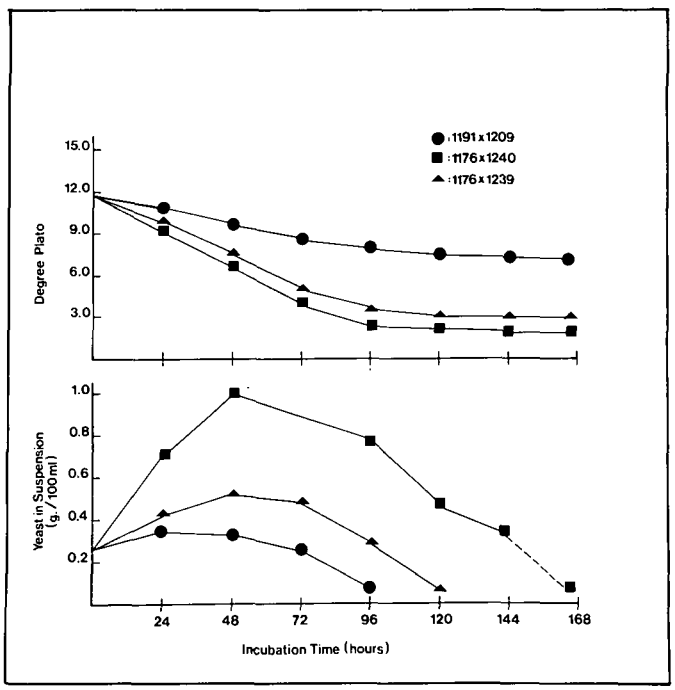

Fig. 5. Static fermentation characteristics of flocculent and nonflocculent hybrids.

produces the extracellular enzyme melibiase which hydrolyzes melibiose into its constituent monosaccharides (*i.e.*, glucose and galactose); these are then taken up and metabolized by the cell. The production of melibiase is controlled by the MEL genes which have been identified in *S. uvarum (carlsbergensis)* (16); in *S. cerevisiae* strains, the melibiase genes are absent or recessive (*i.e.*, mel). To ascertain whether the characteristic "lager" and "ale" flavor could be attributed to the taxonomic difference *per se,* diploids were constructed that were dominant—*S. uvarum (carlsbergensis)*—and recessive—*S. cerevisiae*—for melibiose fermentation, and the beers produced from such strains were studied.

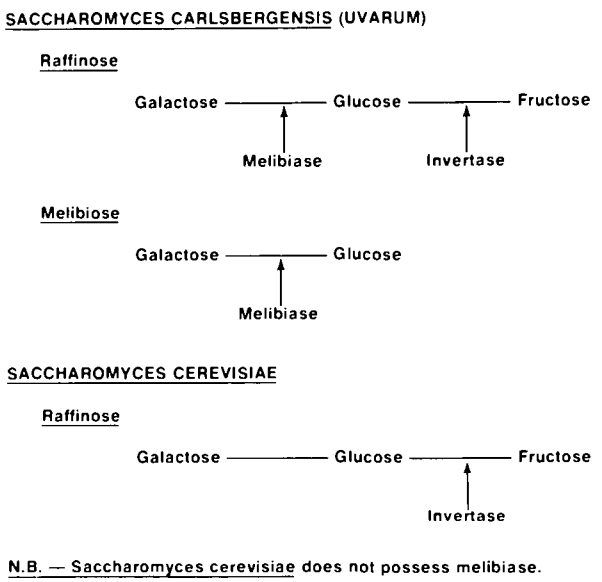

Fig. 6. Taxonomic difference between *Saccharomyces carlsbergensis* and *S. cerevisiae.*

Initially, it was necessary to answer the question, "What are the distinctive characteristics between lager and ale that can be directly attributed to the yeast species (*i.e.*, the genotype)?" A detailed account of studies attempting to answer this question will be published elsewhere and, for the sake of brevity, only a précis of the results will be discussed in this paper. Three typical production ale strains (mel) and three typical production lager strains (MEL) were selected for study. Their fermentation characteristics were examined at 15° and 21°C in an 11.8°P wort; extreme care was taken to adapt the culture to the appropriate fermentation temperature. A comparison of the fermentation rates and sedimentation characteristics of the strains revealed few differences that could be directly attributed to the two species of yeast (Fig. 7). It is interesting to note, however, that all strains exhibited increased sedimentation rates at 15°C, as compared to 21°C; this resulted in a more complete fermentation at 21° than at 15°C.

Analysis of the beers at the point of dropping of the fermentation into aging revealed interesting similarities and differences between those fermented with ale and those fermented with lager yeast strains (Table II). The concentration of diacetyl was below 0.1 mg/l. in all cases, with no apparent influence of yeast genotype or incubation temperature; pH levels were consistently between 4.2 and 4.4. The concentration of free SO_2 revealed an interesting difference between ale and lager yeast strains. The level of SO_2 in fermented wort with lager strains was between 6 and 11 mg/l. whereas, with ale strains, it was consistently below 1 mg/l.; there was no influence of incubation temperature on the SO_2 level at the end of fermentation.

Analysis of the finished beers indicated differences between those produced from ale and lager strains (Table III); there was little variation within a yeast species, *i.e.*, the three strains of both ale and lager yeasts gave similar analytical data. Beer produced from lager strains had higher levels of H_2S, tyrosol, and phenylethanol and lower levels of tryptophol (a finding confirming the earlier report of Szlavko); the levels of DMS, esters, and aliphatic fusel oils were similar in all products.

Profile taste assessment revealed that ale strains are considered to produce beer with a "hoppy" and "estery" note, whereas lager strains produced beer with a "sulfury" and "yeasty" character, this being the case at both fermentation temperatures. Taste panel members were able to distinguish between the beers produced from

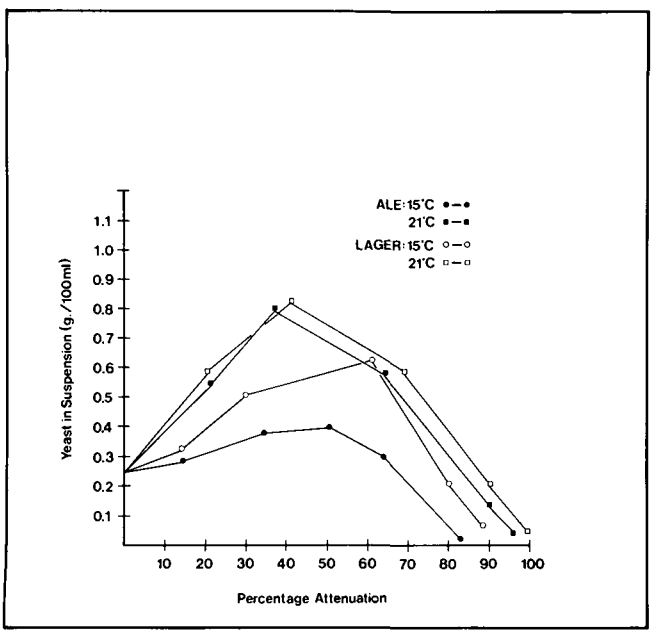

Fig. 7. Static fermentation characteristics of "typical" ale and lager yeast strains.

TABLE II

ANALYSIS OF FERMENTED WORTS PRODUCED FROM "TYPICAL" ALE AND LAGER YEAST STRAINS

	ALE		LAGER	
	15°C[t]	21°C	15°C	21°C
Diacetyl[a]	0.05	0.06	0.07	0.06
SO_2[a]	<1	<1	9	10
pH	4.2	4.2	4.3	4.3

[a] mg/l

[t] fermentation temperature

ale strains but were unable to distinguish between the beers produced from lager strains. Furthermore, the panel was able to distinguish between beers fermented at the two temperatures from the lager strains (more sulfury at 21°C) but differences between beers produced at the two fermentation temperatures with ale strains were undetectable.

These studies would indicate that a single strain of yeast cannot be used to produce both ale and lager beers without significant manipulation of the wort composition (*e.g.*, different malts). Yeast strains contribute to the final product in a major way, with marked differences between ale and lager yeasts. With regard to the flavor of the final product, there is little strain-to-strain variation with lager yeasts, whereas it is quite marked with ale strains. Finally, lager yeast strains would appear to be more sensitive to fermentation temperature changes than ale yeast strains.

With this background information indicative of certain basic differences between ale and lager yeast strains, a number of diploids were constructed, some of which possessed a gene for melibiose fermentation (MEL), whereas others were recessive (mel) for this trait. In other words, diploids of *S. uvarum (carlsbergensis)* and *S. cerevisiae* were produced, and it was of interest to examine their brewing characteristics to see if they reacted as ale or lager strains. To this end, the diploids (both MEL and mel), together with their constituent haploids, were fermented in an 11.8°P wort at 21°C, aged, filtered, and bottled. Analysis of the fermented worts at dropping (Table IV) revealed normal levels of diacetyl and pH. The SO_2 levels were all less than 1 mg/l. indicating that both MEL and mel strains were "ale-like" in this respect. Taste panel assessment deemed the beers to be unpalatable due to their "raw hop" or "papery" aroma and taste. Analysis of the bottled products revealed some interesting differences as compared with beers produced from production ale and lager brewing strains (Table V). The hybridized cultures produced beer with significantly lower levels of aromatic higher alcohols (*i.e.*, tryptophol, tyrosol, and phenylethanol) and H_2S and elevated levels of aliphatic higher alcohols (*i.e.*, the amyl alcohols, methionol, *n*-propanol and 1-butanol). All of the hybridized strains produced atypical products which could not be characterized as either ale or lager, and the ability to ferment melibiose *per se* is of little relevance in determining whether a particular yeast will perform as an ale or lager.

TABLE III

ANALYSIS OF FINISHED BEERS PRODUCED FROM "TYPICAL" ALE AND LAGER YEAST STRAINS

	ALE		LAGER	
	15°C[t]	21 C	15 C	21°C
tryptophol[a]	4	7	1	3
tyrosol[a]	11	20	20	21
phenyl ethanol[a]	24	28	35	37
amyl alcohols[a]	26	36	33	34
methionol[a]	1.6	1.8	1.8	1.5
n-propanol[a]	9	10	5	8
i-butanol[a]	8	11	10	10
ethyl acetate[a]	14	18	19	12
i-amyl acetate[a]	1.8	1.7	2.4	1.7
H_2S[b]	0.50	0.46	0.63	0.67
DMS[b]	79	63	83	68

[a] mg/l
[b] µg/l
[t] fermentation temperature

3. Production of Hybrids Able to Use the Fermentable-Wort Sugars

An acceptable yeast strain for brewing purposes is required to use the fermentable-wort sugars—sucrose, glucose, fructose, maltose, and maltotriose (Fig. 8) (29). Any yeast strain produced via hybridization must, therefore, have the genetic configuration to take up and metabolize these five sugars. The ability to metabolize glucose and fructose is constitutive to yeast and, with the exception of certain unusual mutants (22), all yeast strains are able to use them. Sucrose is only of minor importance because its concentration in wort is very small (approximately 0.25% w/v); nevertheless, it will be shown that the relevant genetic complement is necessary for its use. The disaccharide maltose and the trisaccharide maltotriose are the major wort sugars, accounting for 50–55% and 10–14%, respectively, of the total carbohydrate

TABLE IV

ANALYSIS OF FERMENTED WORTS PRODUCED FROM HYBRIDS AND BREWER'S YEAST STRAINS

	Haploid *MEL*	Diploid *mel*	Diploid *MEL*	ALE *mel*	LAGER[t] *MEL*
Diacetyl[a]	0.09	0.08	0.11	0.06	0.06
SO_2[a]	<1	<1	<1	<1	10
pH	4.2	4.2	4.3	4.2	4.3

[a] mg/l
[t] fermentation temperature = 21°C

NB: Taste panel assessment deemed the beers produced from hybridized strains to be unpalatable due to their "raw hoppy" or "papery" aroma and taste.

TABLE V

FINISHED BEERS PRODUCED FROM HYBRIDS AND BREWER'S YEAST STRAINS

	Haploid *MEL*	Diploid *mel*	Diploid *MEL*	ALE[t] *mel*	LAGER[t] *MEL*
tryptophol[a]	0.5	2	0.5	7	3
tyrosol[a]	2	3	3	20	21
phenyl ethanol[a]	8	8	11	28	37
amyl alcohols[a]	49	29	42	36	34
methionol[a]	2.3	3.3	2.5	1.6	1.5
n-propanol[a]	28	21	23	10	8
i-butanol[a]	17	15	22	11	10
ethyl acetate[a]	11	21	16	18	12
i-amyl acetate[a]	1.9	4.8	3.5	1.7	1.7
H_2S[b]	0.15	0.15	0.37	0.46	0.67
DMS[b]	120	90	64	63	68

[a] mg/l
[b] µg/l
[t] fermentation temperature = 21°C

content in wort. The yeast's ability to use maltose and maltotriose is inducible and depends upon the correct genetic complement.

Studies on the metabolism of maltose in *Saccharomyces* sp. have been conducted for many years by a number of investigators (*e.g.*, 10,12,33,40). The majority of these studies however, were carried out on yeast cultures propagated on a semidefined medium with maltose as sole carbon source under oxygenated conditions (*e.g.*, shaken culture). Further, the yeast was usually harvested for study in logarithmic phase. The results of these investigations, although useful as a basis for further research, have questionable relevance to the environment that a brewer's yeast culture encounters. In the static fermentation conditions of a brewery fermentor, the yeast is expected to ferment a large proportion of the wort's maltose and most of the maltotriose when it is in stationary growth phase in a medium which, by definition (*i.e.*, by virtue of the growth phase), is depleted in assimilable nitrogen, contains no oxygen, has a decreasing pH, and a rapidly increasing alcohol concentration and carbon dioxide tension.

Nonflocculent hybrids were constructed because, although occasional prolonged fermentation times were required in order to sediment the cells out of suspension, the additional complicating factor of premature flocculation due to cell agglutination was prevented. Although hybrids of potential for brewing must be able to use the fermentable-wort sugars in static culture, an initial examination of haploids and hybrids in an agitated environment gave a convenient indication of their sugar-fermenting potential. If a culture was unable to perform adequately in shaken fermentation, it was most unlikely that it would ferment acceptably in the more adverse environmental conditions that prevail in a static fermentor.

Three hybrids were constructed (1176 × 1240, 1176 × 1241 and 1176 × 1242), all of which were able to use under-agitated conditions, the fermentable sugars contained in an 11.8°P wort (Table VI). In static culture, however, two of the hybrids (1176 × 1241 and 1176 × 1242) were only able to ferment 85% of the fermentable extract (Fig. 9), whereas the third (1176 × 1240) out-fermented the wort. The inability of the two hybrids to out-ferment the wort was not a result of premature sedimentation because only after fermentation had ceased did the cells sediment out of suspension (Fig. 10). Analysis of the wort sugars at the end of the fermentation revealed that maltotriose was the major unfermented sugar (Table VI) with a small amount of maltose remaining in the fermentations by the hybrids that failed to out-ferment the wort in static culture.

The fermentation properties of the four constituent haploids were studied in order to elucidate the reason why two of the hybrids were unable to out-ferment wort in static culture but able to do so with agitation. Further, why was the third hybrid (1176 × 1240) competent in both static and agitated conditions? Under agitated conditions, only two of the haploids (1176 and 1240) were able to out-ferment an 11.8°P wort, thereby using all of the wort sugars (Table VII). The other two haploids (1241 and 1242) were unable to use approximately 60% of the available maltotriose and one of them (1242) lacked a gene for invertase production (suc) and was

TABLE VI

PERCENTAGE RESIDUAL "FERMENTABLE" WORT SUGARS AND YEAST α-GLUCOSIDASE ACTIVITY AT THE TERMINATION OF FERMENTATION – DIPLOIDS

STRAINS	Maltose (%)	Maltotriose (%)	α-Glucosidase [B] activity
Agitated			
1176 x 1240	–	4.6	7
1176 x 1241	–	3.9	<1
1176 x 1242	–	2.6	<1
Static			
1176 x 1240	1.1	7.2	3
1176 x 1241	2.0	55.7	<1
1176 x 1242	5.1	47.9	<1

NB: 100 percent glucose, fructose and sucrose fermented in all cases.

[a] μg maltotriose hydrolysed per mgm protein per hour.

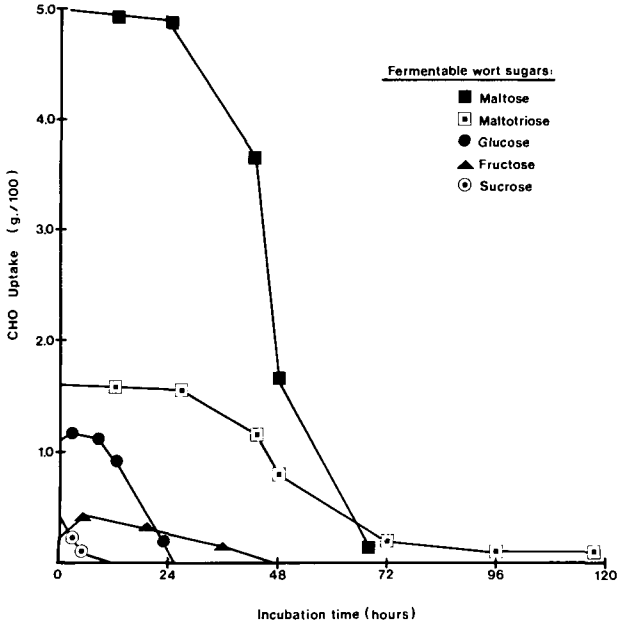

Fig. 8. Carbohydrate uptake during wort fermentation.

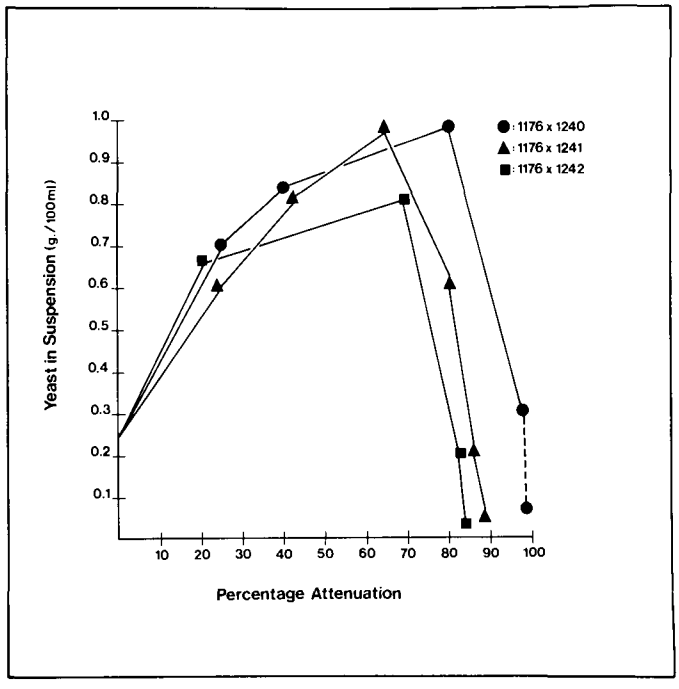

Fig. 9. Static fermentation characteristics of nonflocculent hybrids.

unable to use sucrose. The hybrid, unable to out-ferment in static culture, was a mating of the haploids (*i.e.*, 1176 and 1240) capable of fermenting all wort sugars upon agitation, whereas the hybrids unable to out-ferment wort in static culture were matings of a haploid capable of out-fermenting wort (1176) and haploids incapable in this regard (*i.e.*, 1241 and 1242).

More than one fully competent maltose/maltotriose gene system (MAL genes) in either the homozygous or heterozygous state would appear to be required to fully attenuate a wort in static culture. Although this statement provides a possible genetic solution to the problem, it asks a further question, "How does this polygene theory manifest itself at the biochemical level?" The traditionally accepted uptake mechanism for maltose involves two systems (Fig. 11): first, the maltose uptake system (or maltose permease), which transports maltose across the cell membrane into the cell; and second, maltase (α-glucosidase), which hydrolyzes maltose, once inside the cell, to yield two glucose units/unit of maltose. This is a simplistic approach in that it fails to account for the uptake of maltotriose. Under normal circumstances, it is a prerequisite that, before cells can metabolize maltotriose, they must be capable of maltose metabolism; the converse does not follow. Failure to out-ferment wort in all likelihood is a fault of either maltose permease or α-glucosidase. Measurement of the permease system, although possible (11), is difficult and inaccurate; consequently, the α-glucosidase system was studied. Levels of α-glucosidase activity were found to vary greatly between yeast strains and also within a particular yeast strain, depending upon the growth phase (Tables VI and VII). The strains that failed to out-ferment the wort in static culture (*i.e.*, 1176×1241 and 1176×1242) contained little α-glucosidase activity (using either maltose or maltotriose as substrate) at the cessation of fermentation, whereas the cultures able to out-ferment wort contained significant α-glucosidase activity at the end of the fermentation.

Towards the end of fermentation, it would appear that the activity of α-glucosidase deteriorates—the extent of the deterioration determining the degree of attenuation. At this point in time, it is unclear whether it is the maltose uptake system that deteriorates preventing the α-glucosidase access to its substrate and thus stimulating its decrease in activity or whether the α-glucosidase activity decreases first. However, since the uptake system is situated in the cell membrane and thus exposed to environmental change, it would seem reasonable to speculate that this component deteriorates initially. Further, gene dosage and complement has a part to play because, in strains that contain more than one maltose gene (either in the homozygous or heterozygous condition), the α-glucosidase remains active longer at the termination of fermentation and such strains are better-equipped to ferment the wort maltotriose in static culture. Under agitated conditions, the maltose/maltotriose uptake system remains active longer due, in all probability, to a greater concentration of oxygen thus resulting in an increased yeast crop and a generally more viable and active yeast culture; it should not be forgotten that this uptake system is an active transport system (11) and requires a constant supply of energy.

The foregoing results would indicate that it does not suffice for a strain to possess a single gene for maltose in order to achieve

TABLE VII

PERCENTAGE RESIDUAL "FERMENTABLE" WORT SUGARS AND YEAST α-GLUCOSIDASE ACTIVITY AT THE TERMINATION OF FERMENTATION – HAPLOIDS

STRAINS	Sucrose (%)	Maltose (%)	Maltotriose (%)	α-Glucosidase[a] activity
Agitated				
1176	–	–	3.1	10
1240	–	–	4.8	8
1241	–	0.5	59.4	<1
1242	96	0.5	67.9	2

NB: 100 percent glucose, fructose and sucrose fermented in all cases.

[a] μg maltotriose hydrolysed per mgm protein per hour.

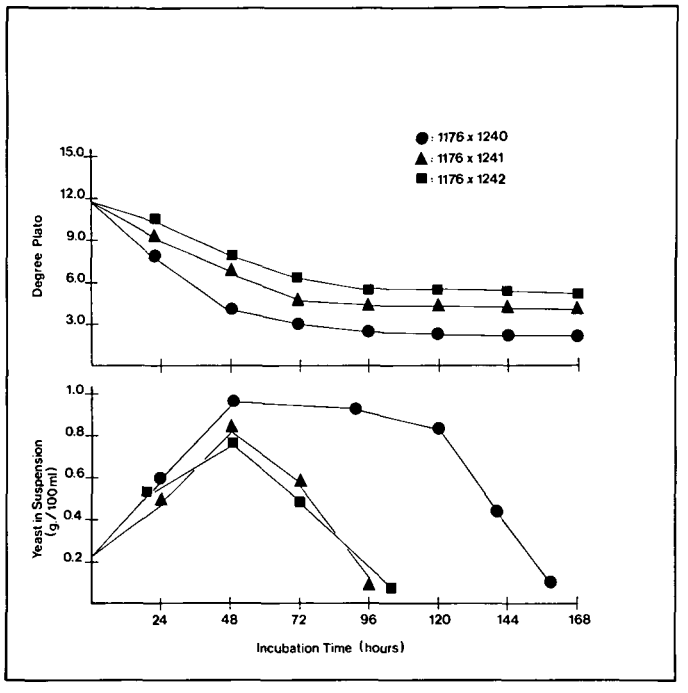

Fig. 10. Static fermentation characteristics of nonflocculent hybrids.

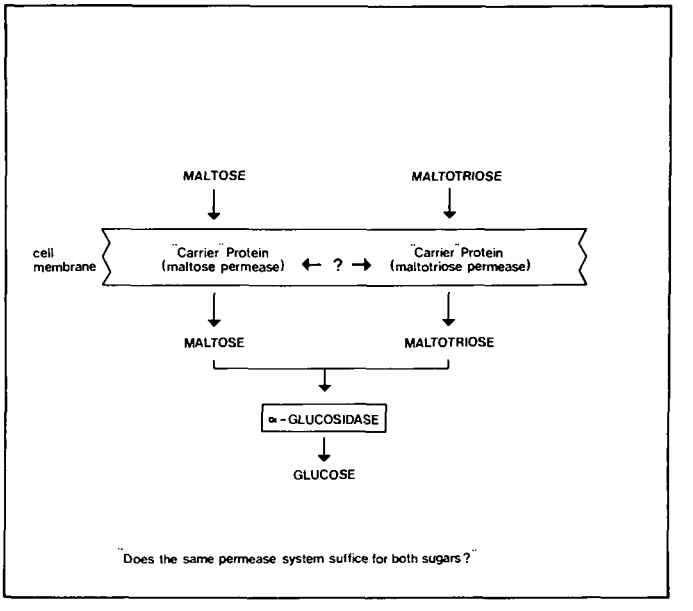

Fig. 11. Uptake and metabolism of maltose and maltotriose.

adequate wort fermentation in static culture; this fact alone highlights one of the reasons why the brewer, many generations ago, empirically, but with a high degree of experience and substantial success, selected polyploid yeasts for use in breweries. Maltose metabolism is under the multiple gene control of a polymeric system. Analysis of interbreeding yeast strains has revealed at least six unlinked genes for maltose, MAL 1-6 (Table VIII). Halvorson *et al.* (10) have compared the α-glucosidases produced in *Saccharomyces* sp. in response to five of these polymeric genes (MAL 5 was not studied) and concluded that the purified enzymes were indistinguishable from one another. The results gave no indication of each MAL gene's performance in a brewing wort because the enzymes were studied in a cell-free system, consequently the uptake system was not considered. Further, Halvorson's studies were conducted on cultures grown in a semidefined medium with maltose as sole carbon source, the cells being harvested to produce the cell-free extract in midlogarithmic growth phase.

The fermentation rates of haploids containing single, but different, genes for maltose (MAL 1-4 and MAL 6) were studied in a typical wort under shaken cultural conditions (Table IX). The haploid containing MAL 6 fermented the wort at the fastest rate and was the only gene to remove all of the fermentable sugars from the wort. Haploid cultures containing MAL 2 and MAL 4 displayed slow-initial-fermentation rates and failed to ferment all the wort sugar, leaving approximately 60% of the maltotriose unfermented. Cultures containing MAL 1 and MAL 3 performed poorly in wort, with slow-initial-fermentation rates and a complete inability to ferment maltotriose.

The above results would indicate that the gene of choice for incorporation into a brewing yeast strain would be MAL 6. However, literature reports (45) would indicate that MAL 1, MAL 2, and MAL 3 have been identified in "brewing" strains of *S. cerevisiae* (in all likelihood they were polyploid in nature). Fermentation studies with haploid strains can be deemed questionable because many such strains have nutritional problems with only a single gene for each trait. To overcome this problem, each MAL gene containing haploid was mated with "standard" haploids (maltose negative, mal, with no detectable nutritional problems) and the wort fermentation properties of the resulting diploids studied (Table X). Again, differences between the MAL genes were apparent but not as accentuated as in the haploid state. The diploid containing MAL 6 fermented at a faster initial rate and out-fermented the wort in 4 to 5 days. Diploids containing either MAL 2, MAL 3, or MAL 4 exhibited slower fermentation rates than the MAL 6 containing diploid, but all out-fermented the wort. It is interesting to note that, in the diploid situation, MAL 3 was able to use maltotriose, whereas it was incapable of doing so in the haploid state. MAL 1 yeast, even in the diploid phase, was unable to ferment maltotriose and there would appear to be a real phenotypic difference between MAL 1 and the other MAL genes studied.

TABLE VIII

MALTOSE (*MAL*) GENES IN SACCHAROMYCES Sp.

Gene	Chromosome Location
MAL 1	Chromosome VII
MAL 2	Chromosome III
MAL 3	unmapped
MAL 4	Chromosome XI
MAL 5	unmapped
MAL 6	unmapped

TABLE IX

PERCENTAGE ATTENUATION AND PERCENTAGE RESIDUAL MALTOTRIOSE LEVELS OF WORT FERMENTED UNDER AGITATED CONDITIONS BY HAPLOIDS CONTAINING DIFFERENT SINGLE MALTOSE GENES

STRAINS	Attenuation at 4 days(%)	Final attenuation (%)	Residual maltotriose (%)
MAL 1	40	79	100
MAL 2	88	91	60
MAL 3	60	84	72
MAL 4	88	90	61
MAL 6	99	100	–
Brewing polyploid	100	100	–

TABLE X

PERCENTAGE ATTENUATION AND PERCENTAGE RESIDUAL MALTOTRIOSE LEVELS OF WORT FERMENTED UNDER AGITATED CONDITIONS BY DIPLOIDS CONTAINING DIFFERENT SINGLE MALTOSE GENES

STRAINS	Attenuation at 4 days(%)	Final attenuation (%)	Residual maltotriose (%)
MAL 1/mal	61	71	100[a]
MAL 2/mal	96	98	3.5
MAL 3/mal	98	98	3.5
MAL 4/mal	98	99	2.0
MAL 6/mal	100	100	–
Brewing polyploid	100	100	–

[a] Residual maltose in addition

Brewer's yeast strains possess multiple genes for maltose (6) and any genetic reconstruction of such cultures should take this fact into account. MAL 3 in the haploid has shown itself to be unsuitable for use in brewery cultures; however, it has been identified as a composite gene of many brewer's yeast strains (6). A study of the fermentation performance of a polyploid series of strains containing MAL 3 alone and in combination with other MAL genes was conducted in an attempt to bring this investigation closer to the "real world" of the brewery. A diploid homozygous for MAL 3 (MAL 3/MAL 3) performed as poorly as the MAL 3 haploid (Table XI), whereas, in a diploid where the MAL 3 was heterozygous (MAL 3/mal), wort was fermented adequately and completely. A tetraploid (MAL 3/MAL 3/mal/mal) was constructed by crossing the diploid homozygous for MAL 3 with a maltose negative (mal/mal) diploid. This culture possessed excellent fermentation abilities with the wort being out-fermented in approximately 36 hr; it also possessed similar fermentation characteristics to an excellent production brewing-ale strain that most certainly is polyploid. A diploid (MAL 2/MAL 3), heterozygous for both MAL 2 and MAL 3, was constructed and it exhibited ideal fermentation properties also. It would appear that polyploid yeast strains containing multiple genes for maltose metabolism display superior fermentation properties in wort. Why then did the diploid homozygous for MAL 3 perform unsatisfactorily? This diploid was not simply homozygous for MAL 3; it was the product of a self-diploidization. Consequently, any problem exhibited in the haploid would be accentuated in the diploid—the perils of inbreeding should not be forgotten!

DISCUSSION

Anyone attempting to produce a brewer's yeast strain via genetic manipulation should be cognizant of the requirements for an acceptable brewing strain, "In order to achieve a beer of high quality, the yeast culture must be effective in removing the desired nutrients from the growth medium (*i.e.*, the wort), it must impart the required flavor to the beer, and, finally, the microorganisms themselves must be effectively removed from the fermented wort after they have fulfilled their metabolic role" (34). This definition, although wide ranging and nonspecific, enables a breakdown of a brewing strain's activities during the fermentation of wort into three areas: 1) nutrition (*e.g.*, sugar uptake), 2) by-product formation (*i.e.*, excretion of compounds that contribute to the flavor of the product), and 3) removal of the culture from the fermented wort (*e.g.*, flocculation). In this paper, genetic aspects of all three areas have been considered and, as mentioned in the **INTRODUCTION**, pose more questions than answers.

The type of genetic experiments described here involve the manipulation of yeast chromosomes and chromosome fragments, *i.e.*, no foreign genetic material has been introduced into the yeast cell; only existing DNA material has been manipulated—a process that has been called homogenetics. In recent years, another approach to genetic experimentation has taken place in various laboratories; this other type of genetic manipulation is called heterogenetics because it involves the synthesis and interjection of replicating DNA molecules from unrelated organisms (14). This latter type of research has certain inherent dangers, which have been grossly overrated, in that mutations may be produced that cannot be controlled; the study of homogenetics contains none of these dangers.

Previous studies from this laboratory (38) have identified and mapped a gene for flocculation (FLO 4), the investigation being reported here attempted to use this gene to produce a strain of yeast possessing the required agglutination characteristics so that it would settle out of suspension at the completion of fermentation. Studies to date have failed to achieve this goal because the hybrids produced thus far have been too flocculent, resulting in a premature flocculation and an under-attenuated wort. A number of hybrids with varying flocculation intensities were produced, however, and further study should result in cultures exhibiting the required phenotypic characteristics. The expression of the flocculation gene has been reported to be modified by inhibitory or suppressing factors (7,18,42), the identities of which are currently unknown. It is interesting to note that Molzahn (24), during a study of different mutagens on brewing yeast strains, found that the flocculation of a moderately flocculent strain was increased following mutagenic treatment. In this case, the mutagen had probably removed a modifier (or suppressor) gene and promoted an increase in flocculation. Suppressor genes for a variety of traits have been identified in yeast (25); at the last count 30 such genes had been identified. If the gene or genes that suppress the expression of the FLO 4 gene could be identified, it would greatly aid attempts to construct hybrids possessing the necessary sedimentation characteristics to ferment out a wort in static culture. The identification of such a suppressor gene(s) is currently ongoing in this laboratory.

Although taxonomically the species *S. cerevisiae* and *S. uvarum (carlsbergensis)* are distinguished by their melibiose fermentation properties (20), the data contained in this paper would infer that this property *per se* has little or no influence upon a yeast strain's ability to produce a "lager-type" or an "ale-type" beer. Typical lagers have been described as "sulfury," whereas typical ales are referred to as "hoppy and estery." Diploid strains, produced via genetic manipulation, dominant and recessive for the melibiose gene (*i.e.*, MEL or mel), failed to produce beers that resembled either the lager or ale stereotype. It would appear that there is a grouping of *S. uvarum (carlsbergensis)* strains, polyploid in nature, that produce a product that can be deemed to be a lager. By the same token, there is a grouping of *S. cerevisiae* strains, again polyploid in nature that produce a product that can be deemed to be "ale-like." There are strains of both *S. cerevisiae* and *S. uvarum (carlsbergensis)* that ferment wort to produce unpalatable beer that bears no resemblance to either ale or lager. The reasons for this discrepancy between strains warrant detailed study because the answer may provide information on the determining factors that influence a yeast strain's metabolism while fermenting wort. It is noteworthy that lager yeast strains appear to be a much more uniform group than ale yeast strains. This is not wholly surprising because most of the lager yeast strains being used for production purposes around the world today are variants of a common source.

TABLE XI

PERCENTAGE ATTENUATION AND PERCENTAGE RESIDUAL MALTOTRIOSE LEVELS OF WORT FERMENTED UNDER AGITATED CONDITIONS BY A POLYPLOID SERIES CONTAINING THE *MAL 3* GENE

STRAINS	Attenuation at 4 days (%)	Final attenuation (%)	Residual maltotriose (%)
MAL 3 (n)	60	84	72
MAL 3/MAL 3 (2n)	55	61	100[a]
MAL 3/mal (2n)	98	99	2.0
MAL 3/MAL 2 (2n)	98	99	2.0
MAL 3/MAL 3/mal/mal (4n)	98	99	2.0
Brewing polyploid (?n)	100	100	–

[a] Residual maltose in addition

In 1883, Emil Christian Hansen (17), working in the Carlsberg Brewery in Copenhagen, successfully isolated four strains of yeast from fermenting wort. When studied from the standpoint of brewery use, only one of the strains proved to be suitable for beer fermentation. This strain, originally described as "Carlsberg Brewery Yeast No. 1," was introduced into the Carlsberg Brewery for use on a production scale on May 13, 1883. Due to its origin, this strain was named *S. carlsbergensis* Hansen 1883, and around it evolved a new species of yeast distinguishable taxonomically from its parent species *S. cerevisiae* in its ability to ferment melibiose. Hansen's strain soon spread to the major lager breweries of Europe and North America. Thus today, most lager strains in production use throughout the brewing world are derivatives of the strain isolated in 1883; their uniform fermentation performance would tend to support this view. Ale strains, however, have not originated in such a centralized manner as lager yeasts and consequently have more diverse fermentation properties, producing beers with a broad spectrum of flavor characteristics.

The uptake of maltose and maltotriose into the yeast cell requires two factors—an uptake or permease system transporting the sugar across the membrane into the cytoplasm (this is an active transport process requiring the expenditure of metabolic energy) and the enzyme α-glucosidase which hydrolyzes the sugar, once inside the cell, to glucose units. The α-glucosidase is a common enzyme for both maltose and maltotriose (23) and, although the evidence is not clearly established, it would appear that each sugar has its own permease system. Studies on the genetic control of maltose metabolism have almost exclusively dwelt on α-glucosidase, with scant attention being paid to the permease systems; this is not surprising because of the inherent difficulties of measuring permeases. Harris and Millin (12) consider that the induction of the permease is probably a prerequisite for the induction of α-glucosidase. They view the system as reminiscent of the *lac* operon (*i.e.*, a number of physically adjacent genes of related function) of *Escherica coli* (15), wherein the role of the permease is to provide a suitable intercellular concentration of the inducer (lactose in *E. coli* or maltose/maltotriose in yeast) leading to the synthesis of the hydrolytic enzyme (β-galactosidase or α-glucosidase). The "operon" mechanism in the maltose/maltotriose (MAL) system of *Saccharomyces* sp. consists of at least three components: 1) a gene for α-glucosidase, 2) a gene for maltose permease, and 3) a gene for maltotriose permease.

Although six MAL systems have been identified as polymeric gene complexes, they are not identical in their mode of action. MAL 1 is not capable of metabolizing maltotriose in the whole cell although, in the cell-free system, its α-glucosidase is able to hydrolyze the trisaccharide, thus indicating that the gene coding for maltotriose permease is abent (*i.e.*, recessive) or suppressed. A diploid heterozygous for MAL 1 was also incapable of metabolizing maltotriose unless the permeability barrier was eliminated, thus indicating that the MAL 1 operon in all probability consists of genes for α-glucosidase and maltose permease. Strains containing the MAL 6 operon are able to take up both maltose and maltotriose with little difficulty, indicating effective permease systems for both of these sugars plus α-glucosidase. Strains containing MAL 2 and MAL 4 were able to metabolize both maltose and maltotriose, but haploid strains were unable to use all of the available maltotriose in an 11.8°P wort. When fermentation ceased, little α-glucosidase activity could be detected in these strains. It could possibly be that toward the end of fermentation α-glucosidase activity declined, thus inhibiting the uptake of the remaining maltotriose via feedback inhibition of the permease. However, a more likely explanation is that maltotriose permease ceased to be active due to adverse environmental conditions or a lack of metabolic energy. Consequently, maltotriose uptake ceased, thus halting the induction of α-glucosidase. The maltotriose permease of the heterozygous diploids of MAL 2 or MAL 4 would appear to be less sensitive to environmental conditions and consequently such strains were capable of fermenting all of the available maltose and maltotriose. A similar situation prevails with MAL 3 in which the haploid is unable to ferment maltotriose whereas a heterozygous MAL 3 diploid fermented this trisaccharide albeit with some difficulty.

The connection between maltotriose fermentation and yeast sedimentation is worthy of comment. Yamamoto and Inoue (46) reported that a yeast culture with a slow maltotriose fermentation was liable to prematurely sediment out of suspension. Studies being reported in this paper would confirm this: namely that in static fermentation a yeast strain requires more than one fully competent MAL operon in either the homozygous or heterozygous condition to ferment out a normal gravity wort (*i.e.*, 11–14°P). Reasons for the multiple-gene requirement can only be speculated upon, but gene dosage, resulting in elevated enzyme levels of both permease and α-glucosidase, would appear to be the most likely answer. Indeed, Rudert and Halvorson (31) have demonstrated a strictly additive increase in α-glucosidase levels with multiple dosages of MAL genes.

The relation between sedimentation and flocculation is a complex one. This study has shown that nonflocculent yeast strains will sediment if they cease fermenting. In this situation, cell agglomeration does not appear to occur; individual cells are subject to gravitational forces which they are unable to resist due to their metabolic inactivity. Yeast strains containing genes for flocculation are subject to an additional factor wherein, at a particular point in the growth cycle, individual cells agglomerate into flocs and thus have a greater tendency to sediment out of suspension. At the point of cell agglomeration, such cells may still be capable of using the fermentable sugars in wort if any remains; a major distinction should be made between cultures that flocculate and those that are subject to premature sedimentation as a result of slow-maltotriose-fermenting ability.

This study has indicated that the widespread use of polyploid yeasts in brewing is by no means an accident. The advantages of polyploid yeasts are illustrated by the study of the MAL 3 polyploid series. Although a haploid containing MAL 3 as the single-gene system for maltose/maltotriose metabolism exhibited relatively poor wort-fermentation properties, a tetraploid strain containing two dominant MAL 3 systems fermented wort with as much ease as an acceptable brewing polyploid strain. Polyploids also, due to their multiple-gene structure, are genetically more stable and less susceptible to mutational forces than either haploid or diploid strains.

The reason for the existence in *Saccharomyces* of six seemingly identical but unlinked gene systems, all coding for maltose metabolism, has always been a perplexing problem for many biochemists and geneticists. This study may shed light on the situation. The results clearly indicate that, in a complex growth medium such as wort, the six gene systems do not react in an identical manner, *e.g.*, MAL 6 is capable of fermenting both maltose and maltotriose whereas MAL 1 is only able to ferment maltose. This would indicate that the natural environments, in which the strains containing these genes evolved, were most probably different. Although it is mere speculation, it is not impossible that strains containing MAL 6 arose in a distinctly different environment, one wherein both the maltose and maltotriose content of the surrounding medium was high (*e.g.*, products of cereal starch degradation); antithetically, strains containing MAL 1 arose under environmental conditions containing minimal levels of maltotriose (*e.g.*, fruit juices).

The finding that each of the six "operons" use or fail to use maltotriose in characteristic fashion may help to resolve a current controversy between yeast taxonomists and brewing microbiologists. In the 1952 edition of *The Yeasts* (21), the maltotriose nonfermenting species *S. uvarum* was maintained as a taxonomic entity distinct from the maltotriose-fermenting species *S. carlsbergensis*. However, in the 1970 edition of the same text (20), the two species were consolidated under the single species *S. uvarum*, reasons being that, "the ability to ferment maltotriose as a basis for species delineation was not considered to be sound." Many brewing microbiologists have challenged this consolidation (8) and

strongly disagree with lager yeast strains forming a part of the species *S. uvarum*. (It must be admitted that much of the disapproval is based on emotional rather than scientific grounds.) Studies in this laboratory have confirmed that strains of the original *S. uvarum* species are unable to ferment maltotriose (indeed, use has been made of this fact to propose a method for the isolation of pure maltotriose from wort (35); however, since they are able to ferment maltose, it is probable that they possess a MAL 1 type system as sole MAL gene complement; if this is the case, the consolidation of the two yeast species is justified.

It can be concluded that in the near future it will be possible to construct yeast strains, probably polyploid in nature, capable of completely fermenting wort in static culture and sedimenting out of suspension at the appropriate time. However, the problem of producing a palatable product still remains. Taste panel opinion however, indicates that one of the major defects of beer produced with hybridized diploids is one of a "wort" or "hoppy" aroma and taste. A major function of yeast during fermentation is to adsorb onto its cell surface a variety of "bittering" hop constituents; if these constituents are allowed to remain in beer, they give the product an undesirable "hoppy" or "papery" note. The cell-surface to cell-volume ratio of polyploid yeasts is larger than those in diploid strains (27). Consequently, the adsorptive power of polyploid yeasts would be greater, leading to more bitter and other undesirable constituents being removed from the wort, resulting in a "cleaner" product.

Fundamental research on brewer's yeast strains should be directed toward obtaining "more information about the enzymatic makeup of the yeast cell, the genetic control of that makeup, the quantitative interplay of its metabolic pathways, and the changes that the latter undergo in response to changes in wort composition during the growth cycle," stated Rainbow (30) at the conclusion of his Horace Brown Memorial Lecture in 1976. The study of yeast genetics has forced research workers to return to the "biochemical drawing board." Biochemical knowledge, until recently taken for granted, is being questioned in the light of novel genetic information. This is particularly the case in the area of metabolic control, where a greater understanding of the factors that govern the expression of gene function (*e.g.*, the suppression of flocculation) will assist in the production of hybridized yeast strains "programmed" to meet the fermentation patterns and flavor characteristics deemed necessary to produce a good beer.

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