CONSTRUCTION OF A HYBRID BREWING STRAIN OF SACCHAROMYCES CARLSBERGENSIS BY MATING OF MEIOTIC SEGREGANTS

by

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A brewing strain of *Saccharomyces carlsbergensis* yielded upon sporulation 1-, 2- and 3-spored asci. The spore viability was low, but a number of clones could be derived from the spores. Among these $\mathbf{a}$ - and α -maters were identified revealing that the parental brewing strain is heterozygous for the two mating type alleles. A number of lines with opposite mating type were crossed pairwise. One of the hybrids produced by mating of two presumably haploid or near haploid clones was equal to the parental strain in brewing performance on a pilot scale. The separation of the nuclear genomes of brewer's yeast into individual cells and their successful mating into hybrid strains opens the possibility to introduce genetic alterations in brewing strains by mutagenesis and genetic transformation of the haploid lines.

1. INTRODUCTION

To our knowledge no controlled genetic alterations of brewer's yeast have so far been carried out to yield a strain used in industrial production of beer. New and genetically impro-

ved strains are desirable to meet the demands of new brewing techniques and the production of beers with specific properties. For the brewer breeding of strains with appropriate flocculation, improved carbohydrate utilization, and reduced

Abbreviations: YPD = yeast extract peptone dextrose, P = Plato.

diacetyl formation are of special interest. This has been attempted by introducing mutations in the brewing strains either by mutagenesis or by crossing with haploid mutant strains. Mutagenesis can only be applied efficiently to traits caused by dominant mutations (8) since brewing strains are diploid or polyploid. Cross breeding of brewing yeasts is complicated by the fact that they are non-maters and that they either do not sporulate or sporulate poorly giving few spores often with low viability. Introduction of characters from non-brewing yeast into brewing yeast has been carried out by rare mating (17) and by

protoplast fusion (4, 13, 15, 16) but unfortunately, in addition to the desired characters, many undesirable traits were transmitted to the somatic hybrids (4). We have tried to overcome this problem by isolating from brewing strains clones exhibiting the *a* and α mating type and investigated whether crossing of these clones can reconstitute brewing strains of good performance. In spite of the low sporulation frequency and the low spore viability it has been possible to isolate from spores, clones showing mating abilities (3, 12). This paper describes the procedure of isolating mating clones from a

Table I

Strains used.

Strain	Genotype	Mating type	Source
244			Production strain of <i>S. carlsbergensis</i> of the Carlsberg Breweries
D286-2A	MATa ade 1 his 1	<i>a</i>	Course manual on yeast genetics. Cold Spring Harbor Laboratory
D273-11A	MAT α ade 1 his 1	α	— » —
C80-CG7, -CG45, -CG46, -CG58, -CG61, -CG65, -S1, -S2		<i>a</i>	Spore derived clones of strain 244 in experiment 1
C80-CG44, -CG47, -CG56, -CG57, -CG59, -CG60, -CG62, -CG63, -CG64, -CG66, -CG67, -S3, -S4, -S5, -S6, -S7		α	— » —
C80-S24, -S25, -S26, -S27, -S28, -S29		bisexual	— » —
C80-S8, -S9, -S10		<i>a</i>	Spore derived clones of strain 244 in experiment 2
C80-CG110, -S13, -S14, -S15, -S16, -S17, -S18, -S19, -S20, -S21, -S22, -S23, -S32, -S33		α	— » —
C80-S30, -S31		bisexual	— » —
C80-CG71			C80-CG44 $\times$ C80-CG45
-CG75			C80-CG7 $\times$ C80-CG56
-CG77			C80-CG56 $\times$ C80-CG58
-CG83			C80-CG56 $\times$ C80-CG65
-CG133			C80-CG65 $\times$ C80-CG110

strain of brewer's lager yeast previously thought to be non-sporulating. From these haploid or near haploid maters hybrid strains were constructed by crossing. After studying the fermentation characteristics of these hybrids the brewing performance of one strain was tested in pilot scale and was found to be equal to that of the parental strain.

2. MATERIALS AND METHODS

2.1. Strains and media

The strains used in this study are listed in Table I. The following media were employed: YPD contained 1 % Difco Bacto Yeast Extract, 2 % Difco Bacto Peptone, 2 % glucose. Sporulation medium contained 1 % potassium acetate, 0.1 % Difco Bacto Yeast Extract, 0.05 % glucose. Wort medium was made up of Pilsener wort. Plates were solidified with 2 % Difco Bacto agar.

2.2. Sporulation and separation of spores

After two days of growth on a YPD plate cells of strain 244 were streaked on a sporulation plate and incubated seven days at 22 °C. Asci are then present on the plate, while this strain fails completely to sporulate at 30 °C. In order to open asci and to kill non-sporulated cells, the washed cell and ascus suspension was treated with 2 % (w/w) β -glucuronidase, Sigma No. G-0751 after pretreatment with thioglycollate (following a procedure for preparing spheroplasts (11)), suspended in water and frozen overnight at -18 °C. The spores were separated by sonication and a drop of Triton X-100 was added to prevent the spores from sticking together. The spores were then plated on YPD plates and incubated at 22 °C.

2.3. Determination of mating type

In principle the procedure of ANDERSON & MARTIN (3) was followed. After seven days at 22 °C on YPD medium colonies were isolated and tested for sporulation ability as described under 2.2. Non-sporulating colonies were considered to be potential mating strains.

Mating was attempted by mixing on YPD plates the strain with the two tester strains,

D286-2A and D273-11A, listed in Table I. After two days of growth the plates were replicated to plates containing sporulation medium. If sporulation occurred it was taken as an indication of a successful mating. A colony was designated as non-mater, when no sporulation occurred, as mating type **a**, when sporulation occurred after mixing with the α tester, as mating type α , when sporulation took place after mixing with the **a** tester and bisexual (bs), when sporulation occurred after mixing with both mating type testers.

2.4. Spore staining

As many of the colonies sporulated poorly it was necessary to stain the spores for detection of sporulation. Since a large number of colonies were to be examined a modification of the malachite green-safranine staining method (14) was applied. Colonies in a replica pattern on a sporulation plate were replicated with velvet cloth to a 10 × 10 cm glass plate. When dry the glass plate was heated gently on a hot-plate and transferred to a 5 % malachite green solution in a 70 °C–80 °C water bath for one minute. After washing in cold water the glass plate was transferred to a 0.5 % safranine O solution for one minute. After washing in cold water the plate was examined under a microscope.

2.5. Isolation of hybrids produced by mating

Some of the isolated strains of opposite mating type were crossed pairwise by mixing them on a YPD plate. Hybrids were selected by growing cells from the mating mixture for approximately 15 generations in liquid YPD. This allows enrichment of hybrids which grow faster than the maters. The cells were then plated on YPD plates and the largest colonies were isolated and tested for sporulation. Sporulating colonies are hybrids resulting from a successful mating. These presumably diploid or near diploid strains were streaked on wort agar and incubated at 10 °C (suitable fermentation temperature for lager beer). The colony size was compared with the colony size of the parental brewing strain grown under the same conditions. Strains giving colonies of about the same size as the parental strain were chosen for further analysis.

2.6. EBC-tube fermentations and analyses of racked beer

Propagation of yeast strains was carried out at 10 °C by successive inoculations of larger and larger volumes of sterile pilsener wort: Ten ml were inoculated with approximately 10^6 yeast cells; after 7 days the culture was transferred into 300 ml wort, which after 7 days was poured into 8 litre wort. After fermentation under stirred conditions for 12 days the yeast was allowed to settle for 2 days unstirred.

The propagated yeast was added to 2 litre sterile aerated pilsener wort in an EBC-tube (6) at a pitching rate of $\frac{1}{2}$ % w/v centrifuged yeast. The fermentations were carried out at 10 °C. The number of yeast cells in suspension was counted using a Coulter Counter model ZB. Specific gravity of the beer during fermentation was measured in gradient-tubes (9). After 7 days of fermentation the beer was separated from the yeast by centrifugation. The beer was analysed for vicinal diketones and their precursors by gas liquid chromatography. (7).

The fermentations were carried out three times, whereby bottom yeast from one fermentation was used as pitching yeast in the following fermentation.

2.7. Pilot brewing and analyses of beer

Propagation was done as mentioned for EBC-tube fermentations, but the strains were propagated and fermented in duplicate (a and b). The pilot scale fermentation and storage of beer as well as the analysis of yeast and beer was done as described earlier (1, 2, 5). However, a pitching rate of $\frac{1}{2}$ % w/v centrifuged yeast was used and the temperature during fermentation was lower-

ed from 10 °C after 6 days and held at 7 °C for 1 day. The fermentations were carried out two times whereby bottom yeast from the first fermentations was used as pitching yeast in the second fermentations. The beers a and b from the second fermentations were mixed at racking.

The bottled beer was also analysed for the head space profile on a Perkin Elmer Head Space Analyzer (F 45 head space injector, F 45 gaschromatograph, column 2m $\times$ 1/8" SS 15 % Carbowax 4000 on Chromosorb W Aw and flame ionisation detector, Sigma 10 data handling station).

3. RESULTS

3.1. Isolation of maters

The sporulation frequency of the brewing strain 244 of *Saccharomyces carlsbergensis* after 7 days on sporulation medium at 22 °C was approximately 20 %. Only one-, two-, and three spored asci were found. The clones derived from individual spores differed in morphology and size. Dilution series of spores revealed that germination percentage was low. In two experiments the clones obtained from the spores were tested for their ability to sporulate (Table II). In experiment 1 small colonies were selected and 71 % of the clones found to be non-sporulating. In experiment 2 large colonies were selected and the frequency of non-sporulating clones dropped to 41 %. The effect of selection for colony size on the frequency of non-sporulating clones (Table II) indicates that haploid cells carrying a single mating type allele are slow growing. The spore derived clones that again can sporulate must carry both the α - and α -mating type. Further investigation will have to show whether this is

Table II

Mating behaviour of non-sporulating clones derived from isolated spores of *S. carlsbergensis* strain 244.

	No. of isolated clones grown from spores	No. of non-sporulating clones	Mating type			
			α	α	bisexual	non-mater
Exp. 1	426	303 (71 %)	8	16	6	273
Exp. 2	224	91 (41 %)	3	14	2	72

due to the actions of genes for homothallism and/or irregular disjunction of chromosomes during meiosis. In the two experiments 30 lines able to mate with the α -tester strain but not with the α -tester strain were recovered. These are thus carrying the α -mating type. Only 11 lines were obtained which carry the α -mating type. 8 non-sporulating clones were obtained which show bisexual behaviour, i.e. ability to mate with both α - and α -tester strains. Both the excess of α -maters and the occurrence of bisexual clones can be explained by the presence of genes for homothallism and/or irregular meiotic segregations. All maters were prototrophs and four were temperature sensitive showing no or poor growth at 30 °C. All the maters were more slowly growing compared to the parental strain. Sporulation of a hybrid between *S. carlsbergensis* and *S. cerevisiae* (C80-CG7 $\times$ D273-11A) yields asci, some of which are 4-spored in contrast to what is found for the brewing strain 244. The viability of spores from these 4-spored

asci were by micromanipulation found to be low (2%).

A number of the fastest growing strains of opposite mating type were crossed pairwise. Five hybrids of five different crosses, C80-CG71, C80-CG75, C80-CG77, C80-CG83 and C80-CG133 giving colonies of about the same size on wort medium as the parental strain were chosen for analysis of their growth and fermentation characteristics as related to brewing performance. All five hybrids sporulated, when brought on sporulation medium.

3.2. EBC-tube fermentations with the hybrid strains

The yeast cell counts and the apparent extract (calculated from the specific gravity) during fermentations of the parental strain and the 5 hybrid strains are shown in Figures 1–6. Variation among strains in the cell concentration curves diminished over the three fermentations

Figure 1. Cell concentration in suspension during first EBC-tube fermentation for parental strain 244 and 5 hybrid strains, measured after 0, 1, 4, 5, 6 and 7 days.

Figure 2. Cell concentration in suspension during second EBC-tube fermentation for parental strain 244 and 5 hybrid strains, measured after 0, 1, 2, 3, 4 and 7 days.

Figure 3. Cell concentration in suspension during third EBC-tube fermentation for parental strain 244 and 5 hybrid strains, measured after 0, 1, 2, 3, 6 and 7 days.

Figure 4. Apparent extract during first EBC-tube fermentation for parental strain 244 and 5 hybrid strains, measured after 0, 1, 4, 5, 6 and 7 days.

Figure 5. Apparent extract during second EBC-tube fermentation for parental strain 244 and 5 hybrid strains, measured after 0, 1, 2, 3, 4 and 7 days.

Figure 6. Apparent extract during third EBC-tube fermentation for parental strain 244 and 5 hybrid strains, measured after 0, 1, 2, 3, 6 and 7 days.

Table III**The amount of vicinal diketones and their precursors in racked beer from EBC-tube fermentations.**

		Parental strain 244	Hybrid strains				
			C80- CG71	C80- CG75	C80- CG77	C80- CG83	C80- CG133
2nd fermentation							
Free diacetyl	ppm	0.05	0.05	0.05	0.05	0.05	0.03
Free 2,3-pentanedione	ppm	0.03	0.02	0.03	0.02	0.02	0.02
Total diacetyl	ppm	0.36	0.39	0.53	0.47	0.37	0.19
Total 2,3-pentanedione	ppm	0.36	0.36	0.51	0.43	0.35	0.20
3rd fermentation							
Free diacetyl	ppm	0.09	0.11	0.10	0.10	0.10	0.08
Free 2,3-pentanedione	ppm	0.04	0.05	0.05	0.05	0.06	0.04
Total diacetyl	ppm	0.63	0.83	1.31	0.94	1.02	0.52
Total 2,3-pentanedione	ppm	0.52	0.62	1.04	0.85	0.91	0.51

(Figures 1–3), especially due to the changes in cell concentration patterns of hybrid strains C80-CG75 and C80-CG83. During the third fermentation the cell concentration curves of the parental strain and the 5 hybrid strains were within the normal range for brewing yeast. Hybrid strain C80-CG133 is however more flocculent than the other strains. The parental strain and the hybrid strain C80-CG133 showed very similar changes of apparent extract during

fermentation whereas the other four hybrid strains were slower in attenuation (Figures 4–6).

The amount of vicinal diketones and their precursors in the racked beer of the second and third fermentation is shown in Table III. Their amount varied among the two fermentations, but the precursors to vicinal diketones were higher in beer from two of the hybrids, while beer made with hybrid C80-CG133 in both fermentations had lower values than the parental

Table IV**The amount of vicinal diketones and their precursors in racked beer from pilot fermentations.**

		Parental strain 244		Hybrid strain C80-CG133	
		ferm. a	ferm. b	ferm. a	ferm. b
1st fermentation					
Free diacetyl	ppm	0.38	0.35	0.25	0.17
Free 2,3-pentanedione	ppm	0.14	0.13	0.09	0.07
Total diacetyl	ppm	1.40	2.92	1.34	1.05
Total 2,3-pentanedione	ppm	0.72	1.55	0.66	0.52
2nd fermentation					
Free diacetyl	ppm	0.22	0.34	0.11	—
Free 2,3-pentanedione	ppm	0.10	0.09	0.04	0.07
Total diacetyl	ppm	0.74	0.81	0.35	—
Total 2,3-pentanedione	ppm	0.39	0.42	0.17	0.33

— not measured.

Figure 7. Cell concentration in suspension during first pilot fermentation of parental strain 244 and hybrid strain C80-CG133, measured after 0, 1, 2, 3, 6 and 7 days. The arrow indicates time of cooling.

Figure 8. Cell concentration in suspension during pilot fermentation of parental strain 244 and hybrid strain C80-CG133, measured after 0, 1, 2, 3, 6 and 7 days. The arrow indicates time of cooling.

Figure 9. Apparent extract during first pilot fermentation of parental strain 244 and hybrid strain C80-CG133, measured after 0, 1, 2, 3, 6 and 7 days. The arrow indicates time of cooling.

Figure 10. Apparent extract during second pilot fermentation of parental strain 244 and hybrid strain C80-CG133, measured after 0, 1, 2, 3, 6 and 7 days. The arrow indicates time of cooling.

Tabel V**Analysis of bottled beer from pilot fermentation.**

Analysis of bottled beer	Parental strain 244	Hybrid strain C80-CG133
Original extract, % Plato	10.70	10.67
Alcohol, % wt/wt	3.70	3.70
Real degree of fermentation, %	67	68
Bitterness, BU	22	18
pH	4.13	4.10
Viscosity, cP 20 °C	1.57	1.58
Head retention, s	91	84
Initial haze, EBC	0.5	0.6
Total haze, 0 °C after 5 days at 60 °C, EBC	9.4	9.3
Colour, EBC	7.5	7.6
Diacetyl, ppm	0.13	0.11
CO ₂ , % wt/wt	0.47	0.45
Total N, mg/l	400	400
Taste Panel score*	2.2	2.2

* 3 = normal, 2 = acceptable, 1 = not acceptable, 0 = with grave faults.

strain. It can be concluded that the EBC-tube fermentations indicate that all 5 hybrid strains have acceptable performance and that the hybrid C80-CG133 is the most promising one.

3.3. Pilot brewing with hybrid strain C80-CG133

Due to the good fermentation characteristics of hybrid strain C80-CG133 comparative pilot scale fermentations were carried out with this strain and the parental strain 244.

Two fermentations were carried out. The changes in cell concentration and extract during the fermentations are shown in Figures 7–10. Slightly higher yeast cell concentrations could be obtained with strain 244 compared to strain C80-CG133, but the attenuation curves are almost identical for the two yeast strains.

The amount of vicinal diketones and their precursors in the racked beer from the pilot fermentations is presented in Table IV. In agreement with the EBC-tube fermentations the hybrid strain C80-CG133 consistently gave

Tabel VI**Head space profile in bottled beer from pilot fermentation.**

		Parental strain 244	Hybrid strain C80-CG133
Acetaldehyde	ppm	2.8	2.5
Dimethyl sulphide	ppm	0.10	0.09
Ethyl formate	ppm	1.3	1.2
Ethyl acetate	ppm	13	14
Isobutyl acetate	ppm	0.07	0.04
1-Propanol	ppm	19	18
2-Methyl-1-propanol	ppm	15	10
3-Methylbutyl acetate	ppm	1.8	1.6
3-Methyl-1-butanol	ppm	83	75
Ethyl hexanoate	ppm	0.19	0.20
n-Hexyl acetate	ppm	0.028	0.025
Ethyl octanoate	ppm	0.18	0.27

slightly lower values for these compounds. After six weeks of storage the bottled beer was analysed with the results given in Tables V and VI.

The analytical data of the beer produced with the parental strain and the hybrid strain are very similar. Except for the total haze at 0 °C after 5 days at 60 °C, all the data are within the specifications for this type of beer by the Carlsberg Breweries. The high haze value is typical for unstabilised pilot made beer. The head space profile is also very similar for the two beers and taste panel scores are equal and acceptable. It can be concluded that a hybrid brewing strain can be constructed by mating of meiotic segregants of *Saccharomyces carlsbergensis*.

4. DISCUSSION

The brewing strain 244 of *Saccharomyces carlsbergensis* previously thought to be non-sporulating was found to sporulate at 22 °C but not at 30 °C, the temperature frequently employed in sporulation. The strain thus is temperature sensitive for sporulation. Consistent with previous findings for other brewing strains (3, 17) the asci contain few spores some of which can germinate and yield clones of α -mating type and of α -mating type as well as bisexual lines. This shows that the brewing strain 244 is heterozygous for the mating type alleles. By crossing vigorous lines of mating type α with lines carrying mating type α it was possible to produce hybrids, five of which showed fermentation behaviours similar to the original strain 244. The hybrid strain C80-CG133 showed a fermentation behaviour and brewing performance very close to strain 244 with slightly stronger flocculation and production of somewhat lower amounts of vicinal diketones and their precursors. It thus has been demonstrated that the nuclear genomes of brewer's yeast can be segregated into spores and clones competent in mating and that they can be recombined into new hybrids by mating. It is important to note that the hybrid C80-CG133 again is a poor sporulator since this could be an industrially important characteristic of brewing strains.

The two strains C80-CG65 (α) and C80-CG110 (α) are presumably haploid or near haploid as recessive auxotrophic mutants can be

readily induced in the latter strain, which will be reported in a subsequent paper. This opens the possibility for genetic characterisation of the brewing strains and for establishing selection markers in brewing strains without which a rational breeding of brewing strains is not feasible (18). It is now known that strain 244 is heterozygous for nucleotide sequences in chromosome III as detailed in a companion paper (10). The extent of heterozygosity and the reasons for the aberrant meiotic behaviour of brewing strains can be studied by means of the analysis of haploid segregants.

Besides recombination breeding using existing variability the haploid maters permit the introduction of new genetic traits by mutation or genetic transformation. The establishment of single gene alterations is equally feasible in heterozygous or homozygous forms.

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