Osmotic Sensitivity and Tolerance and Proteinase Production in a Strain of Saccharomyces

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A brewing yeast strain, NCYC 1085, unusual in that it sporulated freely and produced diploid spores, was sensitive to the degree of osmotic tension induced by the addition of 1.5 M KCl or 2.5 M ethylene glycol to yeast extract-peptone-glucose medium, but its progeny, obtained on sporulation and dissection of the resulting asci, included a number of osmotic-tolerant strains, the percentage of which increased as these strains were also sporulated and dissected. In addition, after repeated isolation of single-spore clones for three or four generations, clones producing zones of liquefaction of gelatin ranging in size from zero to large (approximately 1.5 cm) appeared, with the intensity of hydrolysis increasing in clones obtained from the later generations. The isolation of erythromycin-resistant mutants by manganese treatment was also accompanied by the appearance of osmotic-tolerant and gelatin-liquefying clones.

Introduction

Brewing yeasts *(Saccharomyces cerevisiae)* are only moderately tolerant of elevated osmotic tensions in the growth medium, being able to grow in glucose concentrations of 20% or slightly higher (1 – 1.5 M) and KCl concentrations of 2.0 M [1]. However, to the best of our knowledge, the sensitivity of brewing yeasts to elevated osmotic tensions, and the genetic control of sensitivity and tolerance, has not been investigated in detail. Singh [2] has determined that the presence of suppressor genes in strains of *S. cervisiae* was associated with sensitivity to the presence of 1.5 M KCl or 2.5 M ethylene glycol in the medium.

Likewise, *S. cerevisiae* is not commonly regarded as producing extracellular proteolytic enzymes [3], though Stewart (personal communication) has stated that brewing yeast strains are known which will bring about some degree at least of protein hydrolysis, and Chen and Miller [4] demonstrated that protease formation occurred during sporulation. The production of extracellular proteases would confer advantages in genetic studies [3], since current studies on yeast proteases and their inhibitors are done with broken-cell preparations. On a technological level, yeast strains which produced extracellular proteases world be useful, for instance, in chill-proofing of beer, and possibly in other applications.

In this paper we report the results of our investigations of dominant and recessive genes controlling osmotic tolerance in brewing yeasts, in particular in strain NCYC 1085, and on the appearance of sub-clones of this strain, producing significant quantities of extracellular proteolytic enzymes.

Materials and Methods

Yeast strains: Strain NCYC 1085 and other strains were obtained by courtesy of Mrs. Barbara Kirsop, Brewing Industry Research Foundation, Nutfield, England; AP-1-$\alpha\alpha$, from Dr. Anita Hopper, University of Massachusetts, and other industrial strains from Labatt's Breweries of Canada Limited, Bass-Charrington's Limited, Canadian Breweries Limited; from Dr. R. E. Simard, Department des Vivres, Laval University, Quebec, P. Q., Canada, and other sources. The cultures were maintained on yeast extract-peptone-glucose medium, plus 2% soluble starch.

Other media and procedures: Antibiotic-resistant strains were made by incubating the yeast strains in liquid YEP medium + 8 mM $MnCl_2$, according to the method of Putrament *et al.* [5], and plating the

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resulting cultures on YEP-glycerol medium containing 1 mg/ml of erythromycin, or chloramphenicol, 1.5 mg/ml.

Mass matings were done in still culture using thin layers of YEP broth inoculated with both the desired strains, and using the RD mutant of the prototrophic (brewing) strain as one parent and an auxotrophic laboratory strain as the other [6]. The cultures were incubated for 5 – 6 days at 28 – 30 °C.

Petite mutants were made of the prototrophic strains by incubating for two days in YEP broth + 20 μg/ml of acriflavin in still culture, according to a method suggested by D. C. Hawthorne.

Isolations were made on minimal medium (YNB, w/o amino acids, + glycerol, 2%, and ethanol, 3% (v/v), to prevent sporulation [7], and the colonies were restreaked on the same medium.

Sporulation was done in McClary's medium (liquid), inoculated with 10% (v/v) of a 48-hour liquid culture in YEP medium. The cultures were washed, treated with snail enzyme to dissolve the ascus walls [8], and dissected on YEP agar blocks using a flat-ended needle and a Singer micromanipulator.

Osmotic sensitivity was determined by replica plating on a medium containing 2% glucose, 1% yeast extract, 1% peptone, and 1.5 M KCl or 2.5 M ethylene glycol, using a multipoint inoculator.

Protein hydrolysis was determined by similar replica plating on a medium containing 2% glucose, 1% yeast extract and 10% gelatin, and incubating at 18 – 20 °C. Liquefaction was determined visually.

Results and Discussion

Table I shows the strains of industrial yeasts used and their sensitivity or tolerance to the concentrations of KCl or ethylene glycol used. As was mentioned by Singh [2], the response was not always the same to the two media, some strains being sensitive to KCl-containing medium, some to ethylene glycol-containing medium and some to both. At least half of the strains were tolerant of the osmotic tensions achieved with both compounds. However, it is interesting to note that four clones obtained from dissection of an ascus from a hybrid of AP-1-$\alpha\alpha$, a laboratory mating dipolid, and YS2968, a distiller's yeast, there was 2 : 2 segregation of sensitive and tolerance, so that there was apparently at least one recessive gene for sensitivity, the two parent strains used both being tolerant but diploid

Strain	KCl	Glycol
OK 11	+	–
OK 13	+	–
OK 17	+	–
NBA	+	+
2968-75	+	+
L-1	–	–
L-3	+	+
AP-1	+	+
2968 × AP-1 – 1D		
4 A	–	–
4 B	–	–
4 C	+	+
4 D	+	+
C.A.	+	–
C.F.	–	–
DICH	+	–
DIB	+	–
IFO 2193	+	+
NCYC 1026	+	+
NCYC 1062	–	–
NCYC 1085 *	–	–
NCYC 1139	–	+
NCYC 1329	+	+
NCYC 1330	–	+
U 155	+	–

Table I. Tolerance to increased osmotic tension in some industrial yeast strains.

* Sporulates freely forming diploid spores.

or near-diploid. In addition, strain NCYC 1085, a homothallic brewing yeast, producing viable, diploid spores [9], was sensitive.

Strain 1085 was further investigated by sporulation and dissection of the resulting asci, the clones obtained being tested for sensitivity (Table II). Out of 25 single-spore clones tested, 6 were tolerant and the rest were sensitive to elevated osmotic tensions. Sporulation of the tolerant and sensitive clones thus obtained yielded increasing numbers of tolerant clones from the tolerant clones, and few if any from the sensitive ones. Eventually the ratios of tolerant clones to sensitive reached 2 : 2, 3 : 1 and 4 : 0 on the 3rd or 4th generation progeny from the original tolerant clones, whereas in the first generation obtained by dissection of asci from NCYC 1085 only 0 : 4 and a few 1 : 3 tetrads were obtained.

The frequency of appearance of tolerant clones was increased greatly by the use of erythromycin-resistant mutants of strain 1085. The mutants were obtained by plating a culture of 1085, grown in YEP medium containing 8 mM $MnCl_2$ [5] on YEP-glycerol medium containing 1 mg/ml of erythromycin, and selection of resistant clones. The mutants thus obtained were sporulated, asci were dissected, and the resulting single-spore clones were dissected and tested for tolerance or sensitivity. Segregation ratios for tolerance : sensitivity reached 2 : 2, 3 : 1

Table II. Segregation of tolerance to increased osmotic tension in the progeny of various clones derived from NCYC 1085.

Strain	Colony size Small : Large				Resistant : Sensitive									
					KCl					Glycol				
	2:2	1:3	3:1	0:4	2:2	1:3	3:1	0:4	4:0	2:2	1:3	3:1	0:4	4:0
1085					6:25 (Total)					1:25 (Total)				
1085-E^r *	2	6			5	2					1		6	
1085-C^r *	3	4			5	1				4	2			
1085-1	2		4			1	4	1		1	1		6	
1085-2			4	2	2	4	1	1		1	2	2		2
1085-3			3	3	2:28 (Total)					1:28 (Total)				
1085-4	1			1	0:23					0:23				
1085-2-12	1			7	4	2	2			6	2			
1085-2-13				8	1		5		2	6		2		
1085 (R)-7-3	3			2										
1085 (R)-7-3														
−1 A	2	1		5						2	1			3
−1 B	1	1	1	1						1	1			2
−1 C	4									3		1		
−1 D	1	2		4						2				5

* Obtained by Mn treatment.

and 4 : 0, in the first generation, in the clones from the erythromycin-resistant mutants. No tolerant clones were obtained from chloramphenicol-resistant mutants. The mechanism leading to these results is not known.

Testing of these clones for proteolytic activity, by inoculation on gelatin plates (10% gelatin) with a multi-point inoculator, revealed the somewhat unexpected result that clones having significant proteolytic activity as demonstrated by gelatin liquefaction, had arisen in the progeny both of the original strain 1085, and of the erythromycin-resistant mutants, since the original strain 1085 did not possess this character. Some of the clones from the erythromycin-resistant strain showed relatively strong proteolytic activity (Table IV), as did some of the 3rd and 4th generation clones from the original tolerant strains (Tables IV and V). Sporulation and dissection of all four clones from each of two tetrads showing segregation for proteolytic activity showed increasing numbers of clones producing gelatin liquefaction, as well as increasing strength of

Clone	1085 (R)-7-3			1085-E^r			1085-C^r		
	Gly	Col	Gel	KCl	Col	Gel	Gly	Col	Gel
1 A	+	L	−	w	S	−	−	S	−
1 B	−	S	w	w	S	++	−	S	−
1 C	−	S	+	+	L	−*	+	L	−
1 D	+	L	+	+	S	++	−	S	−
2 A	+	L	+	+	L	−	−	L	−
2 B	+	L	+	−	L	++	−	L	−*
2 C	+	L	w	+	S	++	w	M	−
2 D	+	L	w	+	S	−	w	M	−
3 A	−	S	+	−	S	−	+	S	−
3 B	+	L	+	+	L	++	−	L	−
3 C	−	S	+	+w	M	−*	−	S	−
3 D	+	L	+	w	M	++	+	S	−
4 A	−	S	−	w	S	−	−	S	−
4 B	w	M	+	+	M	++	−	S	−
4 C	−	S	−	+	L	−	w	S	−
4 D	w	L	+	+	S	−	+	L	−

* Sporulated on glucose-yeast extract-gelatin medium.
L, large colony; S, small colony; M, medium colony; w, weak growth; Gly, ethylene glycol; Col, colony size; Gel, gelatin hydrolysis.

Table III. Colony size, gelatin hydrolysis and tolerance to increased osmotic tension in single-spore clones from a strain derived from NCYC 1085.

Table IV. Colony size, gelatin hydrolysis and tolerance to elevated osmotic tension in single-spore clones isolated from a strain derived from NCYC 1085.

Clone	1085(R)-7-3											
	−1 A			−1 B			−1 C			−1 D		
	Gly	Col	Gel	Gly	Col	Gel	Gly	Col	Gel	Gly	Col	Gel
1 A	+	L	w	−	L	+w	+	L	w	w	L	+
1 B	+	L	+	−	S	w	−	S	++	+	L	++
1 C	+	L	+	+	L	+	w	S	w	+	L	+
1 D	+	L	w	+	L	+	w	S	++	+	L	+
2 A	−	S	w	+	S	+w	−	S	++	+	L	+
2 B	−	S	+	+	M	+	+	L	w	+	L	+w
2 C	+	L	+	+	S	+	w	S	++	+	L	w
2 D	+	L	+	+	L	+	+w	L	++	+	L	+
3 A	+	L	+	+	L	+w	+w	S	++	+	L	+
3 B	+w	L	w	+	L	+	+	L	w	−	S	+++
3 C	−	L	+	+	L	+	−	S	+++	+	L	−
3 D	w	L	+	+	L	+	+	L	w	−	L	+++
4 A	−	S	+	−	S	w	+	L	w	+	S	w
4 B	−	S	+	+	L	+++	−	S	+++	+	L	+w
4 C	+	L	+	+	S	w	+	L	+w	+	S	+w
4 D	−	L	−	+	L	+++	−	S	+++	+	L	w
	Co:No			Co:No			Co:No			Co:No		
Tetrads	2:2			1:3			3:1			1:3		
Totals	13:3			12:4			15:1			11:5		

L, large colony; S, small colony; M, medium colony; w, weak growth; Gly, ethylene glycol; Col, colony size; Gel, gelatin hydrolysis.

Table V. Colony size, gelatin hydrolysis and tolerance to elevated osmotic tensions in clones isolated from a strain derived from NCYC 1085.

Clone	1085(R)-7-3											
	−4 A			−4 B			−4 C			−4 D		
	Gly	Col	Gel	Gly	Col	Gel	Gly	Col	Gel	Gly	Col	Gel
1 A	+	L	w	+	L	+w	+	S	w	w	L	−
1 B	+	L	w	+	L	+w	−	L	+	w	L	−
1 C	+	L	w	−	S	+	+	S	w	w	L	−
1 D	+	L	w	vw	S	+	−	L	+	w	L	−
2 A	+w	S	w	+w	L	+	−	L	++	vw	L	+
2 B	+w	L	w	vw	S	+	+	S	−	w	L	w
2 C	w	S	w	+	L	+	−	L	++	w	L	w
2 D	+	L	w	+	L	+	+	S	−	w	L	w
3 A	+	L	+w	+	L	+	+	S	−	+w	L	−
3 B	+	L	+w	w	L	+	+	L	−	+w	L	−
3 C	+	S	+w	+	L	+	+	L	w	+	S	−
3 D	−	S	−	vw	L	+	+	M	w	+	L	−
4 A	+	L	+	+	S	+	+	S	+w	w	M	w
4 B	w	S	−	w	L	−	+	L	+w	+w	M	w
4 C	w	S	−	w	L	−	+	L	w	w	L	w
4 D	+	L	+	+w	S	+	+	S	w	vw	S	+

Gly, ethylene glycol; Col, colony size; Gel, gelatin hydrolysis; L, large colony; M, medium colony; S, small colony; w, weak reaction.

the proteolytic acitivy. However, proteolytic activity did not appear to be linked to osmotic tolerance. Colony size, in the original dissection, was correlated with osmotic tolerance, small colonies, as might be expected, being sensitive to elevated osmotic tensions.

It is interesting to note that some of the clones sporulated on the gelatin medium which contained high enough concentrations of glucose and organic nitrogen to repress sporulation in the original strain. However, unlike the strains studied by Chen and Miller [4], which showed gelatin liquefaction during sporulation, the sporulating clones were not proteolytic.

The significance of the above results lies, first, in the demonstration that both dominant and recessive genes for osmotic tolerance apparently exist in these strains. Crosses of apparently tolerant parent strains show clear segregation of sensitive strains in the progeny, and dissection of the sensitive strain NCYC 1085 yielded increasing numbers of progeny which were tolerant of the moderate osmotic tensions used in these experiments. These strains are being investigated further, to determine the limits of osmotic tolerance which can be obtained, and to determine whether tolerance of other toxic or moderately toxic substances can likewise be increased.

The significance of the production of extracellular proteases by strain 1085 is also being further investigated. G. G. Stewart (personal communication) has remarked that some brewing yeasts do possess gelatin liquefying activity, but this fact is not common knowledge among yeast geneticists, Ogrydziak and Mortimer [3] having stated recently that the lack of strains of *Saccharomyces cerevisiae* possessing this characteristic has impeded the study of proteinase production in yeasts. The formation of extracellular proteases by brewing yeasts may well have been considered of little importance in the past by yeast geneticists, since these strains were held to be not amenable to simple genetic analysis. However, recent studies [10] have shown that most brewing and distiller's strains can be readily hybridized with laboratory strains, and genetic analysis of the resulting hybrids, though more complex than that involved in analysis of hybrids of most laboratory strains, has already yielded interesting information concerning the nature of the mating reaction and the mating-type alleles in some industrial yeast strains. Stewart (personal communication) has achieved similar results using protoplast fusion of brewing and laboratory haploids.

As well, further analysis of the behavior of the progeny of strain NCYC 1085, though complicated by the diploid nature of its spores and its homothallism, may be useful in determining the nature of the genetic control of the synthesis of the major proteases and peptidases of *S. cerevisiae*, as well as of the various proteinase inhibitors. Likewise the nature of the mitochondrial involvement in the phenomenon of extracellular protease production remains to be elucidated, but should yield valuable information concerning these and a number of related problems.

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