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Industrial yeast strain improvement: construction of a highly flocculent yeast with a killer character by protoplast fusion

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Conditions were optimized for rapid release and improved regeneration of protoplasts of *Saccharomyces cerevisiae* NCIM 3458. Rapid protoplast release was also obtained with representatives of several other yeast genera under the modified conditions of treatment. The application of the procedure in construction of a highly flocculent *Saccharomyces cerevisiae* with a killer character is described. Fusion was effected between UV-killed protoplasts of *S. cerevisiae* NCIM 3578 with a killer character and live protoplasts of the highly flocculent *S. cerevisiae* NCIM 3528 in the presence of polyethylene glycol (PEG) 6000. Fusants were selected using benomyl resistance as marker, the killer toxin producer rather than the highly flocculent yeast being resistant to the fungicide at a concentration of 100 μg ml^{-1}. Fusants were also characterized by their DNA contents, capacity for ethanolic fermentation of molasses sugar and levels of invertase, alcohol dehydrogenase and pyruvate decarboxylase activities.

Keywords: protoplast fusion; killer character; flocculence; *Saccharomyces cerevisiae*; industrial yeast

Introduction

Protoplasts find wide applications in biochemical, physiological and genetic studies [6,20,25]. Protoplast fusion has been used extensively for the improvement of industrial strains of yeasts which are generally polyploid and not easily amenable to approaches such as sexual hybridization, mutagenesis and recombinant DNA technology [29]. Efficient protoplast isolation and regeneration are prerequisites for high fusion or transformation efficiencies.

A desirable property to control killer sensitive wild yeasts during industrial fermentation, particularly in continuous operations over prolonged periods is the killer character [38]. The *Saccharomyces* killer character which is encoded on double-stranded RNA (ds RNA) encapsulated within a glycoprotein coat leads to the production of an extracellular protein which is toxic to sensitive wild yeasts [3,34,41]. The transfer of the killer character to laboratory and industrial strains has been achieved in many laboratories by various methods. Hara *et al* [15] bred killer strains of *S. cerevisiae* by back crossing. Construction of killer strains by gene replacement was described by Boone *et al* [2]. Salek *et al* [30] used electrotransformation to get stable hybrids having killer activity.

Flocculation is an important prerequisite in yeasts used in industrial fermentations [7,32]. Highly flocculent yeasts permit continuous ethanolic fermentation without recourse to centrifugation for cell recovery and recycle [22]. Construction of flocculent strains also has been carried out by protoplast fusion [5,39] as well as by electrofusion [35,36].

In this paper, we present data on the optimization of conditions for yeast protoplast formation and regeneration. The modified protocol has been applied for the isolation of intra-species hybrids of *S. cerevisiae* using UV-killed protoplasts of one of the parental strains. The objective of these studies was to transfer the killer property from a killer strain to a highly flocculent strain of *S. cerevisiae*. The characterization of fusants in relation to ethanol productivity, flocculence and intracellular enzyme levels is also reported.

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Materials and methods

Chemicals: Malt extract, yeast extract and peptone were obtained from Difco Chemical Co, Detroit, USA. Novozyme 234 was obtained from Novo Enzyme Products Ltd, Denmark. Dithiothreitol (DTT), 2-mercaptoethanol, thiamine pyrophosphate (TPP) and nicotinamide adenine dinucleotide (NAD) were purchased from Sigma Chemicals Ltd, St Louis, MO, USA. Benomyl (a commercial preparation registered as Benlet) was obtained from local sources. Polyethylene glycol-6000 was from Polysciences Inc, Warrington, USA. Sugarcane molasses was obtained from the Deccan Sugar Institute, Pune, India, with a total sugar content of 51% (w/w), 10% of which was nonfermentable.

Microorganisms and growth media: *Saccharomyces cerevisiae* NCIM 3458, *Candida shehatae* NCIM 3500, *Pachysolen tannophilus* NCIM 3445, *Rhodotorula glutinis* NCIM 3169, *Kluyveromyces marxianus* NCIM 3232, *Pichia stipitis* NCIM 3549, *Hansenula canadensis* NCIM 3414, *Schizosaccharomyces pombe* NCIM 3457, *Yarrowia lipolytica* NCIM 3472, *Cryptococcus albidus* NCIM 3444, highly flocculent *Saccharomyces cerevisiae* NCIM 3528, and the killer strain, *Saccharomyces cerevisiae* NCIM 3578 were from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The killer sensitive strain *Saccharomyces cerevisiae* MTCC 473 was from the Institute of Microbial Technology, Chandigarh, India. A standard haploid strain, *Saccharomyces cere-*

visiae EG103, was a gift from Professor PK Maitra, Tata Institute of Fundamental Research, Bombay, India.

All strains were maintained on MGYP agar slants containing (g L^{-1}): malt extract, 3.0; glucose, 20; yeast extract, 3.0; peptone, 5.0 and agar, 20.0. Protoplast regeneration medium consisted of MGYP liquid medium with 0.8% agar and various osmotic stabilizers such as sucrose, sorbitol, NaCl, KCl, $MgSO_4$, $MgCl_2$ or NH_4Cl at various concentrations. Selection medium for the isolation of fusants consisted of MGYP liquid medium supplemented with agar (0.8%), NaCl (0.8 M), and benomyl (100 μg ml^{-1}). Molasses growth medium (MMYP) consisted of (g L^{-1}): molasses reducing sugar, 50.0; malt extract, 3.0; yeast extract, 3.0 and peptone, 5.0. The pH of all media was adjusted to 6.0. Molasses fermentation medium contained (g L^{-1}): molasses total reducing sugar, 200.0 and urea, 1.0. The initial pH of the fermentation medium was adjusted to 4.5.

Protoplast isolation: The protoplast isolation and regeneration procedure was based on that described by Hamlyn *et al* [14]. Cultures were grown in liquid MGYP medium (10 ml) for 16–20 h at 30° C on a rotary shaker (200 rpm). Cells were harvested by centrifugation at $800 \times g$ for 10 min and washed with potassium phosphate buffer (0.1 M, pH 5.8). The cells were suspended in the same buffer containing 50 mM DTT and incubated for 1 h at 30° C under gentle agitation. DTT-pretreated cells were then recovered by centrifugation and washed repeatedly with phosphate buffer to remove the traces of DTT. Approximately 10^8 cells were suspended in 2 ml phosphate buffer containing Novozyme 234 (1 mg ml^{-1}) and various osmotic stabilizers such as sucrose, sorbitol, NaCl, KCl, $MgSO_4$, $MgCl_2$ or NH_4Cl at different concentrations and incubated at 30° C with gentle shaking (60 rpm). Samples were withdrawn at different time intervals and protoplast formation was monitored by counting the protoplasts in the samples after dilution with phosphate buffer containing osmotic stabilizer.

Protoplast regeneration: Protoplasts centrifuged at $300 \times g$ for 15 min at 30° C were washed with osmotically stabilized buffer to make them free from Novozyme and suspended in the same buffer. After appropriate dilution, the protoplasts were plated on regeneration medium and the plates were incubated at 30° C. Colonies appearing after 72–96 h were counted to assess regeneration frequency.

Protoplast fusion: Protoplasts of *S. cerevisiae* 3578 and *S. cerevisiae* 3528 were isolated using Novozyme 234 in the presence of 0.8 M NaCl. The protoplast fusion procedure was based on that used by Ouchi *et al* [24] using polyethylene glycol as fusogenic agent. Protoplast fusion was carried out between live protoplasts of the highly flocculent *S. cerevisiae* NCIM 3528 and UV-killed protoplasts of the killer *S. cerevisiae* NCIM 3578. The protoplasts of *S. cerevisiae* 3578 were suspended in phosphate buffer (0.1 M, pH 5.8) containing 0.8 M NaCl and the protoplast suspension was subjected to UV irradiation with a 15-W germicidal lamp at a distance of 6 in for 45 min. Killing of protoplasts was monitored by plating approximately 10^8 cells on MGYP regeneration medium. The UV-killed protoplasts of the killer strain (approximately 10^8) were mixed with an approximately equal number of live protoplasts of the flocculent strain. In a final volume of 1 ml of phosphate buffer (0.2 M, pH 5.8) containing 0.8 M NaCl as an osmotic stabilizer and 33% (w/v) polyethylene glycol-6000, the protoplast mixture was incubated for fusion for 5 min at 30° C. After dilution with the osmotically stabilized buffer, the fusion mixture was centrifuged at low speed ($300 \times g$) and washed twice with the same buffer. The mixed protoplasts were then suspended in 1 ml of the buffer and fusants were regenerated on osmotically stabilized selection medium as well as on MGYP regeneration medium at 30° C for 72–96 h.

Measurement of flocculence: Flocculence of the cells was measured by the method of Johnston and Reader [17]. The culture was grown in MGYP medium (10 ml) at 30° C for 48 h and cells were pelleted by centrifugation. The pellet was suspended in sodium acetate buffer (0.05 M, pH 4.5) containing 5 mM $CaCl_2$ and the suspension was mixed vigorously on a vortex mixer, and allowed to stand at room temperature for 30 s. Flocculence was observed and expressed on a subjective scale, ranging from 0 (nonflocculent with no visible flocs, totally turbid suspension) to 5 (highly flocculent with clearly visible flocs, clearing of top 3.5 cm of tube after 30 s).

Assay of the killer activity: Killer activity was tested as described by Phillskirk and Young [26]. The cells of the killer sensitive strain grown at 30° C in MGYP liquid medium for 16 h were washed with citrate phosphate buffer (0.1 M, pH 4.5). These cells (approx. 10^8) were added to a sterile MGYP medium containing 0.5% (w/v) agar and 0.003% (w/v) methylene blue. This soft agar suspension was overlaid on a petri plate of MGYP agar prepared in citrate phosphate buffer (0.1 M, pH 4.5), supplemented with methylene blue, (0.003%, w/v). The yeast strain to be assayed for killer activity was spot inoculated onto the surface of the medium and the plates were incubated at 28–30° C. After 48 h, plates were observed for a clear zone around the inoculum, in which no growth of sensitive strain occurred. The clear zone was bounded by a zone of dead cells which stained dark blue.

DNA extraction and estimation: Extraction of DNA from the cells was carried out using the hot perchloric acid method as described by Farahnak *et al* [9].

Batch fermentation: The inoculum was grown in MMYP medium containing 5% reducing sugar of molasses for 24 h at 30° C on a rotary shaker. Ten milliliters of the inoculum was transferred to 90 ml of fermentation medium containing different concentrations of reducing sugar viz 20%, 25%, and 30%. The fermentation was carried out at 30° C under stationary conditions. Samples were withdrawn after 24 and 48 h and ethanol in the samples was estimated by the cerric ammonium nitrate method [28].

Measurement of specific ethanol productivity: Cultures were grown in 1-L flasks containing 300 ml MMYP

medium at 30° C for 18 h on a rotary shaker. Cells were harvested by centrifugation and about 1 g (dry wt equivalent) of cells were transferred to 100 ml of the fermentation medium containing 20% reducing sugar of molasses. The flasks were incubated under stationary conditions at 30° C and samples were withdrawn at time intervals of 2 h for a total period of 8 h, for determination of ethanol content. Specific ethanol productivity was expressed as ethanol produced per gram (dry weight) of cells per hour.

Preparation of cell extract: Cell extracts were prepared according to the method of Ferguson and Sims [10]. Cells grown aerobically in MMYP medium for 16 h at 30° C were harvested by centrifugation at 6500 × *g* for 20 min and washed twice with saline. About 1 g (dry wt equivalent) of cells were suspended in 20 ml of pre-cooled phosphate buffer (0.1 M, pH 7.0) along with Ballotini glass beads (0.45 mm diameter). Cells were homogenized at 4° C using a cell homogenizer (B Braun, Melsungen, Germany) for 60 s (three cycles of 20 s each). The homogenized cell suspension was centrifuged at 9220 × *g* for 30 min at 4° C and the clear supernatant fluid obtained was used to determine enzyme activities and protein content.

Enzyme assays: Invertase (EC 3.2.1.26) activity was assayed according to the method of Gas'con and Lampen [11]. The total reaction mixture of 0.5 ml contained 0.2 ml acetate buffer (0.1 M, pH 4.5), 0.1 ml sucrose solution (0.5 M) and 0.2 ml of suitably diluted cell extract. The mixture was incubated at 30° C for 10 min. The reaction was stopped by the addition of 0.5 ml of dibasic potassium phosphate (0.2 M) and the reaction mixture was immediately placed in a boiling water bath for 5 min. Suitable aliquots were taken for estimating D-glucose by the glucose oxidase method using Glox reagent. One unit of enzyme activity corresponds to the liberation of one micromole of D-glucose per min.

Pyruvate decarboxylase (EC 4.1.1.1) was assayed by the method of Reed and Williams [27]. The assay system consisted of: 0.15 ml of potassium phosphate buffer (1.0 M, pH 6.0); 0.1 ml thiamine pyrophosphate (2.0 mM); 0.1 ml of $MgSO_4$ (3.0 mM); 0.1 ml of potassium pyruvate (0.5 M); 0.1 ml of cell extract and water to make a final volume of 1.4 ml. The mixture was incubated for 30 min at 30° C. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid and the precipitate formed was removed by centrifugation. An aliquot (0.2 ml) of the supernatant phase was added to the mixture containing 1 ml of TCA (10%), 0.1 ml potassium ferricyanide (0.25 M) and water to a final volume of 2.4 ml followed by the addition of 1 ml of SDS (4%) and 0.5 ml of ferric ammonium sulfate-SDS reagent. The mixture was allowed to stand at room temperature for 30 min and absorbance was measured at 540 nm. One unit of enzyme activity is defined as the amount of the enzyme required to produce two micromoles of ferrocyanide per hour.

Alcohol dehydrogenase (EC 1.1.1.1) activity was assayed according to the method of Barron and Levine [1]. The assay mixture contained Tris HCl (20 mM, pH 8.6), NAD (0.1 mM), L-cysteine-HCl (1 mM) in a total volume of 2.8 ml. The reaction was started by the addition of cell extract followed by ethanol (0.6 M). The change in absorbance at 340 nm was monitored every 15 s for 1 min. One unit of activity is defined as one micromole of NAD reduced per min.

Protein was determined by the method of Lowry *et al* [21] using bovine serum albumin as standard.

Results

Isolation and regeneration of protoplasts

The protoplasting method was designed to enhance the frequency of protoplast release. The protocol was standardized with *S. cerevisiae* NCIM 3458 using Novozyme 234, a lytic enzyme preparation from *Trichoderma harzianum*. No protoplast formation was observed when cells were directly incubated with the lytic enzyme. Pretreatment with DTT (50 mM) was more effective than with 2-mercaptoethanol. The concentration of DTT and the period of pretreatment were standardized and it was found that pretreatment with 50 mM concentration of DTT and 60 min exposure were optimal for protoplast release by Novozyme 234 (data not shown).

Various inorganic salts, sucrose and sorbitol were tried at concentrations ranging from 0.4–1.2 M as osmotic stabilizers for protoplast release. The time course of protoplast release using different osmotic stabilizers is shown in Figure 1. Irrespective of the concentration used, all the osmotic stabilizers gave at least 80–85% yield of protoplasts after 1 h of incubation with the lytic enzyme. In general, inorganic salts were superior to sucrose or sorbitol. Sodium chloride (0.6 M, 0.8 M) in particular yielded almost 100% protoplasts within 15 min while 95% protoplast formation within 15 min was observed when $MgCl_2$ (0.4 M) or NH_4Cl (0.4 M) was used.

Table 1 summarizes the effect of different osmotic stabilizers on protoplast regeneration of *S. cerevisiae* NCIM 3458. In general, protoplasts regenerated efficiently on medium containing sucrose (0.6 M), sorbitol (1.2 M) or any of the inorganic salts tested except $MgSO_4$. Protoplasts prepared in NaCl (0.6 M) showed highest regeneration (81%) when sucrose (0.6 M) was present in the regeneration medium. The protoplasts released in 0.8 M NaCl gave uniformly high regeneration efficiency on all the osmotic stabilizers tested. Protoplasts derived with KCl or NH_4Cl as stabilizers showed poor regeneration irrespective of the osmotic stabilizer used in the regeneration medium. Magnesium sulfate favored neither protoplast isolation nor protoplast regeneration in the case of *S. cerevisiae* NCIM 3458.

In order to test the general applicability of the protoplasting procedure, protoplast release from strains of different genera of yeasts was examined (Table 2). Cells treated with 50 mM DTT for 60 min were suspended in phosphate buffer (0.1 M, pH 5.8), containing 0.8 M NaCl as osmotic stabilizer and 1 mg ml^{-1} Novozyme. The release of protoplasts was virtually quantitative in a period of 15 min with cells of *Candida shehatae*, *Cryptococcus albidus*, *Pachysolen tannophilus*, *Pichia stipitis* and *Hansenula canadensis* strains. Similar yields with *Kluyveromyces marxianus* and *Yarrowia lipolytica* required 30 min and 45 min of

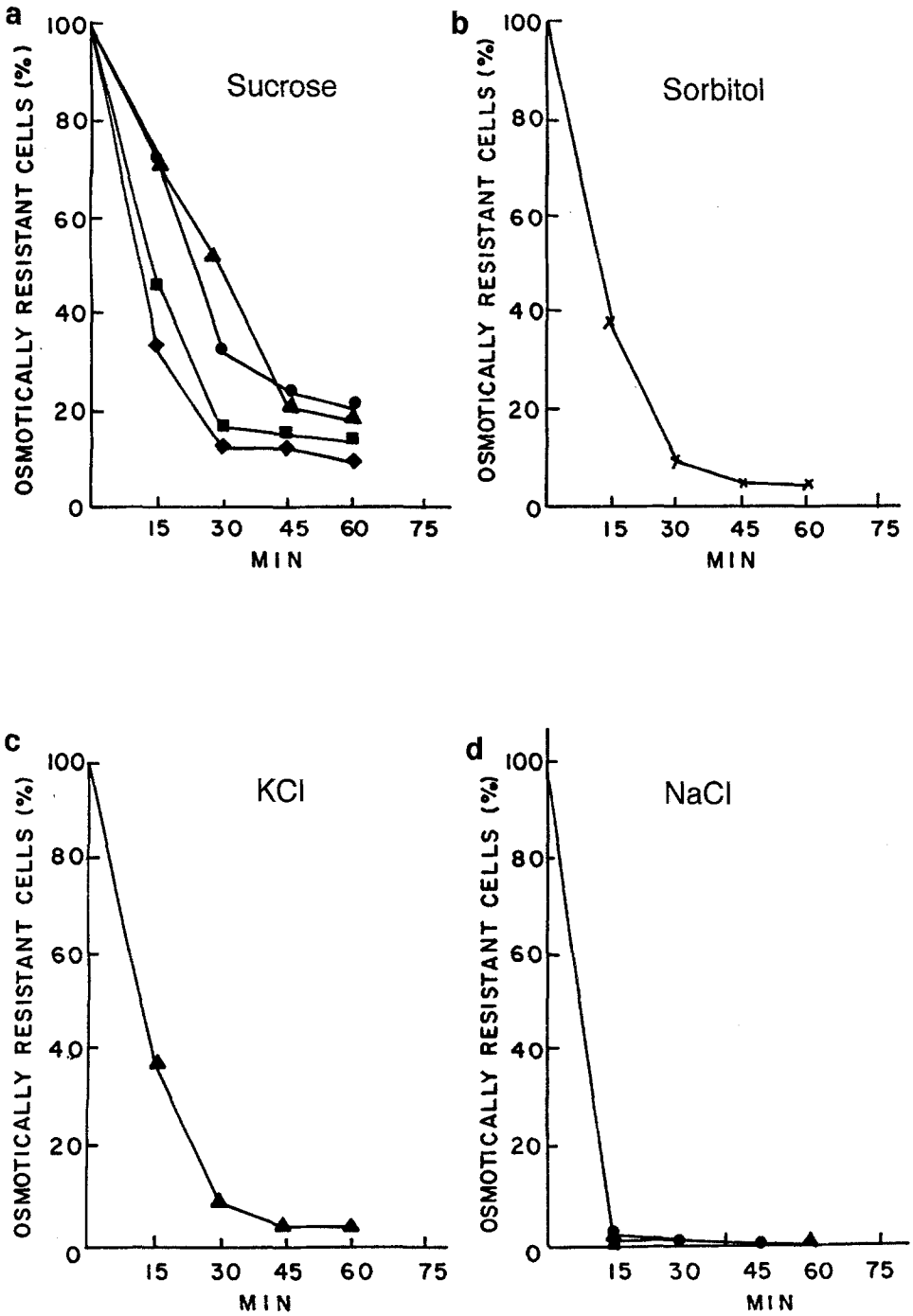

Figure 1a–d Profiles of protoplast formation of *S. cerevisiae* NCIM 3458 in different osmotic stabilizers. (a) Sucrose; (b) Sorbitol; (c) KCl; (d) NaCl; (e) $MgSO_4$; (f) $MgCl_2$; and (g) NH_4Cl. Lytic digestions were carried out using Novozyme 234 (1 mg ml^{-1}) in osmotically stabilized phosphate buffer (0.1 M, pH 5.8). The cells–enzyme–stabilizer mixtures were incubated with shaking at 30° C. –●– 0.4 M; –▲– 0.6 M; –■– 0.8 M; –◆– 1.0 M and –×– 1.2 M

incubation with lytic enzyme respectively. In contrast *Rhodotorula glutinis* and *Schizosaccharomyces pombe* were resistant to Novozyme 234 even after a period of 1 h.

Selection of intraspecies hybrids

Four independent protoplast fusions between *S. cerevisiae* NCIM 3528 (a flocculent, killer-negative, benomyl-sensitive strain) and *S. cerevisiae* NCIM 3578 (a nonflocculent, killer, benomyl-resistant strain) were performed in order to construct an industrially important fusant. The UV killing time for *S. cerevisiae* NCIM 3578 was standardized. All cells were killed after UV exposure for a period of 45 min (data not shown). An exposure time of 45 min was, therefore, chosen to completely inactivate protoplasts of *S. cerevisiae* NCIM 3578. Fusants were recovered on a medium containing benomyl (100 μg ml^{-1}) at a frequency of 2.5×10^{-5}. Controls run with an equal number of protoplasts of the individual parental strains showed no growth

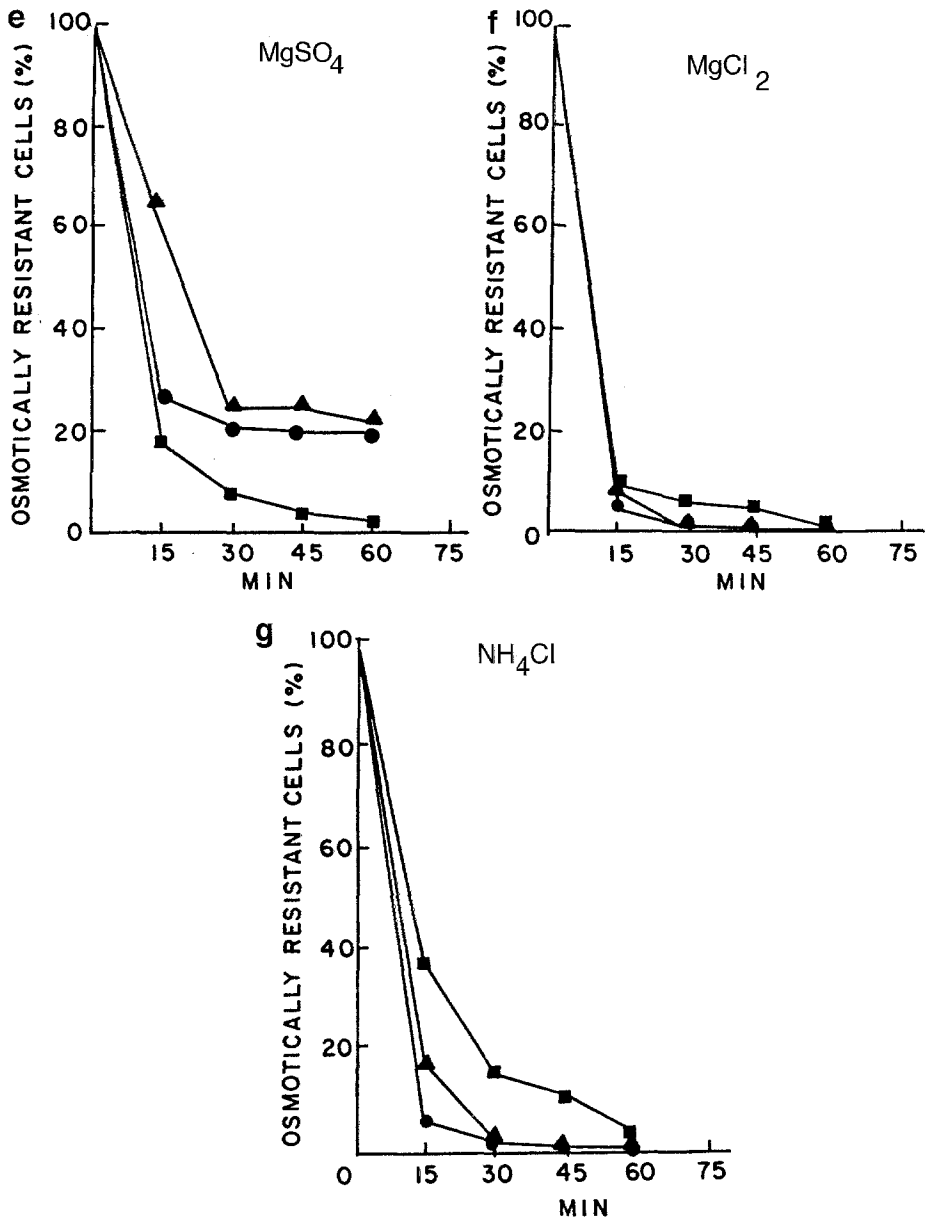

Figure 1e–g

on the selection medium. The 4000 clones obtained were screened for the rough and dry colony morphology characteristic of the flocculent strain, and 320 fusants were picked. Only four clones: F-22, F-26, F-30 and F-31, had both desirable properties of flocculence (Table 3) and killer toxin production (Figure 2). Further work was therefore carried out using these fusants. Although all four fusants showed good flocculence in MGYP medium, no flocculation was observed when they were grown in molasses. The results of DNA extraction and estimation of DNA content are shown in Table 3. The haploid strain *S. cerevisiae* EG103 had a DNA content of 1.12×10^{-8} μg cell^{-1}. The parental strains used in these studies were polyploid as evident from their DNA content. The results indicate that the fusants were also polyploid, having more DNA per cell than the parental strains.

Fermentation performances of the fusants

To study the fermentation performances of the fusants, ethanol production was measured in cane molasses media containing 20–30% total reducing sugar. Ethanol produced by killer strain (*S. cerevisiae* NCIM 3578) was comparatively low irrespective of molasses sugar concentration (Table 4). All the fusants and *S. cerevisiae* NCIM 3528 showed markedly higher ethanol production after 24 and 48 h. Fusants F-30 and F-31 showed improved fermentation ability at 30% molasses sugar concentration. The specific ethanol productivities calculated during initial phase of fermentation in the cane molasses medium are set out in Table 5. All the fusants showed initial specific ethanol productivities similar to that of killer strain *S. cerevisiae* NCIM 3578. Ethanol productivity of the parent flocculent strain was low compared to the values obtained for the fusants.

Table 1 Effect of osmotic stabilizers on regeneration of protoplasts from *S. cerevisiae* NCIM 3458

Osmotic stabilizer in regeneration medium		% Regeneration of protoplasts prepared in				
		NaCl (0.8 M)	NaCl (0.6 M)	KCl (0.6 M)	NH_4Cl (0.8 M)	$MgCl_2$ (0.8 M)
KCl	0.6 M	75.0	52.0	27.0	26.0	60.0
Sucrose	0.6 M	67.0	81.0	23.0	30.0	68.0
Sorbitol	1.2 M	75.4	61.0	24.0	25.0	73.0
NaCl	0.4 M	58.0	55.0	21.0	32.0	59.0
	0.6 M	51.0	56.0	21.0	28.0	52.0
	0.8 M	71.0	56.0	21.0	31.0	36.0
$MgCl_2$	0.4 M	62.0	76.0	22.0	26.0	47.0
	0.6 M	71.0	33.0	20.0	24.0	47.5
	0.8 M	56.0	32.0	26.0	26.0	25.0
NH_4Cl	0.6 M	69.0	0	0	31.2	48.0
	0.8 M	70.0	42.0	19.0	33.7	31.0
$MgSO_4$	0.4 M	0	0	0	0	0
	0.6 M	0	0	0	0	0

Standard deviation of the values ranged from ±3–±5%

Table 2 Protoplast formation from different yeast genera

Strain	% Protoplasting after			
	15 min	30 min	45 min	60 min
Candida shehatae	100	–	–	–
Cryptococcus albidus	100	–	–	–
Pachysolen tannophilus	100	–	–	–
Kluyveromyces marxianus	84	100	–	–
Rhodotorula glutinis	0	0	0	0
Pichia stipitis	100	–	–	–
Yarrowia lipolytica	86	92	100	–
Schizosaccharomyces pombe	0	0	0	0
Hansenula canadensis	100	–	–	–

DTT-pretreated cells were converted to protoplasts using Novozyme (1 mg ml^{-1}) and NaCl (0.8 M) in phosphate buffer (0.1 M, pH 5.8)

Table 3 Flocculation activity and average DNA content per cell for fusants and parental strains of yeasts

Yeast strains	Flocculation activity	Total DNA ($\times 10^{-8}$ μg per cell)
S. cerevisiae NCIM 3578	0	5.00
S. cerevisiae NCIM 3528	5	7.45
F-22	4	9.20
F-26	4	8.34
F-30	3	8.00
F-31	4	8.50
S. cerevisiae EG103	ND	1.12

ND = not determined

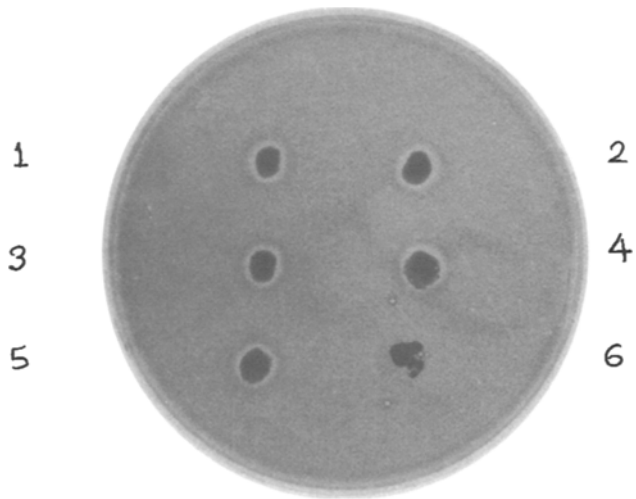

Figure 2 Killer activity of fusants and parental yeast strains 1, F-22; 2, F-26; 3, F-30; 4, F-31; 5, *S. cerevisiae* 3578; 6, *S. cerevisiae* 3528

Enzyme levels in the fusants

We selected three key enzymes: invertase, pyruvate decarboxylase and alcohol dehydrogenase, which play important roles in molasses fermentation and determined their intra-

Table 4 Ethanol production by yeasts at various molasses sugar concentrations

Yeast strains	Ethanol % (w/v) at sugar concentrations of					
	20%		25%		30%	
	24 h	48 h	24 h	48 h	24 h	48 h
S. cerevisiae NCIM 3578	1.13	3.71	0.69	1.57	0.71	1.31
S. cerevisiae NCIM 3528	2.45	4.37	2.25	5.20	1.80	4.60
F-22	2.80	4.90	2.22	4.63	2.12	4.13
F-26	2.48	4.24	2.64	4.93	1.68	3.88
F-30	3.30	5.31	2.47	4.67	2.11	5.81
F-31	2.66	4.84	2.60	5.01	2.94	5.93

Standard deviation of the values ranges from ±5–±8%

Table 5 Specific ethanol productivity of fusants and parental yeast strains

Yeast strains	Specific ethanol productivity[a] ($g\ g^{-1}\ h^{-1}$)
S. cerevisiae NCIM 3578	0.83 ± 0.02
S. cerevisiae NCIM 3528	0.61 ± 0.01
F-22	0.84 ± 0.02
F-26	0.90 ± 0.02
F-30	0.75 ± 0.05
F-31	0.90 ± 0.01

[a]Ethanol productivities were calculated during the initial phase of fermentation using 20% reducing sugar of molasses

cellular levels in the fusants and the parental strains. The results summarized in Table 6 show that invertase activity was higher in the parent flocculent strain and alcohol dehydrogenase activity was higher in the killer strain. Pyruvate decarboxylase activities of the fusants F-26 and F-30 were comparatively higher than those of the parents. The values of the alcohol dehydrogenase and invertase activities in all the fusants were intermediate between the values of the parents. This could be a result of recombination between two genomes after protoplast fusion.

Discussion

Though the experimental conditions for isolation and regeneration of protoplasts from yeasts have been reported, some degree of optimization becomes necessary for individual strains and species. An extensive range of osmotic stabilizers have been used for the isolation of protoplasts from yeasts as well as fungi. Generally, sugars and sugar alcohols are more effective with yeasts than inorganic salts [24,31,37]. Potassium chloride has also been used successfully to get 100% protoplast yield [33,36].

In the present investigation, we tested a wide range of osmotic stabilizers including sugars, sugar alcohols and inorganic salts for rapid protoplasting of DTT-treated yeast cells. Surprisingly, all the inorganic salts tested, except $MgSO_4$, were more suitable than sucrose and sorbitol as osmotic stabilizers. Protoplast formation from representatives of several yeast genera was complete within 15 min when NaCl or $MgCl_2$ was used as stabilizer. This incubation time with the lytic enzyme appears to be the shortest time reported to date for complete protoplast release. The corresponding times reported in the literature vary from 30 min to 2 h [8,18,31]. High concentrations of lytic enzyme

Table 6 Comparison of intracellular invertase, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities of parent and fusant strains

Yeast strains	Invertase ($U\ mg^{-1}$)	PDC ($U\ mg^{-1}$)	ADH ($U\ mg^{-1}$)
S. cerevisiae NCIM 3578	1.68 ± 0.05	0.31 ± 0.04	1.00 ± 0.05
S. cerevisiae NCIM 3528	3.22 ± 0.06	0.42 ± 0.02	0.48 ± 0.08
F-22	2.21 ± 0.08	0.29 ± 0.03	0.65 ± 0.02
F-26	1.88 ± 0.07	0.48 ± 0.04	0.77 ± 0.03
F-30	2.03 ± 0.05	0.49 ± 0.04	0.88 ± 0.05
F-31	3.07 ± 0.08	0.38 ± 0.03	0.99 ± 0.02

[13] and prolonged incubation of yeast cells with the lytic enzyme [8] have been reported to result in decreased regeneration efficiency. The lower concentration of lytic enzyme used in the present studies (1 mg ml^{-1}) and the short period of incubation (15 min) could be the reasons for higher efficiency of regeneration of yeast protoplasts. The high protoplast regeneration frequencies provided the basis for the fusion experiments.

The rapid release of protoplasts, not only from *Saccharomyces*, but also from other yeast genera, including *Hansenula*, *Pichia*, *Pachysolen*, *Cryptococcus*, suggested wide applicability of our procedure described here except for *Rhodotorula* and *Schizosaccharomyces* in which protoplast isolation is not as straightforward as that of *Saccharomyces* [8,16,31].

Ouchi *et al* [23] and Young *et al* [40] used karyogamy-defective mutants of *S. cerevisiae* carrying a killer plasmid in order to breed killer strains having nuclear genes originating from an industrial strain. But both these methods are unsuitable for breeding a killer strain of industrial yeasts due to their polyploid nature and absence of sexual behavior [33]. Ouchi *et al* [24] described a method for transfer of the killer plasmids from UV-killed donor to recipient commercial sake yeast through protoplast fusion. In our studies we employed a similar technique to obtain a flocculent yeast strain producing killer toxin. The polyploid nature of both parental strains caused difficulties in obtaining auxotrophic mutants required for protoplast fusion. Bradshaw and Peberdy [4] used benomyl (a broad spectrum fungal antibiotic) resistance as a marker for the selection of the fusants. Resistance of the killer strain, *S. cerevisiae* NCIM 3578, to benomyl served as the basis for hybrid selection. UV-killed protoplasts of a benomyl-resistant killer strain were used as donor. The time required to kill all the protoplasts of the killer strain was longer (45 min) probably due to the polyploid nature of the strain. The high UV dose possibly damages DNA beyond repair. However, the killer trait might have readily escaped inactivation since it is carried on one or more ds RNA molecules which are considerably smaller than the chromosomes. The protoplasts of the parental strains did not regenerate on the selection medium, proving that the colonies appearing on the selection medium were true fusants and not mutated revertants of one of the parental strains.

All the fusants were benomyl-resistant but only four of them possessed both the desired properties of flocculence and killer toxin production. This suggests the possibility of nuclear fusion, benomyl resistance being a nuclear marker. According to Ouchi *et al* [24] UV-killed protoplast fusion rarely yields nuclear hybrids probably because the nuclear fusion was abortive. When protoplasts of the same mating type fuse together or when one partner is polyploid, nuclear recombination occurs in a frequency two or three orders of magnitude lower than the cytoplasmic mixing. In the present investigation, the selection pressure was in favor of the nuclear hybrids rather than the cybrids. This may be one of the reasons for getting low fusion frequency (2.5×10^{-5}) in the present studies in spite of the very high regeneration efficiency (80%). The fusants contained a slightly higher amount of DNA per cell compared to that of parental strains, possibly due to the deletion of recombinant DNA after nuclear fusion [19]. All four fusants obtained were stable after repeated subculturing over a period of 1 year.

S. cerevisiae NCIM 3578 appeared to be a poor fermenter at high concentrations of molasses sugars even though it exhibited the highest ethanol productivity during the initial phase of fermentation. The flocculent strain *S. cerevisiae* NCIM 3528 fermented molasses efficiently irrespective of molasses sugar concentration. Fusants F-30 and F-31 were better fermenters of molasses compared to both the parents. The fusants also showed improved ethanol productivities as compared to the parental strains. We reported earlier that lower ethanol productivity could be due to the presence of lower alcohol dehydrogenase activity [12]. On this basis, the increased ethanol productivity could be attributed to high alcohol dehydrogenase levels in the fusants.

Acclimatization and clonal selection of a flocculent fusant strain, F-26, by continuous growth in a column fermenter was carried out using 5% molasses medium. Fusant FM-26, showed good flocculence both in MGYP and MMYP media. Further characterization of FM-26 and continuous molasses fermentation using the isolate are in progress.

In conclusion, a killer-toxin producing flocculent yeast strain was constructed by protoplast fusion between a highly flocculent strain and a killer strain. The loss of flocculence in molasses of regenerated fusants remains unexplained at present. Studies on this aspect of the problem are in progress.

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