CHAPTER IV

Strain improvement of highly flocculent *Saccharomyces cerevisiae* NCIM 3528: Construction of flocculent yeast with a killer character by protoplast fusion

SUMMARY

Conditions were optimised 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. Application of protoplast fusion technique 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 6000. Stable fusants were obtained using benomyl resistance as a marker, the killer toxin producer rather than the highly flocculent yeast being resistant to the fungicide at a concentration of 100 μg/ml. Fusants were also characterized by their DNA content, capacity for the ethanolic fermentation of the molasses sugar and the levels of invertase, alcohol dehydrogenase and pyruvate decarboxylase activities.

INTRODUCTION

Killer phenomenon is very common amongst yeasts and is found in many yeast genera. The most studied killer system is that of *S. cerevisiae* which has been divided into three groups (K1, K2, and K28) depending on the molecular characteristics of the toxins secreted, their killing profiles, the lack of cross immunity and the encoding genetic determinants [1]. The killer character is generally encoded by cytoplasmically inherited M dsRNA encapsidated in virus-like particles (VLPs) leading to the production of an extracellular killer toxin which is toxic to the sensitive yeasts, while the producing yeast remains immune to its action. The M dsRNAs are dependent on another group of helper yeast viruses (L-A), which are autonomously replicating viruses, for their replication and encapsidation.

K1 killer toxin is a 19 kDa protein consisting of two distinct disulfide bonded unglycosylated subunits, and is a product of 42 kDa glycosylated precursor molecule. The toxin acts primarily on the cell membrane of the susceptible cells. After binding to the yeast cell wall the toxin is transferred to the cell membrane where it forms voltage independent cation transmembrane channels, causing ion leakage and cell death [2-5]. Constituents of cell wall glucan, mainly β-1,6-D-glucan, have been identified as primary receptors of the toxin which are present at an average of 1.1×10^7 molecules per cell [6-9]. The K2 killer toxin has a similar mode of action as that of K1 while K28 killer toxin inhibits the DNA synthesis of the susceptible cells [10-13].

In industrial fermentations, contamination by wild yeasts could be a serious problem if the starter strain is sensitive to the killer toxin. The occurrence of wild killer yeasts in wine, beer, and even in cane molasses is very high [14-18]. Killer yeasts have been detected in almost 88% of the wine fermentation processes and *S. cerevisiae* is the most predominant one [19]. The introduction of killer property will render the starter strains immune to the killer toxin produced by wild yeast contaminants and also will prevent the contamination with other yeasts, which are susceptible to the killer toxin. The killer property has been transferred in many wine,

brewery and distillery yeasts using classical genetic methods as well as protoplast fusion and transformation [20-28].

Flocculation is an important prerequisite in yeasts used in industrial fermentations. The use of flocculent yeast strain permits easy removal of bulk of the cells resulting in easy downstream recovery of the product making the overall process simple and cost effective. Highly flocculent yeasts permit continuous ethanolic fermentation without recourse to centrifugation for cell recovery and recycle. Also high cell density can be maintained allowing high fermentation rates and high ethanol productivities.

The strain improvement of industrial yeasts has been carried out by various techniques. These include, clonal selection of naturally occurring mutant, physical/chemical mutagenesis, spores hybridization, homologous recombination, plasmid transformation and protoplast fusion [29]. Yeast strains used for industrial fermentations are usually polyploid in nature. Spontaneous or induced mutagenesis cannot be always applied successfully for their improvement since the mutations do not usually reveal themselves because of multiple alleles in them. Their homothallism and inability to sporulate pose problems for sexual hybridization. Cloning of yeast genes coding for desirable characters has been used in constructing new improved strains [30,31] but has some limitations. The cloned fragment might not always be stable in the recipient strain either due to presence of restriction modification system of host cell or plasmid incompatibility. In such cases protoplast fusion becomes indispensable. The simplicity, rapidity and cheapness of this technique makes it many times most preferred one, over other methods. In addition, it allows the transfer of relatively large segments of DNA [32,33].

The flocculent strain used in the present study has been isolated from brewery. It is an efficient fermenter of cane molasses and has been shown previously to be more tolerant to high molasses and ethanol concentration at 37°C as compared to the standard brewing strain [34]. Transfer of killer resistance to the flocculent strain will make the strain more suitable for fermentation as the contamination problems caused by the wild yeasts having killer property will be avoided. In this

chapter, data on the transfer of killer character to a flocculent brewer's strain by protoplast fusion has been presented. The batch fermentation studies carried out using the fusants having both flocculence as well as killer character are also given.

MATERIALS AND METHODS

MATERIALS

Malt extract, yeast extract and peptone were obtained from Difco Chemical Co, Detroit, USA. Novozyme 234 was obtained from the Novo Enzyme Products Ltd, Denmark. Dithiothreitol (DTT), β-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 the 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

Saccharmyces 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 cerevisiae* EG103, was a gift from Prof. P. K. Maitra, Tata Institute of Fundamental Research, Bombay, India.

All strains were maintained on MGYP agar slants containing (g/L) : 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). Molasses growth medium (MMYP) consisted of (g/L) : 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) : molasses total reducing sugar, 200.0 and urea, 1.0. The initial pH of the fermentation medium was adjusted to 4.5.

METHODS

Protoplast isolation

The procedure for isolation and regeneration of protoplasts was based on that described by Hamlyn *et al.* [35]. Cultures were grown in the liquid MGYP medium (10 ml) for 16-20 h at 30°C on a rotary shaker (200 rpm). Cells were harvested by centrifugation at 7000 rpm for 10 min and washed with potassium phosphate buffer (0.1M, pH 5.8). After suspension in the same buffer containing 50 mM DTT the cells were incubated for 1 h at 30°C under gentle agitation. DTT-pretreated cells were then recovered by centrifugation and washed repeatedly with the 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) and various osmotic stabilizers such as sucrose, sorbitol, KCl, NaCl, $MgSO_4$, $MgCl_2$ 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 1000 rpm for 15 min at 30°C were washed with the osmotically stabilized buffer to make them free from novozyme and were suspended in the same buffer. After appropriate dilution the protoplasts were plated on the 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 the method of Ouchi *et al.* [21] using polyethylene glycol as fusogenic agent. Protoplast fusion was carried out using live protoplasts of highly flocculent *S. cerevisiae* NCIM 3528 and UV-killed protoplasts of the killer *S. cerevisiae* NCIM 3578. The protoplasts of *S. cerevisiae* NCIM 3578 were suspended in phosphate buffer (0.1M, 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 inches 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 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) PEG-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 (1000 rpm) 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 [36]. 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 (50 mM, 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 the top 3.5 cm of tube after 30 s).

Assay of the killer activity

Killer activity was tested as described by Phillskirk and Young [37]. 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 (approximately 10^8) were added to the sterile MGYP medium containing 0.5% (w/v) agar and 0.003% (w/v) methylene blue. This soft agar suspension was overlaid on a petriplate 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 clear zone around the inoculum, in which no growth of sensitive strain occurred. The clear zone was bound 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.* [38]. Cells were collected by centrifugation, washed and suspended in 0.5 N perchloric acid at 0° C for 30 min. The suspension was then centrifuged, and cold insoluble material was washed twice with 0.5 N perchloric acid. Nucleic acids were then hydrolyzed by heating at 80°C for 20 min in 0.5 N perchloric acid. The hydrolysate was cooled to 0°C and

centrifuged, and the supernatant was retained. Cells were reextracted at 80°C for another 20 min, and the two supernatant fractions were collected.

DNA concentration was estimated using diphenylamine [39]. Two solutions were used. Solution A contained 1.5 g of diphenylamine in 100 ml of glacial acetic acid and 1.5 ml of concentrated H_2SO_4. This solution was stored in a cool place and protected from light. Solution B contained 1.6 % acetaldehyde. Just before the experiment 20 ml of solution A was mixed with 0.1 ml of solution B to make solution C. A 1 ml portion of the DNA extract (10-50 g/ml) was mixed with 2.5 ml of solution C, and the mixture was sealed and incubated at 30°C for 16-20 h. After colour development the optical density of the solution was read at 600 nm against a DNA standard. Calf thymus DNA was used as the standard (0.4 mg of DNA per ml in 5 mM NaOH).

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 ml 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 [40].

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% of the reducing sugar of molasses. The flasks are 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 [41]. Cells grown aerobically in MMYP medium for 16 h at 30°C were harvested by centrifugation at 7000 rpm 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 10000 rpm for 30 min at 4°C and the clear supernatant 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 Gascon and Lampen [42]. 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 minute.

Pyruvate decarboxylase (EC 4.1.1.1) was assayed by the method of Reed and Williams [43]. 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 puruvate (0.5 M); 0.1 ml of cell extract and water to make 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 of

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 the absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of 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 [44]. 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 was defined as one micromole of NAD reduced per min.

Protein was determined by the method of Lowry *et al.* [45] using bovine serum albumin as standard.

RESULTS

Isolation and regeneration of protoplasts

Protoplasting method was designed to enhance the frequency of protoplast release. For standardization of the method a brewer's yeast *S. cerevisiae* NCIM 3458 was used which is polyploid in nature. The protocol was standardized 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 of the cells with reducing agents such as β-mercaptoethanol and DTT was tried. However, longer incubation (2 h) in presence of β-ME (100 mM) was found to be detrimental to the cells as well as the protoplasts. In addition, pretreatment with DTT (100 mM) for 60 min was more effective than with β-ME. The concentration of DTT and the period of pretreatment were standardized. Pretreatment with 50 mM DTT for 60 min was optimal for protoplast release by Novozyme 234 (Table IV.1).

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 Fig. IV.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 and 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 IV.2 summarizes the effect of different osmotic stabilizers on protoplast regeneration of *S. cerevisiae* NCIM 3458. In general, protoplasts regenerated efficiently in a 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 the highest regeneration (81%) when sucrose was used as osmotic stabilizer in the regeneration medium. The protoplasts released in 0.8 M NaCl had 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. $MgSO_4$ favoured neither isolation nor regeneration of protoplasts of *S. cerevisiae* 3458.

The standardized procedure was applied for the protoplast isolation of *S. cerevisiae* NCIM 3528 (flocculent strain) and *S. cerevisiae* NCIM 3578 (killer strain). As shown in Table IV.3, protoplast formation of flocculent strain was complete within 15 min, killer strain however showed some resistance to cell wall digestion and took 30 min for the complete protoplastation. In order to test the general applicability of the procedure, protoplast release from strains of different genera of yeasts was examined (Table IV.3). 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 incubation with lytic enzyme respectively. In contrast, *Rhodotorula*

glutinis and *Schizosaccharomyces pombe* were resistant to Novozyme 234 even after a period of 1 h.

Protoplast fusion and selection of hybrids

Four independent protoplast fusions between *S. cerevisiae* NCIM 3528 (a flocculent, killer negative, benomyl resistant strain) and *S. cerevisiae* NCIM 3578 (a nonflocculent, killer, benomyl resistant strain) were performed in order to obtain desired yeast strain. The scheme for protoplast fusion is presented in Fig. IV. 3. The UV killing time for *S. cerevisiae* NCIM 3578 was standardized. All protoplasts were killed after UV exposure for a period of 45 min (Fig. IV.2). 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) after 72 h at a frequency of 2.5×10^{-5}. Controls run with an equal number of protoplasts of the individual parental strains showed no growth on the selection medium. About 4000 clones obtained were screened for the rough and dry colony morphology characteristic of the flocculent strain, and 320 fusants were picked. Out of these only six clones exhibited flocculence phenotype comparable to the parental strain. These six clones were tested for killer phenotype and only four clones : F-22, F-26, F-30 and F-31, were found to possess property of killer toxin production (Figure IV.4). All four of them however, were less flocculent than the highly flocculent parental strain (Table IV.4). These fusants showed moderate flocculation in MGYP medium, no flocculation was observed when grown in molasses based medium. Fusants, F-22, F-26, F-30 and F-31 were further characterised with respect to DNA content per cell, fermentation performance and key enzyme levels like invertase, alcohol dehydrogenase and pyruvate decarboxylase.

DNA content of the fusants

The DNA of the fusants, parents as well as a standard haploid strain was extracted using perchloroic acid and estimated with the diphenylamine reagent. The results are shown in Table IV.4. The haploid strain *S. cerevisiae* EG103 had a DNA

content of 1.12 $\times 10^{-8}$ µg/cell. 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 slightly more DNA content 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 medium containing 20-30% total reducing sugar. Ethanol produced by killer strain (*S. cerevisiae* NCIM 3578) was comparatively low irrespective of molasses sugar concentration (Table IV.5). 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 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 IV.6. 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.

Enzyme levels in the fusants

Three key enzymes, which play an important role in molasses fermentation, were selected viz. invertase, pyruvate decarboxylase and alcohol dehydrogenase, for determination of their intracellular levels in the fusants and the parental strains. The results summarized in Table IV.7 show that the invertase activity was higher in the parental 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 the two genomes after protoplast fusion.

Stability of the fusants

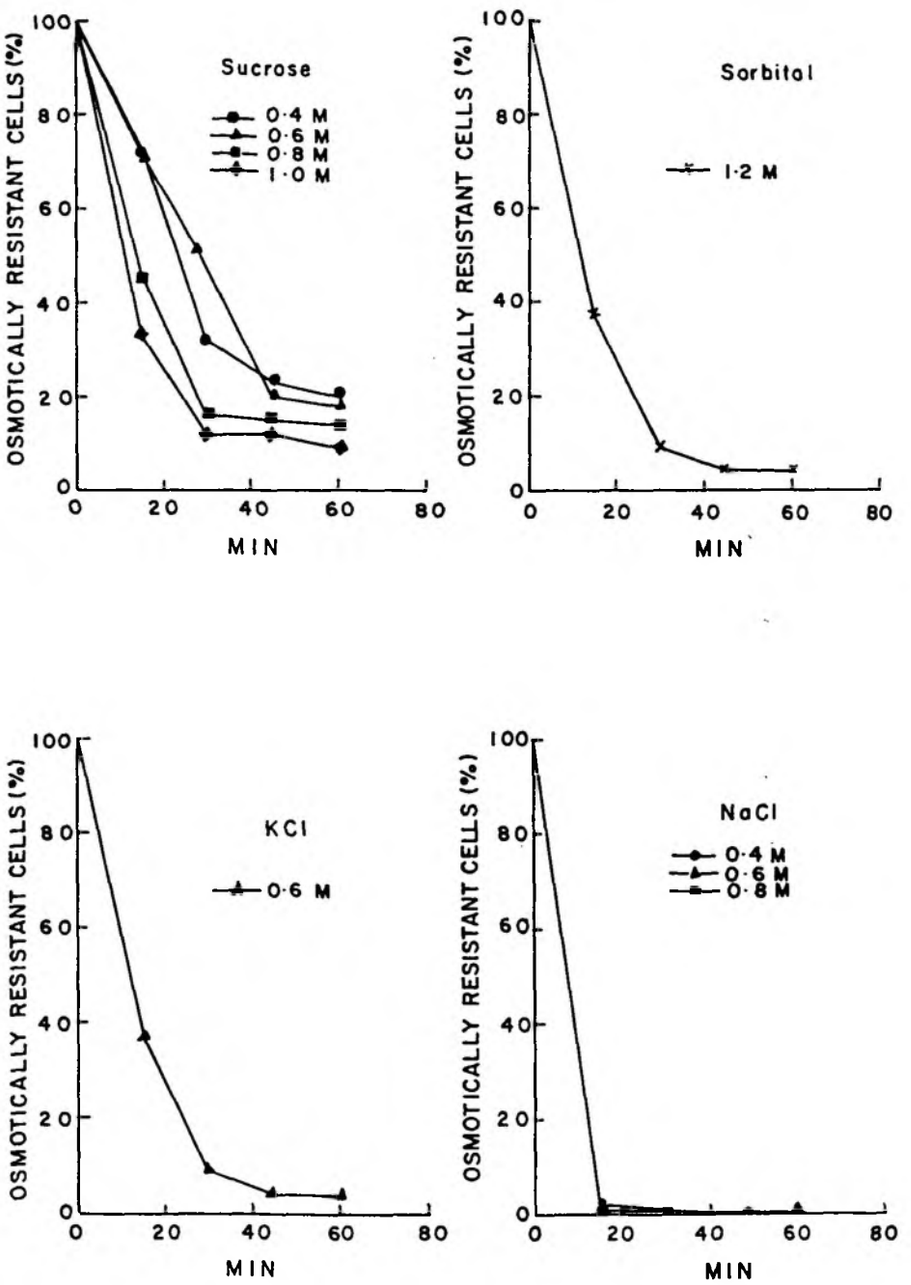
The fusants were maintained by repeated subculturing on the selection medium for first six months and then on MGYP medium for about 3 years. Killer character and flocculation of the fusants was checked after every 6 months. The killer character was found to be stable over the period of four years, but flocculation level of the fusants reduced to some extent.

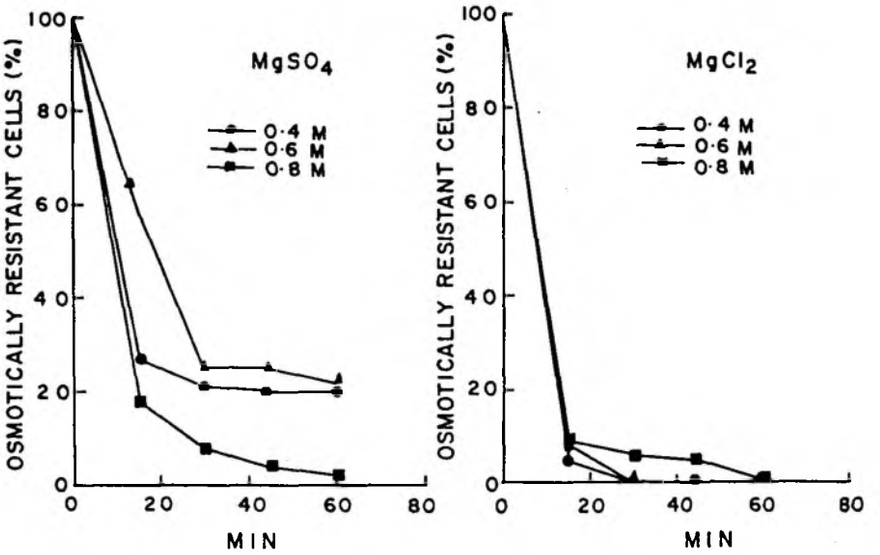
Table IV.1

Effect of DTT concentration on protoplast isolation of *S. cerevisiae* NCIM 3528

DTT (mM)	Time for pretreatment (min)	% protoplast formation after	
		30 min	60 min
50	30	-	-
	60	96.0	100.0
100	30	66.0	80.0
	60	97.0	100.0
200	30	70.0	82.0
	60	97.2	100.0
300	30	66.5	81.0
	60	97.1	100.0

Figure IV.1. 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) in osmotically stabilized phosphate buffer (0.1 M, pH 5.8). The cell-enzyme-stabilizer mixtures were incubated with shaking at 30°C.


Sucrose
0·4 M
0·6 M
0·8 M
1·0 M
Sorbitol
1·2 M
KCl
0·6 M
NaCl
0·4 M
0·6 M
0·8 M
OSMOTICALLY RESISTANT CELLS (%)
MIN
0
20
40
60
80
100


OSMOTICALLY RESISTANT CELLS (%)
MgSO4
0·4 M
0·6 M
0·8 M
MIN
MgCl2
0·4 M
0·6 M
0·8 M
MIN

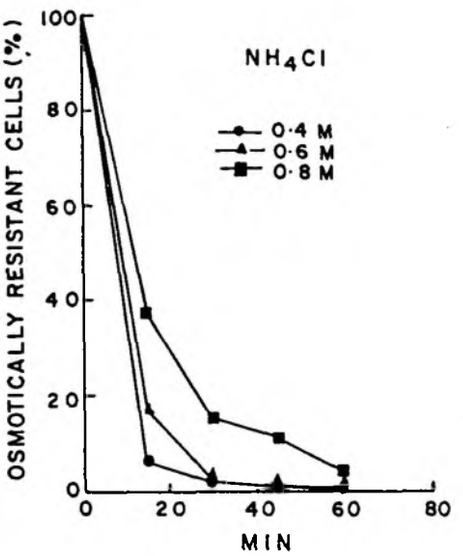

OSMOTICALLY RESISTANT CELLS (%)
NH4Cl
0·4 M
0·6 M
0·8 M
MIN

Table IV.2

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	31.0	36.0
	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 IV. 3

Protoplast formation from different yeast genera

Strain	% protoplasting after			
	15 min	30 min	45 min	60 min
S. cerevisiae NCIM 3528	100			
S.cerevisiae NCIM 3578	91	100		
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) and NaCl (0.8 M) in phosphate buffer (0.1 M, pH 5.8).

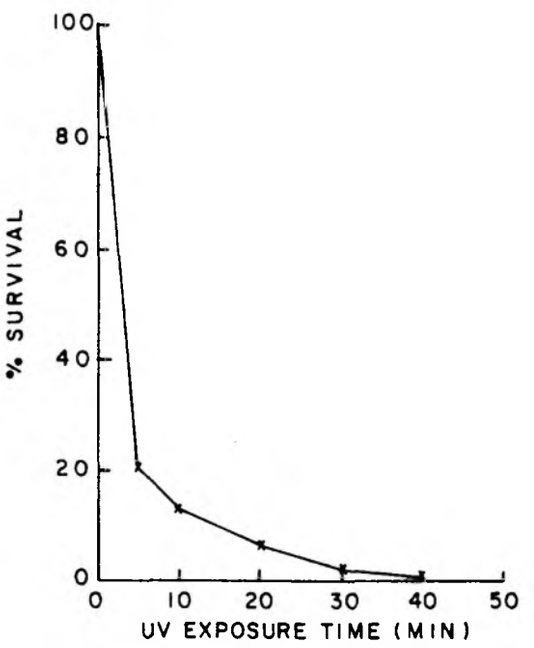


Fig. IV.2 UV survival curve of *S. cerevisiae* NCIM 3578 protoplasts

The protoplasts were suspended in phosphate buffer (0.1 M, pH 5.8) containing 0.8 M NaCl and subjected to UV irradiation with a 15-W germicidal lamp at a distance of 6 inches for 45 min.

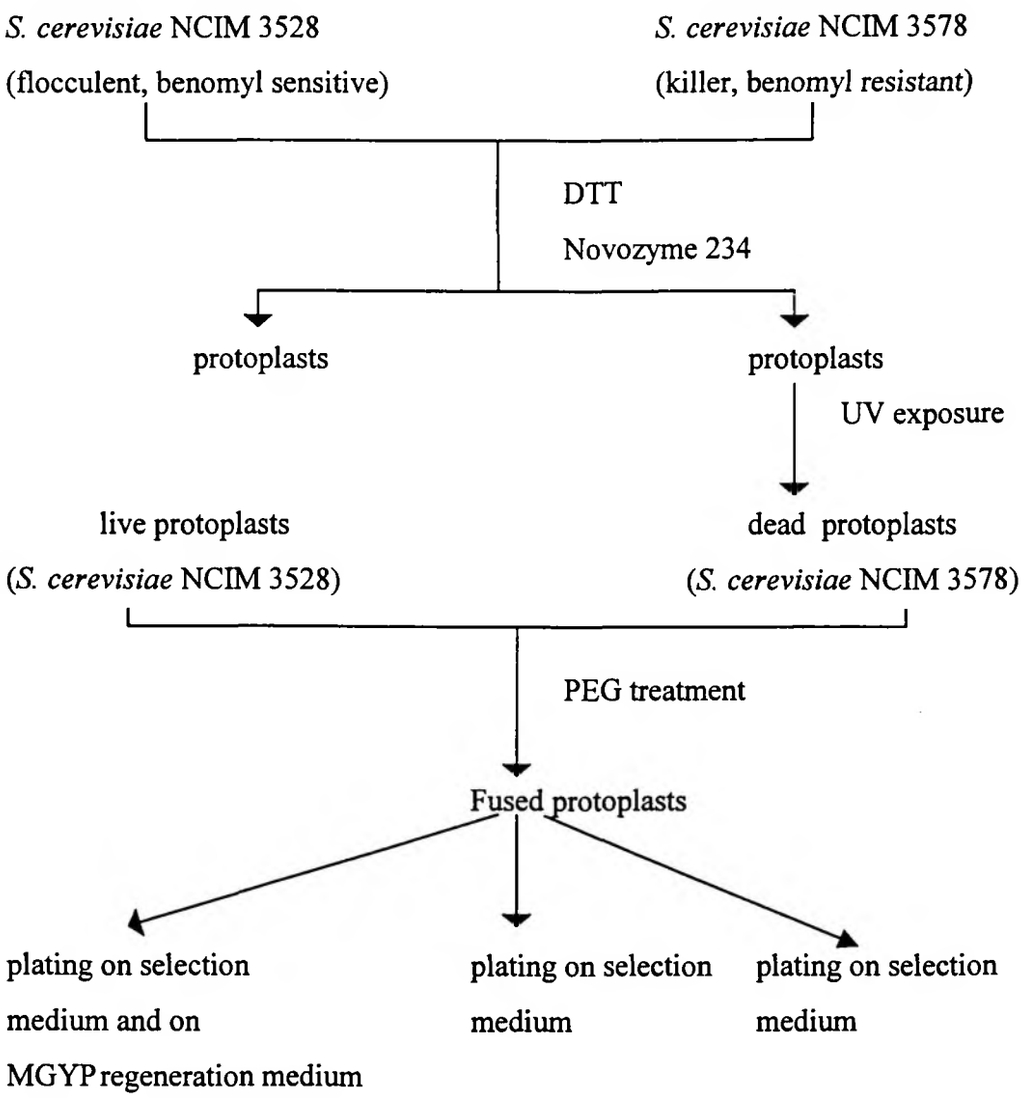


Fig. IV 3. Scheme for protoplast fusion

Table IV. 4

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

Yeast strains	Flocculation activity	Total DNA (x 10^{-8} μg per cell)
S. cerevisiae NCIM 3578	0	5.0
S. cerevisiae NCIM 3528	5	7.45
F-22	2	9.20
F-26	3	8.34
F-30	2	8.0
F-31	3	8.50
S. cerevisiae EG103	ND	1.12

ND = Not determined

Fig. IV. 4 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.

Table IV. 5

Ethanol production by yeasts at various molasses sugar concentration

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

Table IV. 6

Specific ethanol productivity of fusants and parental strains

Yeast strains	Specific ethanol productivity (g/g/h)
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

Table IV. 7

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

Yeast strains	Invertase (U/mg)	PDC (U/mg)	ADH (U/mg)
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	2.03 ± 0.05	0.49 ± 0.04	0.88 ± 0.05
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

DISCUSSION

Strain improvement of flocculent yeast strains has been carried out using various methods [46-49]. Since the highly flocculent *S. cerevisiae* NCIM 3528, used in the present study, was a polyploid brewer's yeast, the protoplast fusion technique was opted for its improvement. Though experimental conditions of the isolation and regeneration of yeast protoplasts have been reported previously, some degree of optimization becomes necessary for individual strains and species. Factors affecting protoplast isolation and regeneration are cell age, pretreatment of the cells, concentration of the lytic enzyme used, time of incubation with the lytic enzyme and the osmotic stabilizer used. Regeneration of the fusants including cell wall regeneration and reversion of walled protoplasts to normal cells is usually carried out in osmotically stabilized medium containing gelatin or agar [50]. The presence of barrier around the protoplasts, provided by the agar or gelatin, prevents the loss of cell wall components into the surrounding medium, facilitates their accumulation on the surface of the protoplast and hence allows the formation of nascent cell wall [51,52].

Requirement of high frequency protoplast formation and mass regeneration has been well documented [33,50,53]. Pretreatment of yeast cells with sulfahydryl agents makes the cell wall more susceptible to the lytic enzymes and thus facilitates the release of protoplasts [54-57]. High concentration of the lytic enzyme [56] and prolonged incubation of yeast cells with the lytic enzyme [58] have been reported to result in decreased regeneration efficiency. An extensive range of osmotic stabilizers have been used for the isolation of protoplasts from yeast as well as fungi. Generally sugars and sugar alcohols are more effective with yeasts than inorganic salts [21,59,60]. Potassium chloride has also been used successfully to get 100 % protoplast yield [26,61].

In the present investigation, we tested wide range of osmotic stabilizers including sugars, sugar alcohols and inorganic salts for rapid protoplasting of DTT-pretreated yeast cells. Surprisingly all the inorganic salts tested, except $MgSO_4$, were

more suitable than sucrose or 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 osmotic stabilizer. This incubation time with the lytic enzyme appears to be the shortest time reported till date for complete protoplast release. The corresponding times reported in the literature vary from 30 min - 2 h [47,58,59,62]. The lower concentration of lytic enzyme used in the present studies (1 mg/ml) 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* [58,59,63].

Introduction of killer property in the highly flocculent brewer's strain was carried out by protoplast fusion. Presence of selection markers on the parental strains is an essential prerequisite for protoplast fusion. Bradshaw and Peberdy [64] used benomyl (a broad spectrum fungal antibiotic) resistance as a marker for selection of the fusants. In the present study, resistance of the killer strain, *S. cerevisiae* NCIM 3578, to benomyl served as the basis for hybrid selection. Attempts to mutagenize the parents in order to generate auxotrophic markers however, failed probably due to their polyploid nature. Ouchi *et al.* [21] described a method for transfer of killer plasmids from UV killed yeast cell (donor) to commercial sake yeast through protoplast fusion. In our studies we employed a similar technique to obtain flocculent yeast strain producing killer toxin. UV killed protoplasts of the benomyl resistant killer strain were used as donor. The time required to kill all the protoplasts of 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 the 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 confirming 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.* [21] 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 however, the selection pressure was in favour 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 [65].

All fusants showed a decrease in flocculation ability when compared with the parental highly flocculent strain. Such decreased flocculation in hybrids has been reported by others also when protoplast fusion or hybridization was used to transfer a flocculent character to the nonflocculent strains [46,47,66]. Flocculation is known to be regulated by several genes and several flocculation suppresser genes have been reported [67]. The suppression of expression of the flocculation genes has been recognized in *MATa/ MATα* diploids [68,69]. According to Shinohara *et al.* [46] this could be one of the reasons for the poor flocculation activity obtained in the hybrids. All of the fusants showed moderate flocculating ability in MGYP medium but showed no flocculence in molasses medium. This could be due to the high salt concentration present in the molasses since high concentration of salt are known to deflocculate the cells of flocculent strain [70,71].

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 NCIM 3528 fermented molasses efficiently irrespective of molasses sugar concentration. Fusants F-30 and F-31 were better fermenters of molasses compared to the parents. The fusants also showed improved ethanol productivities compared to the parental strains. The values of invertase nad alcohol dehydrogenase in the fusants were intermediate between those of the parents indicating the true nature of the hybrids. In case of F-26 and F-30 fusants, the pyruvate decarboxylase activities were higher than the parental strains. It has been reported earlier that the lower ethanol productivity could be due to the presence of lower alcohol dehydrogenase activity [72]. Therefore, the increased ethanol productivity could be due to high alcohol dehydrogenase levels in the fusants.

In conclusion, the method for rapid isolation and improved regeneration of protoplasts was standardized which is applicable to different yeast genera. Protoplast fusion of a highly flocculent *S. cerevisiae* NCIM 3528 and a killer stain, *S. cerevisiae* NCIM 3578 using a dead donor technique was carried out and fusants having both flocculence as well as the killer property were obtained. The fusants which most probably are nuclear hybrids have a good fermentation capacity and improved ethanol productivities as judged by batch fermentation studies.

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