the experiment, the total percent reduction in BOD was calculated.

The results are shown in table 1. Amongst the individual species, the bacterium *P. aeruginosa* removed comparatively larger amounts of BOD. The fecal bacterium *E. coli* also accounted for a considerable reduction in BOD. This is indicative of the fact that the fecal bacteria are not inactive in the stabilization pond medium, as it was thought earlier[7], but also play a significant role in the purification of incoming wastewater. The BOD removal efficiencies of the filamentous *A. niger* and the alga *C. vulgaris* were comparatively poor. Whereas the unicellular yeast accounted for a considerable removal of BOD comparable to that removed by bacteria. When the bacterial species were grown individually in association with *A. niger*, the BOD removal efficiency was slightly reduced, most probably because of the antagonistic activity of the fungus. However, when the bacteria were grown in association with *C. vulgaris*, or when the alga, the two fungi and the three bacteria were grown together or when the pond complex community was inoculated, the BOD removal efficiency was increased considerably. The maximum BOD removal was recorded with the complex pond community. There was a negligible change in the BOD of the control flask. From the foregoing results it becomes evident that a beneficial association between various groups of microorganisms prevailing in the pond is better with respect to the BOD removal efficiency than any of the combinations considered in the present study. Hence it appears that the presence of various groups of microorganisms is essential for a better purification of sewage in stabilization ponds.

Table 1 *BOD removal efficiencies of the microorganisms isolated from the stabilization pond individually and in various combinations.*

Organism(s)	Percent reduction in BOD
P. aeruginosa	68.29
P. vulgaris	65.38
E. coli	63.05
A. niger	45.24
Yeast	65.10
A. niger + *P. aeruginosa*	66.00
A. niger + *P. vulgaris*	63.10
A. niger + *E. coli*	61.57
C. vulgaris	47.81
C. vulgaris + *P. aeruginosa*	77.14
C. vulgaris + *P. vulgaris*	73.90
C. vulgaris + *E. coli*	66.67
C. vulgaris + *P. aeruginosa* + *P. vulgaris* + *E. coli* + *A. niger* + yeast	78.33
Pond community	82.67
Control	04.76

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PROTOPLAST LIBERATION FROM *SACCHAROMYCES CEREVISIAE* USING THERMOMYCOLASE OF *MALBRANCHEA PULCHELLA* VAR. *SULFUREA*

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THE isolation of protoplasts from fungi using lytic enzymes, is now a well-established technique[1]. Initially this was a very useful procedure in preparing cell-free extracts[2] and organelles[3] for biochemical studies. More recently interest has been focussed on the use of protoplasts as genetic tool[4]. However, fusion and transformation system depends upon the availability of protoplasts in large numbers and in most cases protoplasts would seem to provide the means of isolating DNA from fungal cells for transformation. Clearly, a key factor for success in this area concerns the availability of suitable lytic enzymes for cell wall digestion.

During our detailed biochemical investigations of

the thermophiles *Malbranchea pulchella* var. *sulfurea*, the spent filtrate was added to a preparation of *Saccharomyces cerevisiae* which contained novozyme 234. The strong mycolytic activity was visible through rapid liberation of yeast protoplasts which were viable. A preliminary investigation was therefore undertaken to assess the efficacy of this enzyme preparation for protoplast release and to compare it with other known preparations.

Four strains of *S. cerevisiae*, *viz* NCIM 3187, 3288, 3095 (NCL, Pune) and NSI (Sugar Institute, Kanpur) were used and grown in Winge's medium containing 2% glucose; and 3% yeast extract for 12–15 hr at 28°C on a reciprocal shaker. A strain of *M. pulchella* var *sulfurea* isolated from coal spoil tips from Madhya Pradesh, and designated as APM-5 was used as source of crude lytic enzyme. It was grown in glucose, asparagine medium (GAB) containing, glucose 10 g; asparagine 5 g; $MgSO_4$, $7H_2O$, 0.5 g; KH_2PO_4, 1 g; distilled water 1000 ml (pH 6.9) was used. Autoclaved medium was inoculated with a 6-day old culture-bearing agar disc (18 mm diam), and incubated at 45°C. After 14 days of incubation cultures were filtered through Whatman No. 1 filter paper and the filtrate was centrifuged at 10,000 rpm for 30 min. The supernatant was used as extracellular enzyme. For intracellular enzyme preparation, mycelia from 14-day old culture was washed with distilled water and crushed with glass beeds in mortar with pestle. The homogenized extracts were centrifuzed at 10,000 rpm for 30 min at 4°C and the supernatant used as intracellular lytic enzyme. For preparation of *S. cerevisiae* protoplasts (strain NCIM 3187, 3288, 3095 and NSI), yeast cells in logrithmic phase of growth were collected and washed three times with distilled water. The cells were resuspended in a reaction mixture containing 10 mg dried thermomycolase/ml; 0.8 M $MgSO_4$[5], citrate phosphate buffer (pH 5.4) and adjusted to give a cell density of 10^7–10^8 cell ml^{-1}. Novozyme 234 (Novo Industries, Denmark) and cellulase (Merck) were used at a concentration of 5 mg ml^{-1}; Cereflo 200L (Novo) was used at final concentration of 5% (v/v). The lytic mixture was then incubated at 28°C upto 3 hr with gentle shaking. Various alterations in this reaction mixture were made to study the relative action of the thermomycolase and these are listed in table 1. Pretreatment of cells with a thiol compound prior to digestion with lytic enzyme has generally been found to be necessary with *S. cerevisiae*, yeast cells were pretreated with 0.01 M dithiothreitol (DTT) in citrate phosphate buffer (pH 7.8) for 5 min. The absence of cell wall was checked by staining with fluorescent brightner Tinopol[6]. Protoplast yields were determined using an improved Nenbaur haemocytometer counting chamber.

The relative efficacy of therromycolase of *M. pulchella* var *sulfurea* in comparison to some of the known commercial preparations is shown in table 1. Absence

Table 1 *Protoplast liberation efficacy of thermomycolase of Malbranchea pulchella var sulfurea and other known commercial lytic enzyme preparations*

Enzyme system	Pretreatment with DTT*	Protoplast yield (% cells as protoplasts)**				
		NCIM 3095	NCIM 3187	NCIM 3288	NSI	Average % of protoplast yield
Novozyme 234 + cellulase (Merck)	(+)	62.5	62.8	60.2	63.8	62.3
Novozyme 234 + cellulase	(–)	45.0	50.8	44 9	45.5	46.5
Extracellular thermomycolase	(+)	66.2	68.0	67.0	66.8	67.1
Extracellular thermomycolase	(–)	51.0	52.9	54 0	52.0	52.4
Intracellular thermomycolase	(+)	64.0	65.5	65.2	64 8	64 8
Intracellular thermomycolase	(–)	47.0	50.2	50 0	48 9	49 0
Extracellular + intracellular mycolase	(+)	76 0	79.9	76.8	72 9	76 4
Novozyme + cereflo 200L (Novo)	(–)	62.8	63.7	63.9	63 0	63.3
Novozyme 234	(–)	61.9	64.0	63 3	62 5	62.9
Cellulase + cereflo 200L	(–)	39.2	41.0	38 2	36 0	38 6
Cellulase	(–)	9.7	11.0	10 0	8 9	9 9
Extracellular + intracellular mycolase + novozyme	(–)	91.5	92.8	93 5	95 3	93 2

* (+) Pretreated cells; (–) No treatment was given; ** % Protoplast yield from 10^7–10^8 cell ml^{-1}

of a cell wall and osmotic sensitivity were the criteria used in defining protoplasts. Good protoplasts yields were obtained with novozyme + cellulase combination but pretreatment with dithiothreitol (DTT) was necessary to achieve this; thermomycolase (extra and intracellular) of *Malbranchea* produced over 65% protoplasts under these experimental conditions. A combination of extra- and intra-cellular thermomycolase was able to release 70-80% protoplasts from DTT-treated *S. cerevisiae* cells. The generality of this result is supported by the work of Thomas *et al*[7] and Masilkova *et al*[8]. In other combinations described in table 1, novozyme was no better than *Malbrancea* lytic preparation. However, a mixture of extra- and intracellular thermomycolase with novozyme 234 proved most effective as it released 90–95% protoplast from *S. cerevisiae* within 2–3 hr. As one would note all the four strains of the yeast were equally sensitive to mycolytic preparation from *Malbranchea*.

The mycolytic preparation of *Malbranchea* was also noteworthy with regard to the rate of release of protoplasts (figure 1). Thus, a mixture containing extra- and intracellular mycolase plus novozyme 234 released 60–70% protoplasts within 60–120 min; nearly 95% of the cells released protoplasts after 3 hr. In contrast, only 50–60% cells were converted to protoplasts in a similar preparation but which lacked novozyme. However, as evident from the data in table 1, part of this was overcome by the addition of DTT.

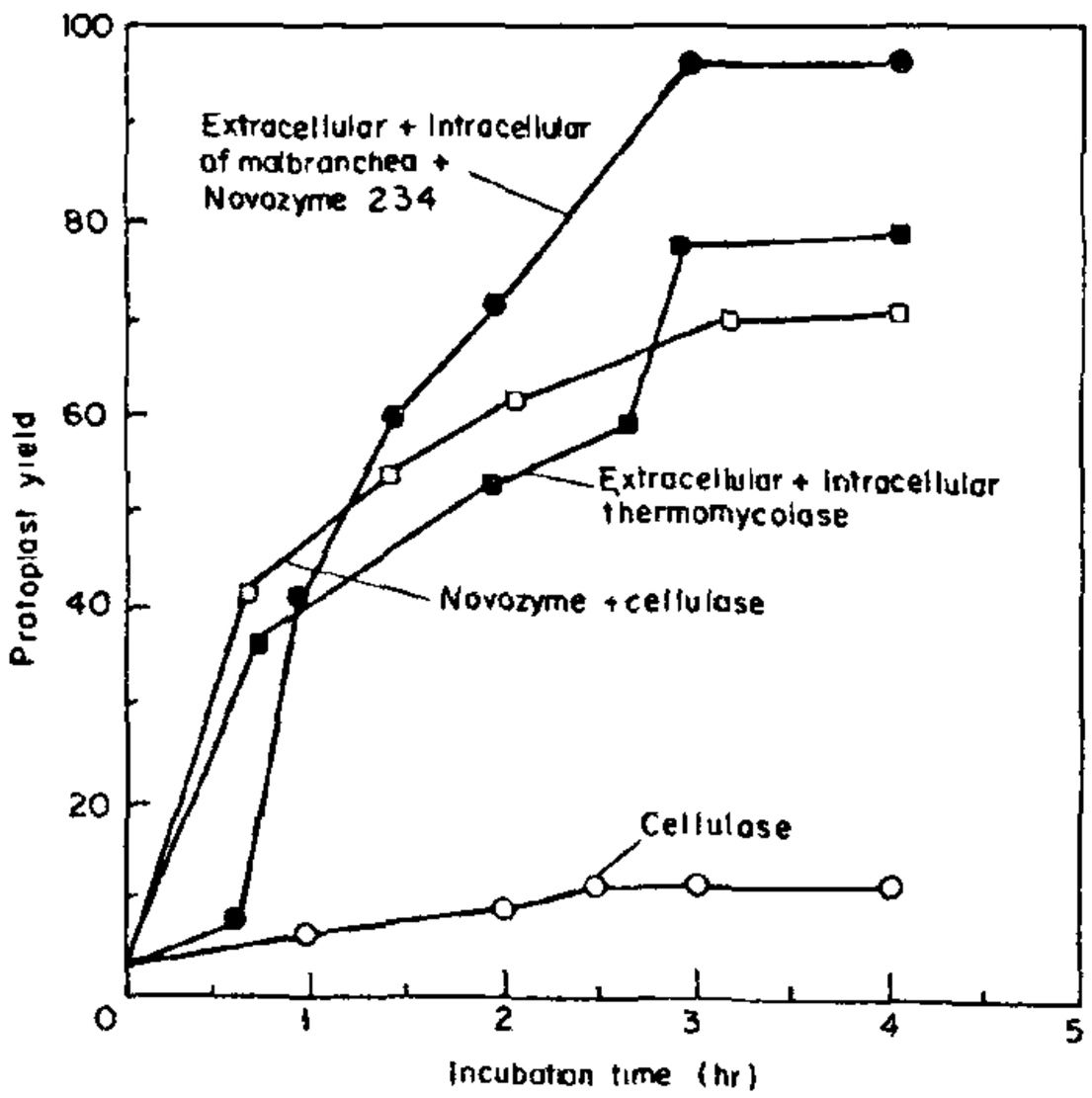

Figure 1. Effect of enzyme combination with incubation time on protoplast release.

The enzyme preparation from the thermophile exhibited considerable proteolytic activity but protoplasts suspended in this solution did not burst even after holding them for 36 hr. Also, protoplasts prepared using the thermomycolase of *Malbranchea* were viable and regenerated to form colonies on agar. In general high level of β-D-Glucanase and chitinase activities were associated with the enzyme that gave 64% protoplast yield (*i.e.* Novozyme 234 and cellulase (Merck)). The importance of these activities in the digestion of fungal cell walls has been reported earlier[9].

Efforts are in progress to separate and characterize some of the more important enzymes involved in the digestion of yeast cell walls with a view to develop a suitable methodology for commercial exploitation. No commercial enzyme is currently manufactured from *Malbranchea* but results of this investigation appear very promising to undertake large scale production of thermomycolase.

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RHIZOSPHERE SOIL NITROGENASE (C_2H_2 REDUCTION) AS INFLUENCED BY RICE VARIETY

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RECENT studies from the temperate and sub-tropic regions of the rice growing countries indicate wide