A RAPID METHOD FOR PREPARATION OF PROTOPLASTS OF *RHODOTORULA GLUTINIS*

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A GOOD preparation of protoplasts is essential for gene fusion and transformation experiments in yeast and *Streptomyces* species. Unlike strains of *Saccharomyces* and *Candida* whose cells are readily converted into protoplasts by treatment with β-glucuronidase, the cells of *Rhodotorula sp* require a long incubation period with a mixture of lytic enzymes. In this report, we describe the development of a rapid method of preparing protoplasts of *Rhodotorula glutinis*. The cells were grown, in a medium[1] containing (gl^{-1}) NH_4NO_3, 0.6; NaCl, 0.5; yeast extract, 0.3; $MgSO_4.7H_2O$, 1.0; $CaCl_2.2H_2O$, 1.0; KH_2PO_4, 1.0; glucose (or glucuronic acid), 10, at 30°C on a rotary shaker. They were harvested by centrifuging at 10,000 g for 10 min at 4°C and washed thrice with distilled water[2]. The cell pellet was suspended in 10 ml of 100 mM Tris.HCl, pH 7 containing 0.5 mg ml^{-1} pronase, 10 mM EDTA and 30 mM 2-mercapto-ethanol to give $3–5 \times 10^7$ cells ml^{-1} and the buffered suspension was incubated at 30°C for 2 hr on a rotary shaker[3]. The cells were centrifuged, washed five times with distilled water, suspended in equal volume of 25 mM citrate-phosphate buffer, pH 5.8 containing 1 M sorbitol and appropriate enzymes (table 1) and incubated at 30°C on a rotary shaker. At times indicated, 0.2 ml aliquots were withdrawn, diluted twenty-fold in 100 mM Tris.HCl, pH 8 containing 35 mM sodium dodecyl sulphate. The initial optical density at 540 nm was ~ 0.4. The protoplasts and cells were identified by microscopy and plating. For the regeneration and growth of protoplasts, 1 M sorbitol was included in the medium. Both protoplasts and intact cells formed colonies on this agar medium. The medium devoid of sorbitol allowed the growth of only intact cells. The results presented in figure 1 show that the treatment of the cells of *Saccharomyces cerevisiae* or *Candida lipolytica* with β-glucuronidase (Sigma) results in 40–60% drop in absorbance within 10 min. Microscopic examination suggested that more than 80% of the cells were converted to protoplasts during this treatment. The effect of incubation beyond 10 min on further drop in absorbance and on the number of cells converted to protoplasts was gradual. For example, after 30 min incubation, about 90% of the cells were found converted to protoplasts. Although exponentially growing cells were used in the experiments, it is likely that a fraction of cells may have entered the stationary phase resulting in their decreased sensitivity to β-glucuronidase treatment. It is known that changes in cell wall structure occur during stationary phase and such cells show resistance to lytic enzymes[4]. This may be attributed to extensive crosslinking of the polysaccharide component or the development of new interactions to hold the structure. The sensitivity of *R. glutinis* cells grown in glucuronic acid (versus cells grown on glucose) to treatment with β-glucuronidase plus driselase (Sigma) is also shown in figure 1. β-glucuronidase alone was not found to protoplast *R. glutinis* cells to any appreciable degree (results not shown). Unlike *Saccharomyces cerevisiae* and *Candida lipolytica* the cells of *R. glutinis* grown in glucose medium were resistant to protoplast formation. The cell wall of *R. glutinis* primarily contains chitin and mannan rather than glucan which is the main cell wall component in *Saccharomyces cerevisiae* and *Candida lipolytica*. In

Table 1 *Formation of protoplasts of Rhodotorula glutinis in medium containing glucuronic acid*

Treatment with	Concentration ml^{-1}	Protoplast formation after 24 hr
β-Glucuronidase	10,000 U	+
Driselase	50 mg	–
Helicase	50 mg	+ +
Zymolyase	50 mg	+
β—Glucuronidase + Driselase	10,000 U + 50 mg	+ + +
β—Glucuronidase + zymolyase	10,000 U + 50 mg	+
β-Glucuronidase + chitinase	10,000 U + 100 U	+
β-Glucuronidase + lytic enzyme	10,000 U + 50 mg	+
Helicase + driselase	50 mg + 50 mg	+ + +
Zymolyase + driselase	50 mg + 50 mg	–

+, Protoplast formation, + + and + + + indicate relatively better protoplasting; –, No protoplast formation.
One unit of β—glucuronidase hydrolyses 1 μg of phenolphthalein from phenolphthalein glucuronide hr^{-1} at pH 5 at 37°C. One unit of chitinase liberates 1 mg of N-acetyl D-glucosamine from chitin in 48 hr at pH 6 at 25°C. Unit definition of other lytic enzymes has not been given by the suppliers.

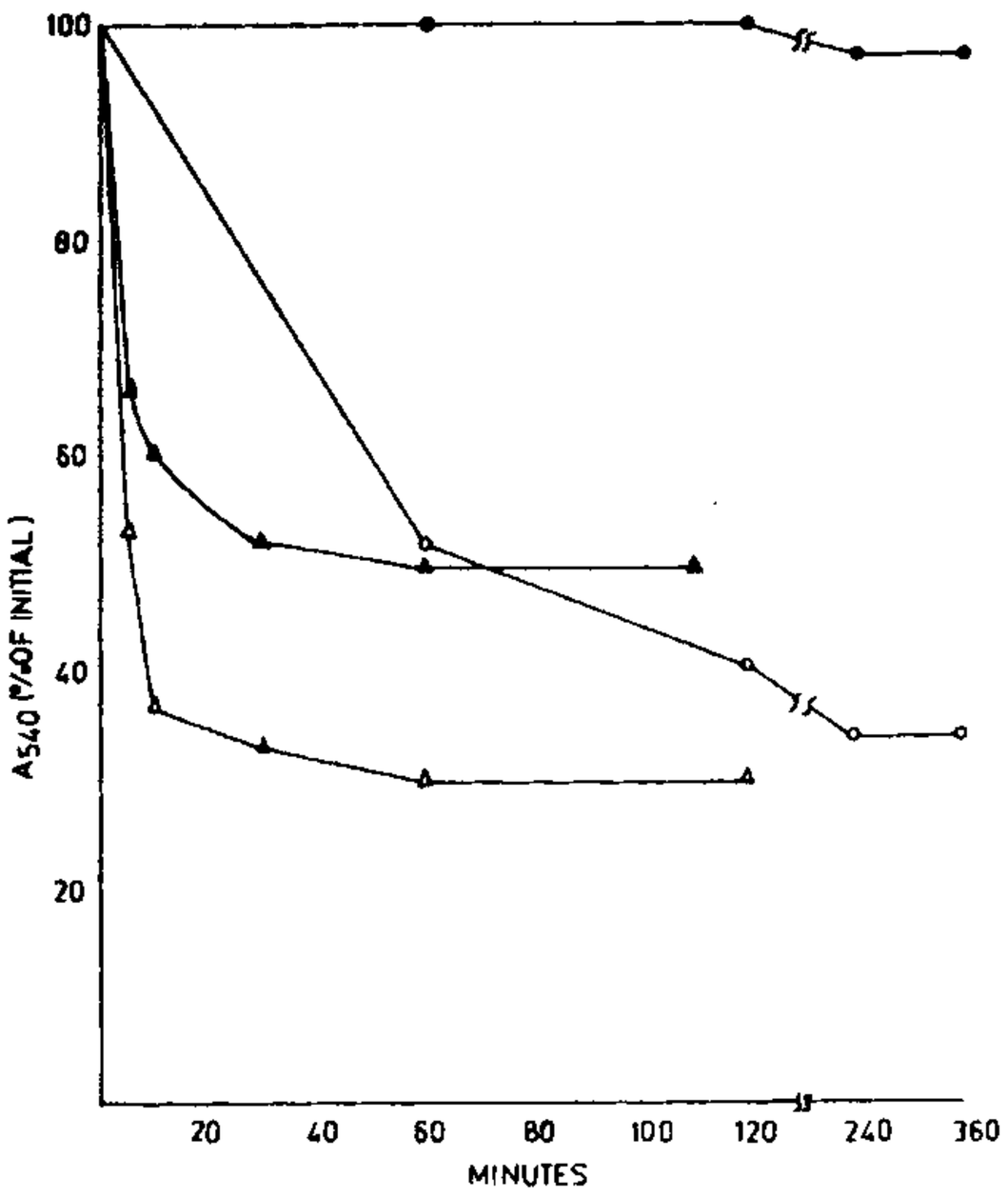

Figure 1. Relationship between treatment time of cells and drop in absorbance due to lysis of cell wall, *Saccharomyces cerevisiae* (▲—▲) and *Candida lipolytica* (Δ—Δ) treated with β-glucuronidase; *Rhodotorula glutinis* grown in glucose (●—●) or glucuronic acid (o—o) and treated with β-glucuronidase plus driselase.

2 hr, greater than 60% glucuronic acid grown cells were converted to protoplasts against less than 10% protoplast formation in glucose grown cells. The growth of *R. glutinis* in a medium containing glucuronic acid results, presumably, in cells whose cell walls contain higher than normal number of glucuronic acid moeities rendering greater sensitivity to β-glucuronidase attack. In addition to β-glucuronidase and driselase, the effect of other lytic enzymes viz zymolyase (Seikagaku Kogya), helicase (Phermindustrie), chitinase (Sigma) and lytic enzyme (Sigma) were examined. The results presented in table 1 show that combinations of β-glucuronidase, helicase and driselase cause significant cell wall lysis in *R. glutinis* grown in glucuronic acid as carbon source. Since β-glucuronidase and helicase, are both isolated from the same source, viz *Helix pomatia*, they might be similar. However, the combination of driselase with helicase was more effective than the combination of driselase with β-glucuronidase. It is likely that helicase is a crude preparation containing some lytic activities in addition to β-glucuronidase, and hence is more effective than the latter. Driselase is also a mixture of several lytic activities (cellulase, xylanase, proteinase, dextrinase etc) and has been used for preparation of protoplasts from plant cells and black yeast, *Aureobasidium pullulans*[5]. We believe that the method described in this paper may be of general use for preparing protoplasts from yeast strains belonging to genus *Rhodotorula*.

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A METHOD FOR ISOLATION OF THERMOPHILIC ACTINOMYCETES

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SINCE thermophilic actinomycetes have been considered to be potential producers of novel antibiotics and a good source of thermostable enzymes, the isolation of this group of microorganisms has gained importance in recent years[1].

For the isolation of thermophilic actinomycetes, several agar media formulations have been recommended[2], but they offer marginal advantages. Incorporation of antifungal antibiotics or bacterial inhibitors has its own disadvantages. For the isolation of mesophilic actinomycetes, a few methods have been suggested, which do not incorporate any extraneous chemical agents. Two of these methods are differential centrifugation (DC) method[3] and the high temperature pre-incubation (HTPI) method[4]. Both these methods do not proclaim their use only for isolation of