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Short Communication: Protoplast formation from the thermophilic fungus *Malbranchea sulfurea*, using the thermostable chitinase and laminarinase of *Paecilomyces varioti*

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Two thermostable enzymes produced by the thermophilic fungus *Paecilomyces varioti*, a chitinase and laminarinase, were used to isolate protoplasts of a thermophilic fungus, *Malbranchea sulfurea*. The frequency of protoplast regeneration observed (35%) was considerably higher than that obtained using commercial lytic enzymes.

Key words: Chitinase, fungus, laminarinase, protoplast formation, thermophilic.

Thermophilic fungi are a useful source of thermostable enzymes. As thermophilic fungi grow optimally at temperatures of 45°C or above, the lytic enzymes required for the isolation of their protoplasts must be active at these temperatures. The commercially available lytic enzymes are of mesophilic origin (Roncal *et al.* 1991) and, to our knowledge, the potential of thermophilic fungi as a source of thermostable lytic enzymes has not yet been explored. The present study was on the production of thermostable chitinase and laminarinase by the thermophilic fungus *Paecilomyces varioti* and the use of these lytic enzymes to isolate protoplasts from a thermophilic fungus *Malbranchea sulfurea*.

Materials and Methods

Microorganisms

The thermophilic fungus *Paecilomyces varioti* IMI 334593 was isolated from soil. *Malbranchea sulfurea* IMI 337352, a thermophilic fungus which produces amylase (Gupta & Gautam 1993) was selected as the target fungus for protoplast formation.

Production of Chitinase and Laminarinase

Two media were used. Medium 1 contained (g/l): colloidal chitin substrate, 2.5; KH_2PO_4, 0.2; $MgSO_4$, 0.2; and yeast extract, 0.5. Medium 2 was Medium 1 supplemented with 0.5% (w/v) glucose. Erlenmeyer flasks (150 ml), each containing 50 ml sterilized medium, were each inoculated with a 0.5-cm agar disc containing mycelial growth of *P. varioti* (cut from a 7-day-old culture on potato/dextrose/agar medium) and incubated at 45°C without shaking. Flasks in duplicate were withdrawn periodically and the mycelia separated by centrifugation at 5000 × ***g*** for 10 min. The supernatant was used for enzyme assay. Extracellular protein was measured by the Lowry method.

Enzyme Assay

The reaction mixture for the chitinase assay (2 ml 0.26% colloidal chitin, 1 ml crude enzyme sample and 1 ml 50 mM acetate buffer, pH 5.2) was incubated at 45°C with intermittent shaking. A_{610} of the assay mixture was measured at zero time and after 45 min. One chitinase unit was defined as the amount of enzyme giving a 1% decrease in absorbance.

The reaction mixtures for the laminarinase assay (0.5 ml 0.26% laminarin, 1 ml crude enzyme sample and 0.5 ml 50 mM acetate buffer, pH 5.2) were incubated at 45°C for 45 min with intermittent shaking and the reducing sugars were measured using the nitrosalicylic acid reagent method. A laminarinase unit was defined as the amount of enzyme liberating 1 μmol reducing sugars from the substrate per min.

Protease activity was measured following the method of Marrink & Grubber (1966). One protease unit was defined as the amount of enzyme mediating an increase of 0.02 A_{280} units.

Preparation of Lytic Enzymes of P. varioti

The crude enzyme sample was dialysed for 24 h against 0.05 M citrate/phosphate buffer, pH 5.0, and then freeze-dried. When required, the resultant powder was dissolved in 0.8 M sorbitol prepared in 0.016 M citrate/phosphate buffer, pH 5.0.

Formation and regeneration of M. sulfurea *protoplasts*

Lytic enzymes (3 mg/ml) were added to approximately 50 mg wet mycelia (48-h-old, suspended in 0.8 M sorbitol). The mixtures were incubated at 45°C for 2 h, filtered through glass-wool (0.5 × 10 cm) and the protoplasts recovered by centrifugation of the suspension at 10,000 × ***g*** for 10 min. Approximately 10^2 protoplasts, suspended in 0.8 M sorbitol, were mixed with the regeneration medium (yeast extract, 1.5%; peptone, 1.5%; sucrose,

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Table 1. Production of chitinase, laminarinase and protease by *P. varioti* in the presence of glucose (Medium 2) at 45°C.

Incubation day	Activity (U/mg protein)			pH
	Chitinase	Laminarinase	Protease	
2	39	0.27	1.5	4.9
3	51	0.40	0.4	4.8
4	16	0.28	0.9	4.5
5	4	0.24	1.1	5.4
6	6.9	0.21	0.9	6.2

0.6 M; and agar, 0.5%) and layered on top of a solidified layer of regeneration medium (3% agar). The plates were incubated at 45°C.

Results and Discussion

Enzyme Production by P. varioti

Maximum production of chitinase and laminarinase was observed on Medium 2 (Table 1). The addition of glucose up to 0.5% (w/v) caused an increase in enzyme production. A further increase in glucose resulted in decreased production. Replacing the glucose with *N*-acetylglucosamine resulted in the repression of enzyme synthesis (data not shown).

Optimization of pH and Temperature

Optimal pH and temperature for chitinase and laminarinase activity were pH 5.0 and 45°C and pH 4.5 & 50°C, respectively. The enzymes were stable between pH 4.0 and 5.0 and for up to 2 h at 60°C.

Isolation and Regeneration of Protoplasts

The formation of protoplasts from *M. sulfurea* mycelia was found to be maximal (1.4×10^7) using lytic enzymes from *P. varioti*. The protoplast yields using control lytic enzymes, those from *Trichoderma harzianum* and commercial chitinase, were 1.25×10^7 and 1.15×10^7, respectively. The protoplasts isolated using the *P. varioti* enzymes also had much better regeneration (34.6%) than those isolated using commercial chitinase (21.3%) or the lytic enzymes from *T. harzianum* (16.5%). The lytic enzyme preparation from *T. harzianum* is known to contain protease and this can decrease the regeneration rate (Roncal *et al.* 1991).

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