ABSTRACT OF THE THESIS ENTITLED

EXOCHITINASE OF THE THERMOPHILIC MOULD
MYCELIOPHTHORA THERMOPHILA

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Chitinases are the enzymes involved in growth, morphogenesis and interactions of chitin containing organisms like fungi, nematodes, arthropods and others. Besides biocontrol potential of chitinases, the applications of soluble hydrolysis products of chitin in various fields like medicine and agriculture have generated immense interest in producing commercially suitable chitinase preparations. The success in using enzymes for different applications depends on the supply of highly active preparations at an affordable and reasonable cost. The thermostable enzymes possess advantages of obtaining higher reaction rates, ability to use higher substrate concentrations, longer shelf life and lower contamination problems. This investigation, thus aimed at production of a thermostable chitinase from thermophilic organisms, particularly fungi.

In the present study, a potent chitinase producing thermophilic fungus *Myceliophthora thermophila* was selected amongst one hundred and thirty four thermophilic fungal isolates, isolated from various environmental samples collected from different geographical regions of India. A cost effective production process with high yields is required for any enzyme to be applicable on an industrial scale. An attempt was, therefore, made to improve the production, and medium was formulated that supports high chitinase yields insubmerged and solid state fermentations. An effort was also made to purify and characterize the enzyme and study its applicability in chitin hydrolysis and biocontrol of fungal, nematode and insect pests. The significant findings of the present investigation are summarized below:

Of the twenty eight chitinase producing strains among 134 thermophilic fungal isolates, *Myceliophthora thermophila* was selected based on higher chitinase titres. The identity of the selected strain was confirmed by amplification and sequencing of the ITS region and its phylogenetic analysis. The chitinase production by this fungus was inducible in the presence of chitin, its hydrolysis products and lactose.

In submerged fermentation, *M. thermophila* produced chitinase optimally in 6 days at 45°C at pH 6.0. Potassium nitrate, magnesium sulphate and incubation days were identified as important factors that influence chitinase production by Plackett-Burman design, and these were further optimized by RSM. Statistical optimization resulted in 7.1-fold improvement in chitinase titres. A sustainable chitinase titre was also
attained in Erlenmeyer flasks of varied volumes and in a laboratory fermentor. The fermentation time was reduced to half (72 h) in the fermentor as compared to that in Erlenmeyer flasks (144 h), with minor enhancement in enzyme yield.

*Myceliophthora thermophila* grew well on wheat bran in SSF and produced chitinase in the presence of an inducer in a growth associated manner. The optimal chitinase titres were obtained in 10 days of incubation at 45°C and pH 6.0. Optimization with Plackett-Burman design revealed the significance of wheat bran, glucose and initial moisture level among others in obtaining higher chitinase titres. Optimizing the values of these variables by response surface methodology resulted in 10.58 -old enhancement in chitinase production. A slight reduction in chitinase titres in shake flasks of higher volume and enamel coated metallic trays was recorded. However, this decline could be circumvented with optimization of bed depth in trays. A 11-fold enhancement in enzyme production was achieved over that in the unoptimized medium. *M. thermophila* secreted other hydrolytic enzymes (cellulose, xylanase, lipase etc.) along with chitinase in SSF. Chitinase production by *M. thermophila* was 7.6-fold higher in SSF (192300 U kg⁻¹ DMR) than that in SmF (25382.4 UL⁻¹).

The crude culture filtrate was concentrated by lyophilisation. The chitinase was purified to homogeneity from the concentrate by using affinity adsorption followed by hydrophobic interaction chromatography. The purified native chitinase is a monomer of 43 kD with a pl of 4.0. It exhibited an optimum pH  4.0 and 55°C, with t₁/₂ of 9.0 and 3.0 h at 60 and at 70°C, respectively. The MALDI–ToF–MS/MS analysis of 11 trypsin digested peptides of the protein showed homology with a glycosyl hydrolase family 18 protein of *M. thermophila* ATCC 42464. One of the two signature sequences was also present in the identified peptides, confirming the identity of the purified protein as a chitinase from *M. thermophila*.

The preferred substrate for chitinase was swollen chitin, although crystalline chitin and chitosan were also hydrolyzed. The maximum hydrolysis rate (V_max) and apparent Michaelis-Menten constant (K_m) for colloidal chitin were 25.25 U/mg protein and 0.386 mg/ml, respectively. Chitinase activity was not affected by most of the organic solvents and detergents. The enzyme does not require any metal ion for activity. Chitinase activity is inhibited by Al²⁺, Hg²⁺ and Fe²⁺ ions. Carboxyl groups and tryptophan residues are important for chitinase activity as the activity is inhibited
by woodward’s reagent K and N-bromosuccinimide. Chitinase activity is inhibited by PMSF, DTT and IAA to varied extent indicating the importance of sulphhydryl groups and serine residues for enzyme activity or stability. The enzyme is quite stable to when preserved in a refrigerator, and retained 82.0% of activity after 7 months storage at 4°C.

Chitin hydrolysis with the chitinase yielded N-acetylglucosamine and chitobiose initially, revealing the exo- mechanism of enzyme action. As the reaction progresses, the chitobiose is hydrolyzed to NAG completely. This chitobiase activity was confirmed by assay with pNP-N-acetylglucosamine. Higher oligosaccharides were not detected in the hydrolysate even after prolonged incubation. Thus the chitinase is useful in generating both NAG and chitobiose.

Chitinase was effective against fungi, nematode and insects. The cell walls of spores/hyphae were disrupted in *Fusarium oxysporum*, *Curvularia* sp. and *Stachybotrys* sp. by chitinase treatment. The chitinase inhibited hatching of eggs in the nematode, *Meloidogyne incognita* with the enzyme treated eggs showing clearance of egg cytoplasm. The chitinase induced mortality of 3rd instar larvae in two insect pests *Aedes aegypti* and *Maconellicoccus hirsutus*.

This investigation has, therefore, been useful in obtaining a thermoacidstable bifunctional chitinase which is suitable for N-acetylglucosamine production at high temperature with increased reaction rates. The enzyme also possesses broad spectrum biocontrol potential against fungi, nematode and insects. Although a significant improvement in chitinase production was achieved in submerged and solid state fermentations, these production levels are far lower than what is required for successful industrial application. Cloning and over expression of the chitinase gene of *M. thermophila* would enable in lowering the cost of enzyme production.

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