Biochem. Cell. Arch. Vol. 10, No. 2, pp. 179 - 184, 2010 | *ISSN 0972-5075*

BACTERIAL PRODUCTION OF CHITINASES FOR THE CONTROL OF PHYTOPATHOGENIC FUNGI

P. Jaganmohan, S. V. Prasad* and B. Purushottam Das*

Harrison Institute of Biotechnology, Shrimp Care Unit, Ramamurthy Nagar, Nellore-524 001, India.
* Department of Biochemistry, Rao's P.G College, Nellore - 524 001, India.
e-mail: pjaganbio@gmail.com.

(Accepted 9 September 2010)

ABSTRACT – Chitinolytic activity of three bacterial species *Bacillus subtilis, Pseudomonas auregenosa* and *Serratia marscencens* (MTCC 2387, MTCC 4676, MTCC 4822 respectively) was studied using different substrates and nitrogen sources under different physiological conditions like temperature and pH. Maximum chitinase activity was seen with *Serratia* at a temperature of 60^{0}C followed by *Pseudomonas* at neutral pH where as *Bacillus* species had shown the maximum activity at pH 9. Both *Pseudomonas* and *Bacillus* had given maximum chitinolytic activity at 45^{0}C.Among the selected substrates colloidal chitin had given maximum production with all species and with different nitrogen sources yeast extract was shown to be best suitable for all species for better production of chitinase. The precipitated chitinase protein from *Serratia* has shown high antagonistic activity against *R. solani* among the tested phytopathogenic fungi with a maximum inhibition ratio of 17.73%.

Key words : Colloidal chitin, antagonistic activity, dialysis, yeast extract, chitinase.

INTRODUCTION

Chitin which is the second-most abundant biopolymer on the planet is an insoluble linear polymer of β-1, 4-linked N-acetyl-β-D-glucosamine (Shahidi and Abuzaytoun, 2005). It is widely distributed in nature as a structural component of crustaceans, fungi, protozoa and insects (Flach *et al,* 1992). Chitinases (EC 3.2.1.14) are glycosyl hydrolases which catalyze the degradation of chitin. These enzymes have a wide range of biotechnological applications such as preparation of pharmaceutically important chitooligosaccharides and N-acetyl-D-glucosamine (Pichyangkura *et al,* 2002; Sorbotten *et al,* 2005), isolation of protoplasts from fungi and yeast (Dahiya *et al*, 2005), preparation of single-cell protein (Vyas and Deshpande, 1991), control of pathogenic fungi (Mathivanan *et al,* 1998) and treatment of chitinous waste (Wang and Hwang, 2001). Chitinases are present in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants and animals and play important physiological and ecological roles (Cody *et al,* 1990; Duo-Chuan, 2006; Gooday, 1990). Chitinases are constituents of several bacterial species; some of the best known include the *Aeromonas, Serratia, Vibrio, Streptomyces* and *Bacillus* genera (Cody, 1989). Bacteria produce chitinases mainly to degrade chitin and utilize it as an energy source. In addition, some chitinases of chitinolytic bacteria are potential agents for the biological control of plant diseases caused by various phytopathogenic fungi (Chernin *et al,* 1997; Downing and Thomson, 2000). Owing to their application in control of phytopathogens the present study was aimed at the production and application of chitinases as phytopathogenic controlling agents.

MATERIALS AND METHODS

Organism :

The bacterial cultures *Bacillus subtilis, Pseudomonas auregenosa* and *Serratia marscencens* (MTCC 2387, MTCC 4676, MTCC 4822 respectively) selected for this study were obtained from Microbial type collection centre, Chandigarh and the phytopathogenic fungi *Aspergillus niger, Alternaria solani, Fusarium solani* and *Rhizopus stolanifera* were kindly provided by Centre for Biotechnology, Acharya Nagarjuna University, Guntur, Andhra Pradesh.

Culture media :

The basic medium used for fermentation contained the following constituents (g L^{-1}): Na_2HPO_4 (0.65), KH_2PO_4 (1.5), NaCl (0.25), NH_4Cl (0. 5), $MgSO_4$ (0.12), $CaCl_2$ (0.005) and colloidal chitin or swollen chitin (10), pH 6.5 (Wen *et al*, 2002). Cultivation was done using 500 mL Erlenmeyer flasks containing 100 mL medium. Chitin agar medium containing the basic medium and 20 g L^{-1} of agar was used for screening the bacterial strains in plates.

Preparation of colloidal and swollen chitin

For the present study colloidal chitin for *P. auregenosa* and *S. marscencens* were prepared according to Lingappa and Lockwood (1962) and Gernot respectively and to prepare swollen chitin for *B.subtilis* a variation of the Monreal and Reese (1969) procedure

has been used.

Production of Chitinase

About 5ml of sterile distilled water is added on to the well grown culture and was vertexed on a vertex shaker for few minutes to make uniform bacterial suspension. One ml of this suspension was used to incubate 50ml of sterile seed medium present in 250ml Erlenmeyer flask. These inoculated flasks were kept on a shaker, maintained at 37°C for 24 to 36 hours and this seed is used for further experimentation. 2ml of this seed was used to inoculate 500ml Erlenmeyer flasks containing 100ml sterile productive medium. The inoculated flasks were incubated at 37°C for 96 hours using aerated and agitated condition on a rotary shaker with 250 rpm for 96 hours. At the end of the fermentation cycle 5ml of the fermented broth was aseptically removed and centrifuged at 2000rpm for 10 minutes. The clear supernatant containing the enzyme was used in the enzyme assay. Triplicates were maintained in all the experiments.

Chitinase Assay

Chitinase activity was assayed by dinitrosalicylic acid (DNS) method (Miller, 1959). The colored solution was centrifuged at 10,000 rpm for 5 min and the absorption of the appropriately diluted test sample was measured at 530nm using UV spectrophotometer (UV-160 A, Shimadzu, Japan) along with substrate and enzyme blanks.

Optimum pH for chitinase production

In the present investigation the chitinase enzyme production was optimized at various temperature range, pH, substrate concentration (chitin) and nitrogen sources. The experiments were conducted in 250 ml Erlenmeyer flasks containing the production medium. While optimizing the pH of the basal medium, the pH of aqueous solution has varied from 4, 5, 6, 7, 8 and 9 by using 1N HCl or 1N NaOH. All the experiments were carried out in the triplicate at 40°C and average values has been reported. The optimum pH achieved by this step was fixed for subsequent experiment.

Optimum temperature for chitinase production

The fermentation has carried out at various temperatures such as 30ºC, 35ºC, 40ºC, 45ºC, 50ºC, 55ºC, 60ºC and 65ºC to study its effect on enzyme production. All the experiments were carried out in triplicate and average values were reported. The optimum range of temperature achieved by this step was fixed for subsequent experiments.

Effect of different types of substrate

The effect of different substrates like chitin powder, colloidal chitin, flaked chitin, and shrimp shell waste on chitinase production has been studied with different sources of substrates which were added into 250ml production medium.

Effect of different types of nitrogen additives

The effect of different nitrogen sources such as peptone, yeast extract, soybean meal, urea, ammonium chloride and ammonium sulphate was studied. All the experiments were carried out in triplicate and average values were reported. The suitable nitrogen source achieved by this step was fixed for subsequent steps.

Preparations of crude antifungal extract

Serratia marscencens species was cultured under optimal production condition for chitinase and incubated for 7-8 days at 37°C. After incubation the culture was centrifuged at (8000 g) at 4°C for 20 min, the supernatant was subjected to ammonium sulfate precipitation (70% saturation). The resultant precipitate was collected and dissolved in a small amount of 50 mM Sodium Phosphate buffer (pH 7) followed by dialysis against the same buffer overnight. The resultant dialyzate was filtered aseptically through membrane filters and used for the bioassays.

***In vitro* antifungal assays**

The effect of chitinase on the mycelial growth of fungi were assayed using the plant pathogenic fungi like *Aspergillus niger, Alternaria solani, Rhizopus stolanifera,* and *Fusarium solani* which were grown at 28°C on Potato dextrose agar, until the colonies were at least 5 cm in diameter. The diameters of the largest and smallest fungal colonies were recorded and the averages were calculated. The inhibition ratios were calculated with the following formula.

$$\text{Inhibition ratio } (\%) = (C-E)/C \times 100$$

Where

C = The average diameter of largest and smallest colonies of the control groups

E = The average diameter of largest and smallest colonies of the experimental groups.

RESULTS AND DISCUSSION

1. Optimization of pH for chitinase production

The pH ranging from 4 – 9 was studied for the detection of optimum pH for high chitinase production. However, the production of enzyme was maximum at a pH of 7 with an activity of 2.76 U/ml for *Serratia,* 1.8 U/ml for *Pseudomona*s but 1.2 U/ml for *Bacillus* at pH–9 (Fig-1). It has been also supported by previous reports (Wen *et al,* 2002). Wang and Chang (1997) have explained the chitinase secretary efficiency of *Psuedomonas auregenose*. The same efficiency has also

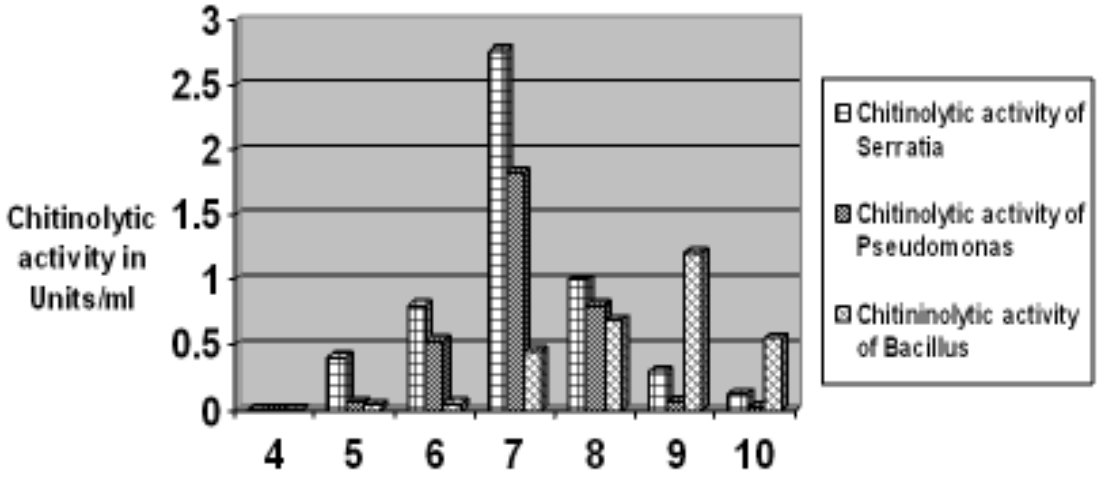

Fig. 1 : Effect of pH on chitinase activity

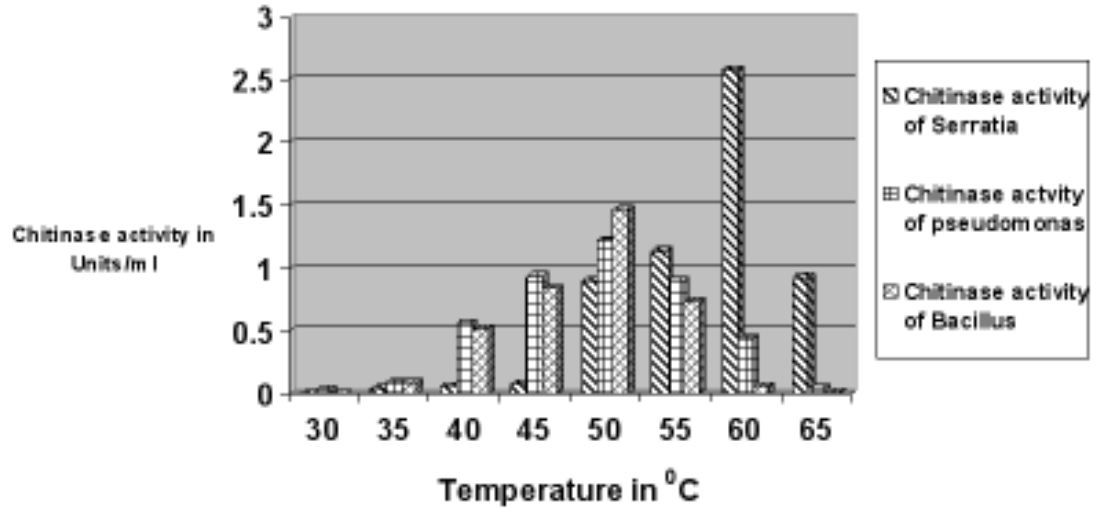

Fig. 2 : Optimization of temperature for maximum chitinolytic activity.

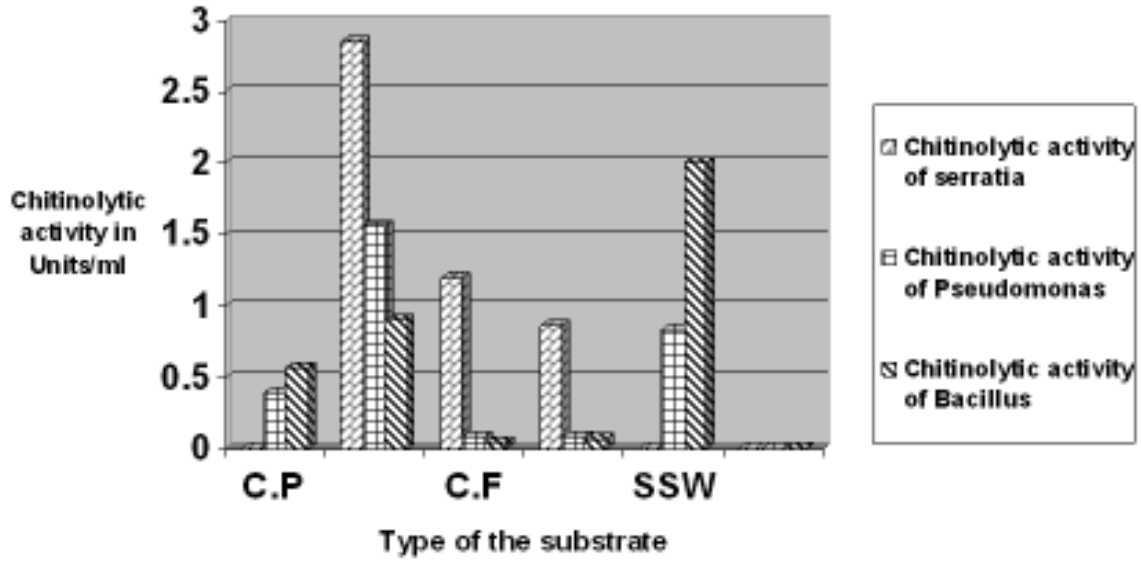

Fig. 3 : Effect of different substrates on chitinolytic activity (In fig-C.P-Chitin purified, C.C-colloidal chitin, C.F- chitin flakes, C.Pw- chitin powder, SSW- Shrimp shell waste and C-control)

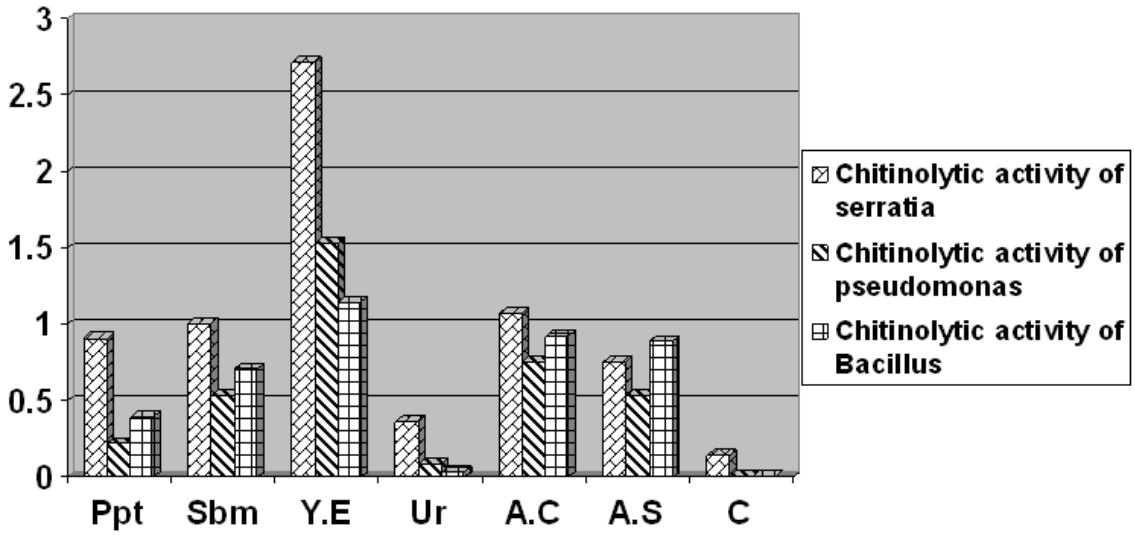

Fig. 4 : Effect of different nitrogen sources on chitinase activity (in fig Ppt-Peptone, Sbm-Soya bean meal, Y.E- Yeast extract, Ur-urea, A.C-Ammonium chloride, A.S- ammonium sulphate and C-control)

been attained from *Psuedomonas auregenose* which secretes 1.8 U/ml at neutral pH in the present study. Even though maximum activity was shown at neutral pH in the present study chitinase produced from same species had shown stability at different pH in different studies. The chitinase from *Serratia, Pseudomonas* and strain were stable over wide pH range i.e. from pH 4.5 to 8.0. Other bacterial Chitinases stable over broad pH range were pH 4.0 to 9.0 of *Aeromonas* sp.No.10s-24 chitinase, pH 6.0 to 9.0 of *Pseudomonas aeruginosa* K-187 (Wang and Chang,1997), pH 5.0 to 8.0 of *Aeromonas hydrophila* H2330 chitinase, pH 4.0 to 9.0 for *Vibrio* sp (Zhou *et al,* 1999), pH 6.8 to 8.0 of *Bacillus* sp. NCTU2 (Wen *et al,* 2002) chitinase and pH 4.0 to 8.5 of *Bacillus cereus* strain 65.

2. Optimization of temperature for chitinase Production

Temperatures varying from 30°C – 65°C were examined for the detection of optimum temperature required for the production of the enzyme. The results showed that the optimum temperatures required for chitinase production were related to species. At 60°C *S.marscencens* have shown the activity of 2.590 U/ml (Fig-2) where as *B.subtilis* and *P.auregenosa* had given an activity of 1.467 U/ml and 1.231 U/ml respectively at 45°C. Chitinase production by some soil bacteria e.g., *Bacillus laterosporous* (Shanmugaiah *et al*, 2008) appears to be less sensitive to temperature variations. The same can be said for some bacteria isolated from marine habitats including *Aeromonas* (Jami Al Ahmadi *et al,* 2008). Temperature and pH affect biological processes through several mechanisms including enzyme induction or suppression, protein denaturation and altering cell viability (Nampoothiri *et al,* 2004). Hence, bacteria isolated from similar habitats may show different patterns of enzyme production.

3. Effect of different Substrate sources on the production of chitinase

Substrate being the most important factor for any enzyme production, an array of substrate such as chitin powder, colloidal chitin, flaked chitin and shrimp shell waste was thoroughly studied. On observing the results colloidal chitin gave a maximum enzyme activity as high as 2.840 U/ml with chitin flakes being the closest at 1.203 U/ml for *S. marscencens* species and colloidal chitin gave a maximum yield as high as 2.000 U/ml with swollen chitin being the closest at 0.901 U/ml when taken for *B. subtilis* species. But whereas for *P. auregenosa* species, the maximum enzyme activity was given by colloidal chitin as high as 1.565 U/ml with purified Chitin being the closest at 0.563 U/ml (Fig-3).

4. Effect of different Nitrogen sources on the production of chitinase

Maintaining the physical factors and the carbon source at optimized condition, nitrogen sources of varying nature were studied. Of all the sources studied, yeast extract gave the maximum enzyme production yield with an activity of 2.709 U/ml for *S. marscencens* species and 1.532 U/ml for *P. auregenosa* species and 1.147 U/ml for *B. subtilis* species (Fig-4). Carbon and Nitrogen source plays vital role in upstream and downstream process. Yeast extract has been used as an ideal substrate for *Bacillus, Pseudomonas* and *Serratia* species. Though the efficiency of enzymes examined against pH, temperature, Carbon and Nitrogen ratio present result

Table 1: Antagonistic activity of Chitinase on Plant Pathogens.

Ogranism	Chitinase activity (E)	Control (C)	Inhibition ratio (%)
Aspergillus niger	8.5467	0.005	17.08
Alternaria solani	5.37	0.005	10.73
Fusarium solani	6.1367	0.005	12.62
Rhizopus stolanifera	8.87	0.005	17.73

clearly distinct the individual efficiency of substrate concentration of *Bacillus species* distinct from *Pseudomonas* and *Serratia* species. In contrast to this result, yeast extract has been reported to enhance chitinase production in some bacterial and fungal species including *Beauveria bassiana, Serratia marcescens, Streptomyces cinereoruber, Alcaligenes xylosoxydans and Trichoderma harzianum* (Monreal and Reese, 1969; Sherief *et al,* 1991; Vaidya *et al,* 2001; Nampoothiri *et al,* 2004). According to Nampoothiri *et al* (2004) this may be due to the presence of chitin or growth factors in yeast extract. However, as stated above, such low concentrations of chitin fail to induce chitinase production in most microorganisms. In addition, some other nitrogen sources including peptone and urea are reported to enhance chitinase production by *A. xylosoxydans* and *Myrothecium verrucaria* (Vaidya *et al,* 2001), indicating that other mechanisms are involved. Fructose, GlcNAc, glucose, lactose and maltose slightly decreased chitinase production by *M. timonae* ($p<0.05$).

5. Antagonistic activity of chitinase from *S. marscencens*

The precipitated chitin from Serratia species was tested for its antifungal activity against selected phytopathogenic fungi like *A. niger, A. solani, F.solani* and *R. stolanifera*. The results have shown that the inhibition activity of chitinase was high with *R.stolanifera* with an inhibition ratio of 17.73% followed by 17.08%, 12.62% and 10.73% for *A.niger, F.solani* and *A.solani* respectively. Bacteria produce chitinases to meet nutritional needs. They usually produce several chitinases, probably to hydrolyze the diversity of chitins found in nature. These enzymes operate by a mechanism leading to overall retention of the anomeric configuration of the hydrolysed residue.

Anti-fungal properties of chitinolytic bacteria have been attributed to the combined action of chitinases and other lytic enzymes, e.g.β-1, 3-glucanases and proteases. There were many tests in which fungi were apparently not affected despite the production of both chitinase and protease, indicating that a combination of these enzyme activities is not generally anti-fungal. Actinomycetes that were producing β-1, 3-glucanase in addition to chitinase and protease did reduce mycelium formation of all fungi studied. In contrast, the few unicellular bacteria that produced these enzyme activities were only inhibitory to some of the fungi. Hence, there is also little evidence that a combination of lytic enzyme activities is generally inhibitory for fungi. The chitinolytic bacteria had selective rather than general anti-fungal activities. Selective antagonism indicates the existence of differences between the chitinolytic bacteria with respect to the compounds that cause inhibition of fungi.

The Chitinase activity plays an important role as biocontrolling agent against deadlier fungus, like – *Aspergilllus* species, *Fusariuim* species*, Trichoderma* species, *Alterneria* species. High chitinase activity sounds in *Trichoderma* i.e., 8,870, *Aspergillus* species – 8.546, *Fusarium* species – 6.136 and *Alternaria* species - 5.370 and shows inhibitaion ratio respectively (Table-1). Though the action of enzyme depends on substrate concentration, Carbon and Nitrogen, Temperature, it could act as effective antifungal agent. Present investigation clearly states the biocontrol and antifungal efficiency of chitinase activity depends on the respective inhibition ratio of the examined fungus.

REFERENCES

Chernin L S, De-la-Fuente L, Sobolev V, Haran S, Vorgias C E, Oppenheim A B and Chet I (1997) Molecular cloning, structural analysis and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans. Applied Environ. Microbiol.* **63**, 834-839.

Cody R M (1989) Distribution of chitinase and chitobiase in *Bacillus. Curr. Microbiol.* **19**, 201-205.

Cody R M, Davis N D, Lin J and Shaw D (1990) Screening microorganisms for chitin hydrolysis and production of ethanol from amino sugars. *Biomass* **21**, 285-295.

Dahiya N, Tewari R, Tiwari R and Hoondal G (2005) Production of an antifungal chitinase from *Enterobacter* sp. NRG4 and its application in protoplast production. *World J. Microbiol. Biotechnol.* **21**, 1611-1616.

Downing K J and Thomson J A (2000) Introduction of the *Serratia marcescens* chiA gene into an endophytic *Pseudomonas fluorescens* for the biocontrol of phytopathogenic fungi. *Can. J. Microbiol.* **46**, 363-369.

Duo-Chuan L (2006) Review of fungal chitinases. *Mycopathologia* **161,** 345-360.

Flach J, Pilet PE and Jolles P (1992) What's new in chitinase research? *Experientia* **48**, 701-716.

Gooday GW (1990) The ecology of chitin decomposition. *Adv. Microb. Ecol.* **11**, 378-430.

Jami Al-Ahmadi K, Tabatabaei Yazdi M, Fathi Najafi M and Shahverdi A R (2008) Optimization of medium and cultivation conditions for chitinase production by the newly isolated: *Aeromonas* sp. *Biotechnology* **7**, 266-272.

Lingappa Y and Lackwood J L (1962) Chitin media for selective isolation and culture of actinomycetes. *Phytopathology* **52,** 317-323.

Mathivanan N, Kabilan V and Murugesan K (1998) Purification, characterization and antifungal activity of chitinase from *Fusarium chlamydosporum* a mycoparasite to groundnut rust, *Puccinia arachidis. Can. J. Microbiol.* **44,** 646-651.

Miller G L (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem.* **31,** 426–429.

Monreal J and Reese E T (1969) The chitinase of *Serratia marcescens*. *Can J Microbiol.* **15**, 689-696.

Nampoothiri K M, Baiju T V, Sandhya C, Sabu A, Szakacs G and Pandey G (2004) Process optimization for antifungal chitinase production by *Trichoderma harzianum. Process Biochem.* **39**, 1583-1590.

Pichyangkura R, Kudan S, Kuttiyawong K, Sukwattanasinitt M and Aiba S (2002) Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carbohydr. Res*. **337**, 557-559.

Shahidi F and Abuzaytoun R (2005) Chitin, chitosan and co-products: Chemistry, production, applications and health effects. *Adv. Food Nutr. Res*. **49**, 93-135.

Shanmugaiah V, Mathivanan N, Balasubramanian N and Manoharan P T (2008) Optimization of cultural conditions for production of chitinase by *Bacillus laterosporous* MML2270 isolated from rice rhizosphere soil. *Afr. J. Biotechnol.* **7**, 2562-2568.

Sherief A A, El-Sawah M M A and Abd El-Naby M A (1991) Some properties of chitinase produced by a potent *Aspergillus carneus* strain. *Applied Microbiol. Biotechnol*. **35**, 228-230.

Sorbotten A, Horn S J, Eijsink V G and Varum K M (2005) Degradation of chitosans with chitinase B from *Serratia marcescens*. Production of chito-oligosaccharides and insight into enzyme processivity. *FEBS J.* **272,** 538-549.

Vaidya R J, Shah I M, Vyas P R and Chhatpar H S (2001) Production of chitinase and its optimization from a novel isolate *Alcaligenes xylosoxydans*: Potential in antifungal biocontrol. *World J. Microbiol. Biotech*. **17,** 691-696.

Vyas P and Deshpande M V (1991) Enzymatic hydrolysis of chitin by *Myrothecium verrucaria* chitinase complex and its utilization to produce SCP. *J. Gen. Applied Microbiol*. **37,** 267-275.

Wang S L and Chang W T (1997) Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in shrimp and crab shell powder medium. *Applied Environ. Microbiol*. **63**, 380-386.

Wang S L and Hwang J R (2000) Microbial reclamation of shellfish wastes for the production of chitinases. *Enzyme. Microb. Technol.* **28**, 376-382.

Wen C M, Tseng C S, Cheng C Y and Li Y K (2002) Purification, characterization and cloning of a chitinase from *Bacillu*s sp. NCTU2. *Biotech Applied Biochem.* **35**, 213-219.

Zhou S N, Yang C Y, Lu Y J, Huang L, Cai C U and Lin Y C (1999) Isolation and characterization of chitinase from marine bacterium *Vibrio* sp. *World J Microbiol Biotech.* **15,** 745-746.