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Isolation and identification of marine chitinolytic bacteria and their potential in antifungal biocontrol

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Chitinolytic marine bacterial strains (30) were isolated from the sea dumps at Bhavnagar, India. They were screened as chitinase producers on the basis of zone of clearance on chitin agar plates incorporated with calcofluor white M2R for the better resolution. Out of these, three strains namely, *Pseudomonas* sp., *Pantoea dispersa* and *Enterobacter amnigenus* showed high chitinase production. They were also found to produce proteases and therefore have a good potential for use as antifungal biocontrol agents for the control of fungal plant pathogens. These strains could degrade and utilize the mycelia of *Macrophomina phaseolina* (Tassi) Goidanich and *Fusarium* sp. *In vitro*, these strains could inhibit the growth of *Fusarium* sp. and *M. phaseolina*. The culture filtrate inhibiting hyphal elongation was observed microscopically.

Keywords: Biocontrol, *Cajanus cajan*, Chitinase production, *Fusarium sp.*, *Macrophomina phaseolina*, Marine bacteria

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Chitin is one of the most abundant biopolymer in nature particularly in marine environment, as many marine organisms including zooplankton and several phytoplankton species have it as a structural skeletal component[1]. Chitinase seems to play an important role in decomposition of chitin. Agricultural pests identified so far include approximately 10,000 species of insects and 8000 species of fungi[2]. Agricultural output as crop yield is severely affected by phytopathogenic fungi, which is higher than the diseases caused by nematodes, protozoa and bacteria[3]. Pigeonpea (*Cajanus cajan*) is one of the major legume crop of tropics and subtropics. It is grown in all the states of India[4], cultivated on 3816 ha of land and production is about 2876 thousand tonnes per annum. *Fusarium* wilt is the most destructive disease of pigeonpea. The annual loss due to wilt in pigeonpea in India and eastern Africa had been estimated to be 36 million and 5 million US $, respectively[5]. Studies are required to control pigeonpea infection.

The objective of the present investigation was to isolate and identify high chitinase producing bacterial strains from marine habitat, to study chitinase production and proteases and to determine their potential for the inhibition of the growth of the fungal plant pathogens like *Fusarium* sp. and *Macrophomina phaseolina* (Tassi) Goidanich.

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Materials and Methods

Isolation and cultivation of bacteria—The medium used for the isolation and cultivation of bacteria was (g/l): chitin, 5.0; yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.3; and KH_2PO_4, 1.36. The *p*H of the medium was adjusted to 7.2. The medium was sterilized by autoclaving at 121°C for 15 min[6].

Preparation of acid-swollen chitin—Phosphoric acid-swollen chitin was prepared according to the method described by Hackman[7].

Isolation of chitinase producer by plate assay methods—Medium (50 ml) was inoculated with soil and water samples from various locations of sea at Bhavnagar (the distance for each sample collection was one kilometer away). These were incubated at 30° ± 2°C on rotary shaker at 180 rev/min for 7 days for the enrichment of chitinase producers. Three such serial enrichments of chitinolytic bacteria with 5% of inocula from the earlier flasks were carried out. The enriched bacteria were streaked on the chitin agar plates containing calcofluor white M2R[8]. The plates were incubated at 30° ± 2°C and examined under UV

for formation of clear zone (CZ) around colonies up to 7 days. The size of clear zone (CZ) and colony size (CS) were measured and the colonies were transferred to chitin agar slants.

Selection of high chitinase producer—Isolates which showed a higher CZ/CS ratio in the plate assay were inoculated into 50 ml of liquid medium and incubated at 30° ± 2°C on rotary shaker at 180 rev/min. The culture was sampled at every 24hr interval to check the maximum chitinase production, and biomass in terms of total cell protein. The culture filtrates were collected by centrifuging the culture broth at 7200 × *g* for 20 min and were used for chitinase assay.

Chitinase assay—Chitinase was assayed as described by Vyas and Deshpande[9]. One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 µmole of *N*-acetyl-D-glucosamine equivalent at 50°C per hr.

Method for the determination of the growth—Growth was determined in terms of cell protein, which was estimated as described by Waterborg and Matthews[10].

Protease activity of chitinase producer—Proteases activity was performed only at optimum period of chitinase production of selected chitinase producing bacteria.

Proteases assay—Proteases activity was assayed according to the method of Ong and Gaucher[11]. The assays were done for acid, alkaline and neutral proteases. The substrates used were—(a) 0.5% hemoglobin in 0.1*M* of Na-acetate buffer (*p*H 5.5) for acid protease; (b) 0.5% casein in 0.1*M* of Tris-HCl buffer (*p*H 7.2) for neutral protease; and (c) 0.5% casein in 0.1*M* of Tris-HCl buffer (*p*H 8.6) for alkaline protease. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmole of tryptophan equivalent at 50°C per hr.

Utilization of fungal biomass as source of chitin for the production of chitinase—*M. phaseolina* and *Fusarium* sp. were grown in Sabouraud's broth (Himedia, India) for 7 days and then the fungal biomass was harvested, washed with sterile distilled water and dried in an oven at 50°C till constant weight. These dried fungal mycelia were then used at 5 g/l in replacement of chitin[12].

Fungal plant pathogen inhibition assay—Effect of each culture and culture filtrate of *Pseudomonas* sp., *P. dispersa* and *E. amnigenus* were seen on the growth of *M. phaseolina* and *Fusarium* sp. For that, *Fusarium* sp. (5×10^5 spores/ml). and *M. phaseolina* (5×10^5 sclerotia/ml) were grown in medium (50 ml) containing 2 × concentration of Sabouraud's broth (Himedia, India) and incubated at 30° ± 2°C on rotary shaker at 180 rev/min for 48hr. After 48hr, 10ml of culture (1×10^8 CFU/ml) and culture filtrate (10ml) of *Pseudomonas* sp., *P. dispersa* and *E. amnigenus* were inoculated and incubated at 30° ± 2°C on rotary shaker at 180 rev/min for 48hr. The inhibition in the growth of plant pathogens was monitored in terms of the dry weight. The control consisted of 10ml of medium alone, inactivated culture or culture filtrate.

Inhibition of growth of fungal mycelia—1 ml suspension of *Fusarium* sp (5×10^5 spores/ml) and *M. phaseolina* (5×10^5 sclerotia/ml) were transferred to 25 ml double strength potato dextrose broth and incubated at 30°C for 6hr. After 6hr, mycelial suspension (10µl) was mixed with enzyme (10µl) on cover glass. The cover glass was kept inverted on silicon grease applied neoprene 'O' ring (12 mm diam). Neoprene 'O' ring was kept on glass slide (Silicon grease applied to prevent evaporation). The slide was observed under Olympus CX 41 microscope (Japan) and photographs were taken at different time intervals with Epson PhotoPC 3100Z digital camera (Japan).

All the experiments were done in triplicate and the values presented were the means of three independent determinations.

Results and Discussion

Screening of chitinase producing marine bacteria—For isolation of chitinolytic bacteria, samples were collected from sea dumps of Bhavnagar. Thirty cultures were isolated on the selective chitin agar plates. Selection of higher chitinase producing cultures was done on the basis of the higher ratio of the zone of clearance (CZ)/colony size (CS) and high chitinase activity (Table 1). Zone of clearance by isolate nos 1, 14 and 15 was seen as 34.43±0.35, 44.43±0.28 and 16.56±0.26 units/ml chitinase activity, respectively (Fig. 1), they were selected for identification and further study for their potential to control fungal plant pathogens.

Identification of bacteria—The marine isolates 1, 14 and 15 were identified using Rapid ID –32 E kit (manufactured by Biomerieux Company, France) for gram-negative bacteria (data not shown). We also followed our results with Bergey's manual of determinative bacteriology[13]. Isolate nos 1, 14 and 15 were gram negative rods, which were identified to be *Pseu-*

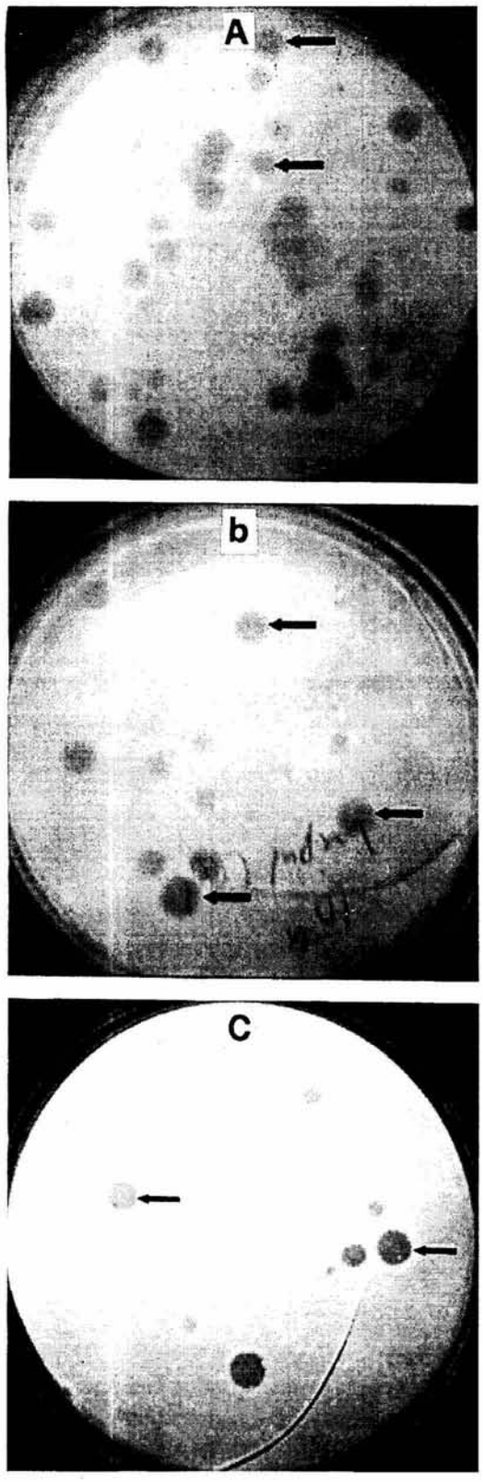

Fig. 1—Zone of clearance by (A) - *Pseudomonas* sp; (B) - *Pantoea dispersa*; and (C) *Enterobacter amnigenus* on chitin agar plate incorporated with calcofluor white M2R in UV light.

domonas sp., *Pantoea dispersa* and *Enterobacter amnigenus*, respectively.

Production profile of chitinase—The production of extracellular chitinase was monitored during the growth of *Pseudomonas* sp. (Fig. 2A), *Pantoea dispersa* (Fig. 2B) and *Enterobacter amnigenus* (Fig. 2C). The protein was also determined as a parameter

Table 1—Ratio of CZ/CS and chitinase acitivity of marine isoaltes
[Values are mean±SD of 3 replications]

Isolate no.	CZ/CS ratio	Chitinase acivity (units/ml)
1	3.50±0.22	34.43±0.35
2	2.33±0.15	6.96±0.46
3	2.50±0.36	11.92±0.26
4	3.00±0.15	11.14±0.46
5	2.00±0.26	14.08±0.26
6	2.50±0.10	6.81±0.13
7	2.50±0.09	10.68±0.46
8	2.00±0.13	6.65±0.26
9	2.66±0.11	5.10±0.46
10	3.25±0.19	13.77±0.27
11	2.00±0.11	3.33±0.13
12	1.33±0.12	3.17±0.12
13	1.33±0.2	2.25±0.12
14	4.00±0.1	44.43±0.28
15	3.50±0.1	16.56±0.26
16	2.50±0.13	1.32±0.2
17	2.00±0.15	1.89±0
18	2.00±0.11	4.15±0
19	2.00±0.12	5.28±0
20	3.50±0.10	1.70±0.2
21	1.66±0.21	5.28±0
22	2.00±0.12	11.41±0.13
23	2.00±0.11	1.70±0.2
24	2.00±0.1	7.54±0.53
25	2.00±0.09	3.39±0
26	2.50±0.12	1.13±0
27	2.00±0.31	13.572±0
28	2.00±0.12	7.54±0
29	2.00±0.09	7.27±0.5
30	2.00±0.1	4.79±0.2

to monitor the growth of these bacteria. The production of chitinase and growth was found to be maximum at 144hr in both *Pseudomonas* sp. And *P. dispersa*, whereas the growth and chitinase production for *E. amnigenus* was found to be maximum at 120hr. The production of chitinase by *S. marcescens* has been reported to be maximum at 144hr[6], whereas for *Bacillus circulans* no. 4.1 at 96hr[14] and for *Alcaligenes xylosoxydans* at 72hr[12]. There is no report for chitinase production by *P. dispersa* and *E. amnigenus*.

Proteases production—Mycolytic enzymes based formulations have been used to control fungal plant pathogens. Mycolytic enzymes consist of chitinase, proteases and glucanase[2]. *Pseudomonas* sp., *P. dispersa* and *E. amnigenus* have been found to produce chitinase and protease, when they are grown in chitin medium as a sole carbon source[6]. The production of extracellular proteases was monitored only at 144hr in *Pseudomonas* sp. and *P. dispersa*, whereas at 120hr in *E. amnigenus*. All the three isolates were found to produce acidic, neutral and alkaline proteases. The activity of neutral protease was found to be higher as compared to acidic and alkaline protease of all the three isolates (Table 2).

Effect of fungal mycelial biomass on chitinase production—As the fungal cell wall contains chitin as the major component, and chitinases are well known to lyse the cell walls of both live and dead fungi[15]. To check the ability of *Pseudomonas* sp., *P. dispersa* and *E. amnigenus* to utilize dead fungal mycelia, fungal biomass of *Fusarium* sp. and *M. phaseolina* were washed, autoclaved, dried and used for substitution of chitin for chitinase production. The fungal mycelia were found to be utilized significantly by *Pseudomonas* sp., *P. dispersa* and *E. amnigenus*, since significant chitinase activity was observed (Fig. 3). Beyer and Diekmann[16] have reported cell wall degradation of *Penicillium chrysogenum* by chitinase system of *Streptomyces* sp. ATCC 11238. Ordentlich *et al.*[17] have reported degradation of cell walls of the phyto-

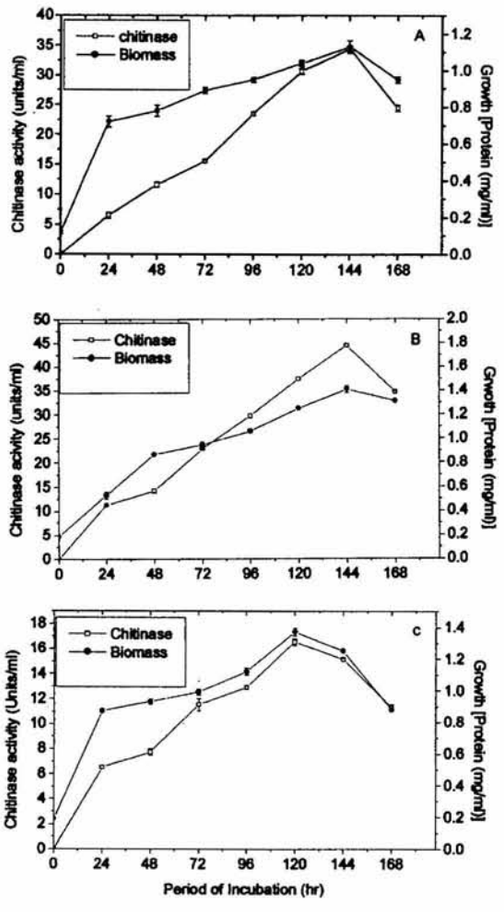

Fig. 2—Production of chitinase and growth of (A) - *Pseudomonas* sp; (B) - *Pantoea dispersa*; and (C) *Enterobacter amnigenus*.

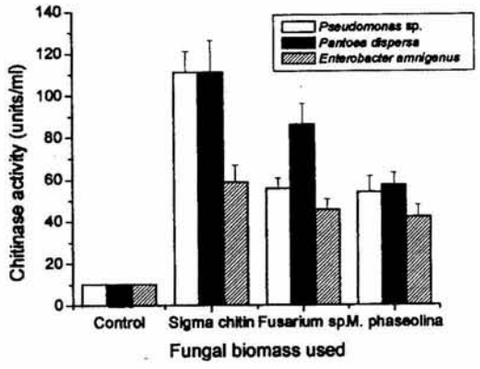

Fig. 3—Effect of replacement of chitin with dried fungal mycelial biomass of two fungal species on chitinase production by *Pseudomonas* sp, *Pantoea dispersa* and *Enterobacter amnigenus*.

Table 2—Activities of proteases of *Pseudomonas* sp. *Pantoea dispersa* and *Enterobacter amnigenus*.

[Values are mean±SD of 3 replications]

Proteases	Protease activity (units/ml)		
	Pseudomonas sp. at 144 hr	*Pantoea dispersa* at 144 hr	*Enterobacter amnigenus* at 120 hr
Acid protease	2.84±0.31	2.30±0.43	1.70±0.17
Neutral protease	7.16±0.21	8.36±0.6	9.36±0.21
Alkaline protease	5.87±0.45	4.61±0.34	5.37±0.15

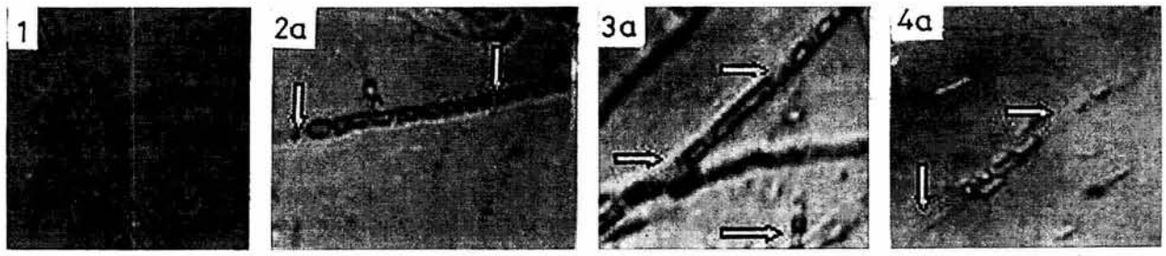

Fig. 4—Effect of chtinase from *Pseudomonas* sp *(2a), Pantoea dispersa (3a) and Enterobacter amnigenus (4a)* on growth of *Fusarium* sp. [(1) Untreated mycelium (magnification 400×); (2a) Treated mycelium using culture filtrate of *Pseudomonas* sp. showing degraded hyphae (arrow) 4hr (magnification 400×); (3a) Treated mycelium using culture filtrate of *Pantoea dispersa* showing degraded hyphae (arrow) 2hr (magnification 400×); and (4a) Treated mycelium using culture filtrate of *Enterobacter amnigenus* showing degraded hyphae (arrow) 3hr (magnification 400×)]

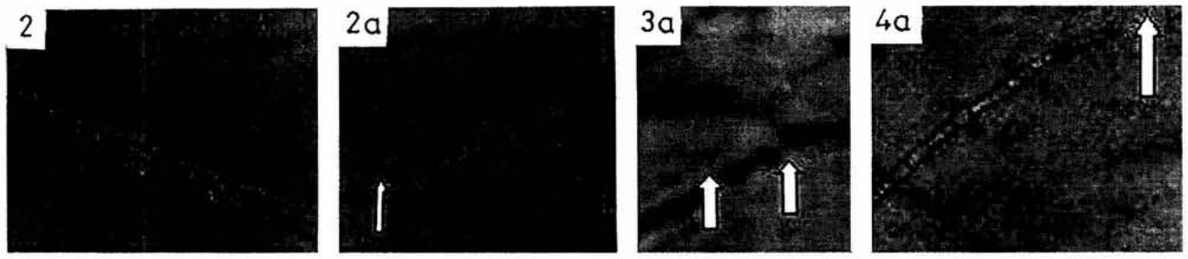

Fig. 5—Effect of chtinase from *Pseudomonas* sp *(2a), Pantoea dispersa (3a) and Enterobacter amnigenus (4a)* on growth of *M. phaseolina* [(2) Untreated mycelium (magnification 400×); (2a) Treated mycelium using culture filtrate of *Pseudomonas* sp. showing degraded hyphae (arrow) 6hr (magnification 400×); (3a) Treated mycelium using culture filtrate of *Pantoea dispersa* showing degraded hyphae (arrow) 3hr (magnification 400×); and (4a) Treated mycelium using culture filtrate of *Enterobacter amnigenus* showing degraded hyphae (arrow) 4hr (magnification 400×)]

pathogen, *S.rolfsii*, by chitinase produced by *S. marcescens.* Vyas and Deshpande[9] have reported the utilization of *Sclerotium rolfsii* mycelia for production of chitinase from *M. verrucaria.* Gupta *et al.*[18] have reported cell wall lysis of many fungal pathogens by chitinase produced by *S. viridificans.* Vaidya *et al.*[12] have reported chitinase production by *Alcaligenes xylosoxydans* by utilization of dead mycelia of *Fusarium* sp. and *M. phaseolina.*

Inhibition of the growth of fungal plant pathogens—In vitro studies were carried out to see the effect of culture and culture filtrate of *Pseudomonas* sp., *Pantoea dispersa* and *Enterobacter amnigenus* on the growth of *Fusarium* sp. and *M. phaseolina.* It was observed that there was 47 ± 1.4 and 82 ± 3.2%, 55 ± 2.1 and 93 ± 2.5%, 31 ± 1.05 and 68 ± 2% inhibition of the growth of *Fusarium* sp. in the presence of *Pseudomonas* sp., *Pantoea dispersa* and *Enterobacter amnigenus* culture and culture filtrate, respectively. For *M. phaseolina*, the inhibition in the growth was observed to be 38 ± 1 and 74 ± 1.5%, 60 ± 2.4 and 95 ± 1.6%, 42 ± 1 and 75 ± 3.2% by *Pseudomonas* sp., *P. dispersa* and *E. amnigenus* culture and culture filtrate, respectively. Vaidya *et al.*[12] have reported 44 and 74% inhibition of the growth of *Fusarium* sp. as well as 69 and 81% inhibition of growth of *M. phaseolina* in presence of *A. xylosoxydans* culture and culture filtrate, respectively.

Inhibition of fungal growth—As for fungal growth, the chitinase from *Pseudomonas* sp., *Pantoea dispersa* and *E. amnigenus* tested individually, showed inhibition of hyphal elongation. In general, the enzyme from *Pseudomonas* sp., *Pantoea dispersa* and *E. amnigenus* affected *Fusarium* sp. and *M. phaseolina.* Culture filtrate from each of these bacteria was found to inhibit growth of *Fusarium* sp., (Fig. 4) and *M. phaseolina* (Fig. 5). Chitinolytic enzymes from *Penicillium janthinellum* P9 cause mycelial damage in *Mucor plumbeus* and *Cladosporium cladosporioides*[19] after 24 hr. Purified chitinase from *Fusarium chlamydosporum* lyse the walls of uredospores and germ tubes of *Puccinia arachidis*[20] after 24 hr.

In summary, these results suggested that *Pseudomonas* sp., *P. dispersa* and *E. amnigenus* show the high level of chitinase production, proteases activity

and inhibition of the growth of fungal plant pathogens. These cultures, therefore, have potential to be exploited for use as an antifungal biocontrol agent.

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References

1 Amy L S, Sinead M C, Jessica A M & David L K, Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin, *Appl Environ Microbiol,* 63 (1997) 408.

2 Deshpande M V, Mycopesticide production by fermentation: Potential and challenges, *Crit Rev Microbiol,* 25 (1999) 229.

3 Stefan K & Hubert B, Biological control of fungal straberry diseases by *Serratia plymuthica* HRO-C48, *Plant Dis,* 85 (2001) 529.

4 Nene Y L & Sheila V K, Pigeonpea: Geography and importance, *in The Pigeonpea,* edited by Y L Nene, S D Hail and V K Sheila (CAB International, Oxon) 1990, 1[ISBN 0-85198-657-9].

5 Reddy M V, Sharama S B & Nene Y L, Pigeonpea: Disease management. *in The pigeonpea,* edited by Y L Nene, S D Hall, and V K Sheila (CAB International, Oxon) 1990, 303 [ISBN 0-85198-657-9].

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

7 Hackman R H, Studies on Chitin V: The action of mineral acids on chitin, *Aus J Biol Sci,* 15 (1962) 526.

8 Vaidya R J, Macmil S L A, Vyas P R & Chhatpar H S, The novel method for isolating chitinolytic bacteria and its application in screening for hyperchitinase producing mutant of *Alcaligenes xylosoxydans, Lett Appl Microbiol,* 36 (2003) 129.

9 Vyas P & Deshpande M V, Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation, *J Gen Appl Microbiol,* 35 (1989) 343.

10 Waterborg, J H & Matthews H R, The Lowry method for protein quantitation. *in The protein protocols handbook*, edited by J M Walker, Totowa (Humana Press, New Jersey) 1996, 7 [ISBN 0-89603-338-4].

11 Ong P S & Gaucher G M, Protease production by thermophilic fungi, *Can J Microbiol,* 19 (1973) 129.

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

13 Holt J G, Krieg N R, Sneath P H, Staley J T & Williams S T, International edition: *Bergey's manual of determinative bacteriology* 9th edition (Williams and Wilkins, Maryland) 1994, 184 [ISBN 0-683-00603-7].

14 Wiwat C, Siwayaprahm P & Bhumiratana A, Purification and characterization of chitinase from Bacillus circulans no 4.1, *Curr Microbiol,* 39 (1999) 134.

15 Ueno H, Miyashita K, Swada Y & Oba Y, Purification and some properties of extracellular chitinase from *Streptomyces* sp. S-84, *J Gen Appl Microbiol,* 36 (1990) 377.

16 Beyer M & Diekmann H, The chitinase system of Streptomyces sp. ATCC 11238 and its significance for fungal cell wall degradation, *Appl Microbiol Biotechnol,* 23 (1985) 140.

17 Ordentlich A, Elad Y & Chet I, The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii, Phytopathology*, 78 (1988) 84.

18 Gupta R, Saxena R K, Chaturvedi P & Virdi J S, Chitinase production by Streptomyces virdificans: Its potential in fungal cell wall lysis, *J Appl Bacteriol,* 78 (1995) 378.

19 Giambattista R D, Federici F, Petruccioli M & Fenice M, The chitinolytic activity of *Penicillium janthinellum* P9: Purification, partial characterization and potential applications, *J Appl Microbiol,* 91 (2001) 498.

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