RA Publications
Journal of Medical Science & Technology

Brief Communication Open Access

Production of chitinase by using A*cremonium sporosulcatum* from shrimp biowaste

B Vinusha [1], Kotlapudi Udai Kumar[2], N Mallikarjuna Rao [3], V Srihari [4], A Raveendra[5], M Prasad Naidu [6]
1 M.Sc biotechnology, S. V university, Andhra Pradesh, India
2 M.Sc Biochemistry Muthayamal College, Tamilnadu, India
3 M.Sc, Acharaya Nagarjuna Univerity, Andhra Pradesh, India
4 M.Sc biotechnology, Darvidian university, Andhra Pradesh, India
5 M.Sc Biochemistry, SV PG college, Andhra Pradesh, India
6 M.Sc Medical Biochemistry, Narayana Medical College, Nellore, Andhra Pradesh, India

Abstract:

Chitin is an essential structural component of the fungal cell wall and is present in the exoskeleton of arthropods and the microfilarial sheath of nematodes, acting as a protective layer against the harsh conditions that may be endured by the pathogen or arthropod, Mammals do not synthesize chitin, yet it is the second most abundant glycopolymer on earth, with an estimated 1010 tonnes of chitin produced each year. It was generally assumed that mammals lacked the ability to produce chitinase proteins, the enzymes responsible for chitin degradation. In the present study we aimed to produce chitinase enzyme by using A.*sporosulcatum* from shrimp biowaste. Chitinase activity was determined by a DiNitroSalicilic acid (DNS) method.

*Corresponding Author: B. Vinusha, M.Sc Biotechnology, S.V University, Tirupathi, India. Email: vinushabellamkonda@gmail.com

Received: October 10, 2012 Accepted: November 20, 2012. Published: January 20, 2013.

Introduction

Chitin, poly β-(1-4)-N-acetyl-D-glucosamine, is a natural polysaccharide of major importance, first identified in 1884. The importance of chitin was not realized for long time. The chitin is synthesized by enormous number of living organisms next to cellulose, chitin available abundantly in crystalline micro fibril form as a preventive mechanism. It has been as a part of exoskeleton of arthropods and in the cell walls of fungi and Yeast. It is also produced by a number of living organisms in the lower plant and animal kingdom, useful in many functions where reinforcement and strength are required.

Chitin occurs naturally as partially deacetylated form (with low content of glucosamine units) depending on its source; α and β chitins could not dissolved in usual solvents due to its distinguished crystalline nature. The insolubility is a major problem that confronts the development of processing and usage of chitin. Solid-state transformation of β chitin into α chitin occurs by treatment with strong aqueous HCL and washing with water. In addition, β form has found to be more reactive than the α form, a distinguished property of chitin with regard to enzymatic and chemical transformations.

Chitins are a large family of glycans that are β-1-4 linked, insoluble linear polymers of N-acetylglucosamine. They are present in the walls of higher fungi, exoskeleton of insects, arachnids,

invertebrates and as an extra cellular polymer of some bacteria. It is estimated that the rate of annual formation of chitin is on study state increase year after year. Bioaccumulation of non degradable chitin leads to great disaster in the world. Therefore, the application of thermo stable chitin-hydrolyzing enzymes (Chitinases) could be alternative for the effective utilization of this abundant biomass. In view of this, various chitinase genes and their phenotypic expressions from Eukaryotes and Bacteria have been well investigated (Patil. 1999).

Chitinases are digestive enzymes that break down glycosidic bonds in chitin.[1] Because chitin composes the cell walls of fungi and exoskeleton elements of some animals (including worms and arthropods), chitinases are generally found in organisms that either need to reshape their own chitin[2] or to dissolve and digest the chitin of fungi or animals. Chitinase is an extracellular enzyme which is capable in hydrolyzing insoluble chitin to its oligomeric and monomeric components. The enzyme produced by thermophilic bacteria was screened and isolated from Sulili hot spring in Pinrang, South Sulawesi, Indonesia. The gram positive spore forming rod shape bacteria was identified as Bacillus sp. HSA,3-1a through morphological and physiological analysis. The production of chitinase enzyme was conducted at various concentration of colloidal chitin at a pH of 7.0 and a temperature of 55 °C. The bacteria optimally was produced the enzyme at a colloidal chitin concentration of 0.5% after 72 h of incubation. The optimum temperature, pH and substrate concentration of chitinase were 60 °C, 7.0 and 0.3%, respectively. The enzyme was stable at a pH of 7.0 and a temperature of 60 °C after 2 h of incubation. The chitinase activities was increased by addition of 1 mM Mg^{2+}, Ca^{2+} and Mn^{2+} ions, whereas the activities were decreased by 1 mM Co^{2+}, Fe^{2+} and Zn^{2} ions. The molecular weight of the crude enzyme was determined using SDS-PAGE analysis. Five protein fractions were obtained from SDS-PAGE, with MWs of 79, 71, 48, 43 and 22 kDa Chitinivorous organisms include many bacteria (Aeromonads, Bacillus, Vibrio,[4] among others), which may be pathogenic or detrivorous. They may attack arthropods, zooplankton or fungi; or they may degrade the remains of these organisms.

Fungi, such as Coccidioides *immitis,* are known to possess chitinases. This may be related to their typical role as detrivores and also to their potential as arthropod pathogens.

Production of chitinase: Single-parameter optimization was carried out for the production of chitinase using a soil isolate belonging to '*Trichoderma harzianum'* in solid-state fermentation. Maximum chitinase activity (3.18 U/gds) was obtained after 96 h of incubation at 30 °C when wheat bran moistened (65.7%) with salt solution was supplemented with colloidal chitin (1%, w/w) and yeast extract (2%, w/w) used as the substrate. The inoculum contains 4×10^{7} spores of *T. harzianum* (TUBF 781). The growth of the fungus on wheat bran particle was visualized by a scanning electron microscope. Chitinase activity was measured as the amount of *N*-acetyl glucosamine (NAG) liberated in µmol/min under reaction conditions. The crude extract showed antifungal activity against a wide range of fungal strains belonging to *Aspergillus*, *Rhizopus* and *Mucor* sp., and was found significant against *Aspergillus niger.*

Objectives of the study

- Isolation of Microorganisms
- Collection of shrimp biowaste
- Optimization of sucrose
- Optimization of sodium nitrate
- Optimization of pH
- Optimization of temperature
- Optimization of different substrates

Materials and methods

Microorganism and inoculum preparation: A fungal isolate *Acremonium sporsulcatum* obtained from the HIB Nellore. This was used in the present study. The culture was maintained on nutrient agar medium and sub cultured every 30 days slants were prepared and incubated for two days at 30˚c and stored at 4˚c. The spores after fully sporulation, slants were dispersed in 10ml of 0.1% tween 80 solution by dislodging them with a sterile loop under aseptic conditions. The spore suspension obtained was used as inoculum viable spores present in the suspension were determined by serial dilution followed by plate count.

Shrimp biowaste: The waste was kindly provided by Shrimp care unit. The shrimp waste was washed with tap water then distilled water thereafter, exposed to water vapour and air dried at room temperature.

Chitinase assay: Chitinase activity was determined by a DiNitroSalicilic acid (DNS) method. This method works on the concentration of N-acetyl glucosamine which is released as a result of

enzymatic action. The 2ml reaction mixture contained 0.5ml of 0.5% colloidal chitin in phosphate buffer (pH-5.5), 0.5ml crude enzyme extract and 1ml distilled water. The well vortexed mixture was incubated in a orbital shaker at 50°C temperature for 1hr. The reaction was arrested by the addition of 3ml DNS reagent followed by heating at 100°C for 20mins. The absorption of colored solution was measured at 540nm using UV-spectrophotometer along with substrate and enzyme blanks. Colloidal chitin was prepared by the modified method of Robert's and Selitrenkoff. One unit of the chitinase activity is defined as the amount of enzyme that is required to release 1µM of N-acetyi D-glucosamine per minute from 0.5% of dry colloidal chitin solution under assay conditions.

Preparation of n-acetyl d glucosamine standard curve:

100mg of was N-Acetyl D Glucose amine weighed. It was made up with 100ml of distilled water. Different concentrations of N-acetyl D glucose amine standard solutions were taken in different test tubes that are 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1ml.

Preparation of dintro salicylic acid (DNS):

- Take 0.5 gm of DNS and chopped in a mortar pistle.
- And weigh 2 gm of NAOH, is dissolved in 50ml of distilled water.
- Then to chopped DNS add NAOH drop by drop, to avoid crystal like substances.
- And then weigh 30 gm of Sodium. Potassium tartarate and dissolved in 40 ml of distilled water.
- Then filter the DNS solution and add sodium potassium tartarate to it.make it 200ml with distilled water.

Composition of Czapec - dox medium (250 ml):

Sucrose	:7.5 grams
NaNO	:30.5 grams
MgSO4	:0.125 grams
Kcl	:0.125 grams
KH2PO4	:0.0875 grams
Fe So4	:0.0025 grams
Distilled water	:250 ml

After this procedure the following steps have been performed

1. Preparation of colloidal chitin
2. Preparation of standard curve
3. Optimization of carbon sources
4. Optimization of nitrogen sources
5. Optimization of ph for maximum production of chitinase
6. Optimization of temperature for maximum production of chitinase
7. Production of chitinase by using different substrates

Results:

A graph was plotted between N-acetyl glucose amine and OD values and a straight line was obtained (Table -1 & Figure 1). By using the standard estabished protocols colloidal chitin was prepared and it was stored at 4˚c for the usage in the experiments.

Chitinase was produced in czapecdox medium with *Acremonium sporosulcatum:* The maximum activity of chitinase was obtained after 72 hrs is 15.08µg at a concentration of 2000mgs (Table-2& Fig-2). The minimum activity of chitinase was obtained after 24 hrs is 0µg at a concentration of 2000mgs (Table-2& Fig-2).

The maximum activity of chitinase was obtained after 72 hrs is 12.43µg at a concentration of 50mg (Table-3& Fig-3). The minimum activity of chitinase was obtained after 48 hrs is 0.87µg at a concentration of 100mg (Table-3& Fig-3).

The maximum activity of chitinase was obtained after 72 hrs is 15.29µg at a pH 7 (Table-4& Fig-4). The minimum activity of chitinase was obtained after 24 hrs is 0.25µg at a pH 8 (Table-4& Fig-4).

The maximum activity of chitinase was obtained after 72 hrs is 10.04µg at room temperature (Table-5& Fig-5). The minimum activity of chitinase was obtained after 24 hrs is 0µg at 4˚c (Table-5& Fig-5).

The maximum activity of chitinase was obtained after 72 hrs is 15.02µg at a concentration of 1000mg from shrimp biowaste(Table-6&Fig-6). The minimum activity of chitinase was obtained after 24 hrs is 1.36µg at a concentration of 1000mg (Table-6& Fig-6).

Sl.No	Conc of N-acetyl glucosamine(ml)	Distilled water(ml)	DNS(ml)	OD VALUES AT 540nm
1	Blank	-	1	0.00
2	0.2	1.8	1	0.4
3	0.4	1.6	1	0.8
4	0.6	1.4	1	0.12
5	0.8	1.2	1	0.14
6	1.0	1.0	1	0.18

TABLE-1: Preparation of n-acetyl glucosamine standard curve

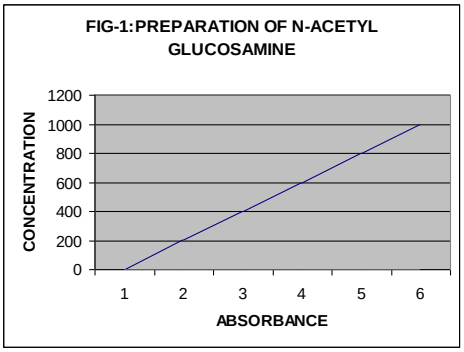


TABLE-2: Optimization of sucrose

S.no	Conc of sucrose(gm)	Activity for 24hrs (ug/ml/min)	Activity for 48 hrs (ug/ml/min)	Activity for 72 hrs (ug/ml/min)
1	Control	0.12	10.04	13.81
2	1.0	0.25	12.55	13.82
3	1.5	0.12	11.80	15.06
4	2.0	0.0	11.30	15.08
5	2.5	0.37	12.05	13.81

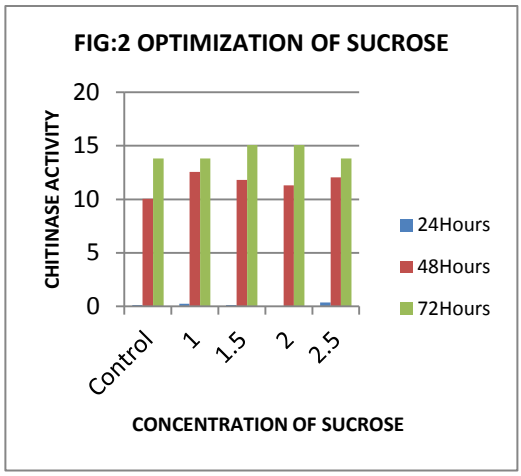
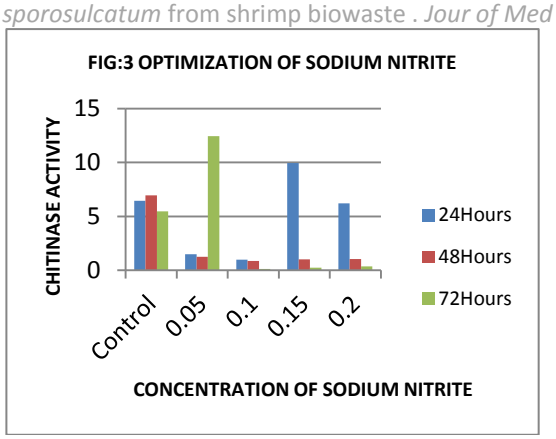


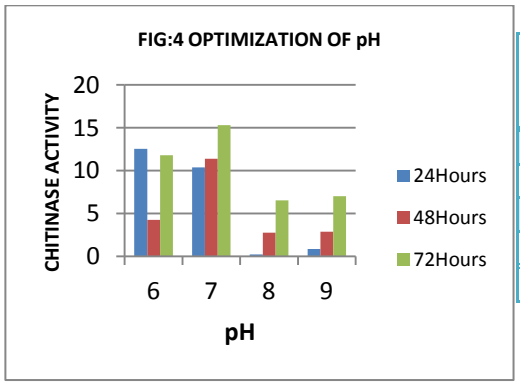
Table-3: Optimization of sodium nitrite

S.No	Conc of sodium nitrate(gm)	Activity for 24hrs (ug/ml/min)	Activity for 48hrs (ug/ml/min)	Activity for 72hrs (ug/ml/min)
1	Control	6.46	6.96	**5.47**
2	0.05	1.49	1.24	**12.43**
3	0.10	0.99	0.87	**0.12**
4	0.15	9.94	1.00	**0.25**
5	**0.20**	**6.21**	**1.13**	**0.37**



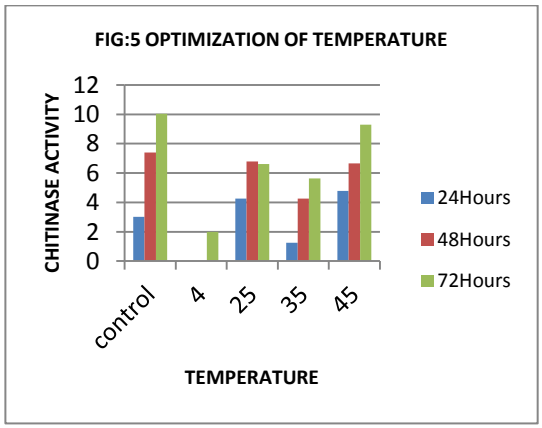
S.No	Different P^H	Activity for 24hrs (ug/ml/min)	Activity for 48hrs (ug/ml/min)	Activity for 72hrs (ug/ml/min)
1	Control	3.01	7.40	10.04
2	6	12.55	4.26	11.80
3	7	10.37	11.38	15.29
4	8	0.25	2.76	6.53
5	9	0.87	2.88	7.03

Table-4: Optimization of P^H



S.No	Different temperature	Activity for 24hrs (ug/ml/min)	Activity for 48hrs (ug/ml/min)	Activity for 72hrs (ug/ml/min)
1	Control	3.01	7.40	10.04
2	4	0.00	0.00	2.00
3	25	4.26	6.78	6.62
4	35	1.25	4.26	5.63
5	45	4.77	6.65	9.29

Table-5: Optimization of temperature



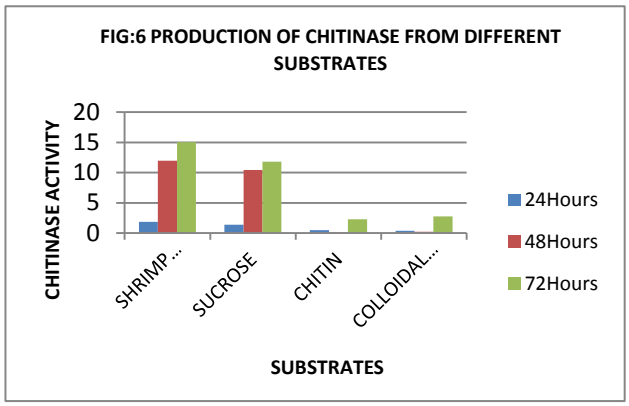


Table6: Production of chitinase using different substrates

S.No	Substrate	Activity for 24hrs (ug/ml/min)	Activity for 48 hrs (ug/ml/min)	Activity for 72 hrs (ug/ml/min)
1	Shrimp biowastes	1.86	11.98	15.02
2	Sucrose	1.36	10.44	11.80
3	Chitin	0.50	0.12	2.26
4	Colloidal chitin	0.37	0.24	2.76

Discussion:

Chitin is one of the most abundant biopolymers widely distributed in the marine and terrestrial environments. The role of the chitinolytic enzymes in nature is to degrade the huge quantities of chitin for recycling. Renewed commercial interest in the production and utilization of chitin and chitinolytic enzymes has demonstrated the need for inexpensive reliable sources of stable chitinase and chitobiase. Presently, commercially available purified and semipurified chitinases are obtained from microorganisms at a high cost, yet of unreliable specific activity. It is imperative to increase the supply of active chitinase while reducing the cost of production. This challenge may be met by extracting both chitinase and chitobiase from low cost readily available nonmicrobial sources (such as soybean seeds), and by recombinant DNA technology to develop chitinase/chitobiase - overproducing microbial strains. Furthermore, this paper will consider the important role of chitinases for the biological control of soil-borne plant pathogenic pests as well as biomass recovery from shellfish and cheese manufacturing industries.

It was generally assumed that mammals lacked the ability to produce chitinase proteins, the enzymes responsible for chitin degradation. However, recent findings have not only demonstrated that mammals produce chitinases, but also that increased secretion of chitinases is closely associated with T-helper type 2 (Th2)-dominated pathophysiological conditions including infection, fibrosis, allergy and asthma. Chitinases belong to the glycoside hydrolase family 18, which also encompasses enzymatically inactive chitinase like proteins (CLPs). Only the true chitinases have a functioning catalytic domain, which facilitates the hydrolysis of glycosidic bonds, resulting in chitin degradation.

While the role of chitinases and CLPs in settings of human allergic inflammation and other pathologies have only recently been highlighted, more information is available on the function and evolution of chitinases in other organisms. This review gives a broad perspective of the effects and functions of chitinases and CLPs in the context of their association with allergy and asthma.

Chitinases are digestive enzymes that break down glycosidic bonds in chitin. Because chitin composes the cell walls of fungi and exoskeletal elements of some animals (including worms and arthropods), chitinases are generally found in organisms that either need to reshape their own chitin[2] or to dissolve and digest the chitin of fungi or animals. Chitinase is an extracellular enzyme which is capable in hydrolyzing insoluble chitin to its oligomeric and monomeric components.

Chitinase and chitin deacetylase are enzymes capable of degrading chitin into chitin oligomers and chitosan. The chitinases characterized and purified in this study were extracted from *Acromonium s*p. When grown in media containing colloidal chitin. The optimum chitinase activity of *Acremonium* was reached after 2-3 days of incubation. The optimium temperature and pH of the chitinase was found at 37°C and 7 and shrimp biowaste as substrate.

Conclusion:

Crustacean shells constitute the traditional and current commercial source of chitin. Conversely, the control of fungal fermentation processes to produce quality chitinase makes fungal mycelia an attractive alternative source. Therefore, the exploitation of both of these sources to produce chitinase in a concurrent process should be advantageous and is reported here. *Acremonium sporsuicatum* was selected from a screening for chitinase activity. The concurrent production of chitinase from shrimp shells and fungi by placing shrimp shells in direct contact with the fermentation of filamentous fungi was studied. The maximum production of chitinase was observed at room temperature and at pH- 7 by taking shrimp biowaste as substrate. Chitinase is having wide industrial applications. Particulary in the protease and other food industries use chitinase in vaste amounts. To meet the requiremnts an attempt to produce chitinase was tried in the present work.

Chitinase was produced by optimizing different carbon and nitrogen sources. Apart from the above sources chitinase was also produced using shrimp biowaste. The results have shown that chitnase can be effectively produced by using shrimpbiowaste using *Acremonium sporosulcatum.*

References:

1. Vannini, C.Caruso, L.Leonordi, E.Rugini, E.Chiarot, 1999, Antifungal properties of chitinases from Castanea sativa against Hypoviruleut & Virulent strains of the Chestnut

blight fungus Cryphonectria parasitica, Physiological & Molecular plant pathology, 55, 29-35.
2. A.Ippolito and F. Nigro 2000. Impact of pre-harvest application of biological control on post-harvest diseases offresh fruits and vegetables. Crop protection 19; 715-723
3. A.Kapat, Sk.Rakshit, T.Panda 1996. Optimization of carbon and nitrogen sources in medium and environmental conditions for enhanced production of chitinase by Trichoderma harzianum. Bioprocess Eng; 15:13-20
4. A.W. Cooke and K.K. Jacobi Control of post harvest diseases of mangoes, 2000, Available from http : // www.tropical –seeds. Com / tech _ forum / fruits _ anon / mango _ ph_diseases.html.
5. Ace M .Baty III, Amanda E.Goodman, Gill G.Geesey, Callie C.Eastburn, Somkiet etal; 2000, Differentiation of chitinase-Active and non-chitinase, Applied and environmental Microbiology, 66,3566-3573.
6. Akhihiro Saito, Masumi Ishizaka, Perigio francisco, Takeshi fujii, K.Yolaka M.Yoshita, 2000, Transciptional co-regulation of five chitinase genes scattered on the streptomyces welicolr A3 (2) chromosome, Microbiology, 146.
7. B.Gerhardson, 2002 Biological substitutes for pesticides. Trends in Biotechnology 8: 338-343
8. B.Henrissat and A.Bairoch, 1996. Updating the sequence-based classificatioin of glycosyl hydrolases. J. Biochem. 316, 659-696
9. B.Henrissat, 1991. A classification of glycosyl hydrolases based on amino acide sequence similarities. Biochem. J., 280, 309-316
10. B.Henrissat, and A.Bairoch, 1993. New families in the classificatioin of glycosyl hydrolases based on amino acid sequence similarities. J .Biochem. 293, 781-788
11. Brinda Mahadevan and Don.L.Crawford, 1997, Properties of the chitinase of the antifungal biocontrol agent streptomyces lydicus, Enzyme & Microbial Technology, 20, p489-493.
12. Jai and J.Kuc, 1996, Antifungal activity of cucumber b-1, 3-Glucanase & chitinase, Physiology & molecular plant pathology, 49, 257-265.
13. C.L.Wilson and M.E.Wisniewski 1994. Biological control of post harvest diseases theory and practice, CRC Press Inc., London.
14. Chandrashekar, R and Mehta, K. 2000. Transglutaminase catalyzed reactions in the growth, maturation and development of parasitic nematodes. Parasitol Today, 16, 11-17.
15. CJ.Ulhoa, JF Peberdy 1991 Regulation of Chitanase synthesis in Trichoderma harzianum.J Gen Micro Biology 14:2163 – 69
16. D.Spadaro and M.L. Gullino 2003. State of the art and future prospects of biological control of post harvest fruit diseases. International Journal of Food Microbiology 24:1-10
17. Diazo Koga, Yuji sasaki, Youichiro uchiumi, Nobuya Hirai, Yasuyuri Arakane, Yasuyuri Nagamatsu, 1997, Purification & Characterization of Bombyx mori Chitinases, Insect biochem and Molecular biology, 27 ; pages 757-767.
18. E.A.B.Emmert and J. Handelsman 1999 Biocontrol of plant disease a Gram positive Federation of European Microbiological Societies Letters171; 1-9
19. E.A.B.Emmert, A.K.Klimowiez, M.G.Thomas and J. Handelsman 2004 Genetics of Zwittermiein a produced by Bacillus cereus, Applied and Environmental Microbiology 70:104-113.
20. E.E.De Villiers and L. Korsten. 1994. Biological treatments for the control of mango post harvest diseases. South African Mango Growers Association Year book 14; 48-51.
21. E.Konbrink, I.E.Sommssieh – 1995. Defence responses of plants to pathogens, adv. Botany Research, 21:2-34.
22. Elisane longhinotti, Fabiola Pozza, Ligia Furlan etal, 1997, Adsorption of Anionic Dyes on the Biopolymer chitin, J.Braz, chem.Soc.Vol.No:9, 5, 435-440.
23. Emilio Benitez, Raquel Melgar, Rogelio Nogales, 2004, Estimating soil resilience to a toxic organic waste, by measuring enzyme activities, soil biology & biochemistry, 36; 1615-1623.
24. ES Mendonsa, PH Vartak, JU Rao, MV Dehpande , 1996, An enzyme from Myrothecium verrucaria that degrades insect cuticles for bio control of Aedes aegypti mosquito. Biotechnol. Lettt. 18: 373:376
25. F.Cladera Olivera, G.R.Caron and A.Brandelli 2003. Bacteriocin like substance production by Bacillus licheniformis strain P40. Letters in Applied Microbiology 38:251-256
26. G.N.Agrios 1997. Plant pathology, Singapore: Harcourt Asia Pvt Ltd. ISBN 981-4033-820.
27. H.Deising, T.Siegrist 1995; Chitin deacetylase activity of the rust uromyces viciae-fabae is controlled by fungal morphogenesis. FEMS microbial letters; 127:207-12.

28. HS.Kelkar, V.Shankar, MV.Deshpande.1990. Rapid isolation and regeneration of *Sclerotium rolfsii* protoplasts and their potential application for starch hydrolysis. Enzyme Microb Technol; 12: 510-4.
29. IA. Stoyachenko, VP. Varlamov, VA. Davankov.1994. Chitinases of *streptomyces kurssanovii*: Purification and some properties. Carbohyd Polym; 24: 47-54.
30. J.Cleveland, T.J.Montville, I.F.Nes and M.L. Chikindas 2001 Bactericins safe, natural antimicrobials for food preservation. International Journal of Food Microbiology 71: 1-20
31. J.H.Lonsdale 1993. Strategies for the control of post harvest diseases of mango South African Mango Groweres Association Year Book 13:109-115
32. J.P.Metraux, L.Streit & Th.Staub, 1987. A Pathogenesis related protein in cucumber is a chitinase, physiology & molecular plant pathology, 33, 1-9.
33. Jaroslav smr, 2000. Modified test for Chitinase and cellulase activity in Soil mites, Pedobiologia 44, 186-189.
34. John M.Pembeston, Stephen P.Kidd, Radonies schmidt, 1997. Secreted enzymes of Aeromonas, FEMS Microbiology letters, 152, 1-10.
35. K.Heungens and J.L. Parke 2001. Postinfection biological control of oomycete pathogens of pea by Burkhlderia cepacia Ammdri Phytopathology 91:383-391.
36. M.Rinaudo, 2006, chitin & chitosan : properties & applications, science Direct, 31, 603-632.
37. Muzzarelli, R.A. A., 1996, Chitosan-Based dietary foods. Carbohydr Polym; 29: 309-16.
38. Naoki Takaya, Dausuke Yamazak, et al; 1998, Intra cellular chitinase gene from Rhizopus molecular cloning & characterization, Microbiology, 144.
39. Neetu Dahiya, Gurinder singh Hoondal, Rupinder Tewari, Ram P.Tiwari, 2005., Chitinase from Enterobacter sp. NRGL, Its Purification, Characterization and Reaction pattern, Electronic Journal of Biotechnology, 8, 3450-3458
40. Patil RS, Ghormade V, Deshpande MV. 2000. Chitinolytic enzymes: an exploration.Microbial Technol. 26: 473-83.
41. Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A.B., 1994. Crystal structure of a bacterial chitinase at 2.3^{o}A resolution. Structure 2, 1169-1180.
42. Perrakis, A., Wilson,K S., Chet, I, Oppenheim, A.B.,1993. Phylogenetic relationships of chitinases . IN Chitin Enzymology. European chitin society.217-232.
43. Quoqingxia, chunshang Jin, Juzhou, shonjun yang, 2001, A noval chitinase having a unique mode of action from aspergillus fumigatus Y J-407, Microbiology, 268.
44. R.E.Camphell, 1989. Biological control of microbial plant patnogens Cambridge University Press London.
45. RA.Laine, WCJ. Lo, 1998. Diagnosis of fungal infections with a chitinase. PCT Int Appl WO 9802742 AI 22, 128: 861-84.
46. Reetarani S. Patil, Vandana Ghormade, Mukund V. Devi ; 1999, Chitinolytic Enzymes: An Exploration, Enzyme and Microbial Technology, 26,473-483.
47. Rochel cohen-Kupiec and ilan chet, 1998, The molecular biology of chitin digestion, Environmental biotechnology, Vol:9, 270-277.
48. Rossl Tellam, Gene Wijttele, peter Willadren, 1998, Petritrophic matrix proteins, Insect Biochemistry & Mol.Biology, 29;87-101
49. S.Orme and S. Kegley 2002. PAN Pesticide Database Pesticide Registration status. Available from http://preview.pesticideinfo.org.
50. T.B. Ng, 2004, Antifungal protiens & peptides of leguminous & non leguninous origins, peptides, 25, 1215-1222.
51. V.A.Edwards, 2001 Selecting a bacterial formula to compete with Vibrio species availablefrom http://www.alken-murray.com/Vibrio.html.
52. W.J.Janisiewicz and L.Korsten 2002. Biological control of post harvest diseases of fruit Annual Review of Phytopathology 40: 411-441.
53. Y.A. Batta, 2004 Post harvest biological control of apple gray mold by Trichoderma harzianum Rifai formulated in an invert emulsion. Crop protection 23:19-26
54. Y.Elad,2000, Biological control of foliar pathogens by means of T.harzianum & potential modes of action, Crop Protection, 19, pages 709-714.
55. Y.M.Jiang, Zhu X.R. and Y.B. Ali 2001. Post harvest control of litchi fruit rot by Bacillus subitlis, Lebensmittel Wissenschaft and Technologies 34: 430-436.

