



# PRODUCTION, OPTIMIZATION AND CHARACTERIZATION OF CHITINASE ENZYME BY *BACILLUS SUBTILIS*

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## ABSTRACT

Chitinases are digestive enzymes that breakdown glycosidic bonds in chitin. Chitin, a linear  $\beta$ -1,4-N-acetylglucosamine (GlcNAc) polysaccharide, is the most abundant renewable natural resource after cellulose. In the present study, an industrial enzyme chitinase produced by *Bacillus subtilis* was optimized and characterized. The bacteria *Bacillus subtilis* was isolated from soil sample. The production of chitinase by *Bacillus subtilis* was optimized under different range of pH, temperature and different incubation time. The maximum chitinase production was observed in Nutrient Broth (NB) amended with 1% colloidal chitin at pH 7.0 and temperature 35°C after 72 h of incubation. Along with optimization, influence of additional carbon and nitrogen sources on chitinase production revealed that chitin and yeast extract served as good carbon and nitrogen sources to enhance chitinase production. The enzyme was characterized under substrates specificity, various range of pH, temperature and different incubation time. Maximum chitinolytic activity was observed with chitin as best substrate at pH-7.0 and temperature 35°C after 1h of incubation.

**Key Words :** Chitinase, Chitin, *Bacillus subtilis*..

Enzyme technology is an interdisciplinary field, and enzymes are routinely used in many environment- friendly industrial sectors. With the advancement in biotechnology especially in the area of genetics, protein engineering, developments in bioinformatics, and the availability of sequence data have opened a new era of enzyme applications in many industrial processes. A number of organisms have been reported to produce chitinase. Chitinase producing organisms (i.e. Bacteria) have been isolated from a number of sources such as air, water, soil, marine water etc. (Wang *et al*; 2010). Chitinase is one of the most important mycolytic enzyme with industrial significance. Chitinase are hydrolytic enzymes that breakdown glycosidic bonds in chitin to its monomer N-acetyl glucosamine. As chitin is a component of cell walls of fungi exoskeletal elements of some animals (including worms and arthropods). Chitinase, a group of enzymes capable of degrading chitin directly to low molecular weight product and found in broad range of organisms including bacteria (*Bacillus*, *Aeromonas*, *Vibrio*, *Pseudomonas*, *Serratia*, *Enterobacter*, *Actinomyces*), fungi (*Trichoderma*,

*Aspergillus*), and higher plants, insects, crustaceans, invertebrates and some vertebrates.

Nevertheless, Chitinases have been isolated from the stomachs of certain mammals including humans. Although, mammals do not produce chitin, they have two functional chitinases; chitoriosidase – CHT1 and acidic mammalian chitinase – AMCCase that have high sequence similarity but lack chitinase activity. Bacteria produce a huge amount of chitinase e.g. *Stenotrophomonas maltophilia* (Khan *et al*; 2010), *Aeromonas hydrophillus strain SUWA-9*, (Xiqian Lan *et al*; 2010), *E.coli* (Ryoji Mizuno *et al*; 2008), *Paenibacillus spp. D1* (Kyoung-Ja Kim, *et al*; 2003), *Streptomyces spp. M-20*, *Streptomyces cyaneus spp-27* (Shigekaju Yano *et al*; 2008) *Aeromonas*, *Bacillus*, *Vibrio spp.* Fungi such as *Coccidioides immitis* are known to posses chitinase. Chitinase is also bifunctional, the medium composition and the character of crude chitinsae from *Bacillus spp.* excrete chitinase and  $\beta$ -1,3 glucanases to digest the hyphae of various fungi to provide a carbon sources, and the products of digestion are used as energy source (Goody, 1990). Almost all of the chitinase

producing *Bacillus spp.* use chitin derivatives as a major carbon sources for chitinase production (Wang *et al.*; 2009).

Chitin is a modified polysaccharide that contains nitrogen related chemical to cellulose that forms a semi-transparent horny substances. Chitin (C<sub>8</sub>H<sub>13</sub>O<sub>5</sub>N)<sub>n</sub>, the (1-4)- $\beta$ -linked homopolymer of N-acetyl-D-glucosamine, is produced in enormous amounts in the biosphere. It is the most abundant biopolymer next to cellulose with an annual production of 1010 to 10 11 tons per annum. Therefore, chitin may be described as cellulose with one hydroxyl group on each monomer substituted with an acetyl amine group. This allows for increased hydrogen bonding between adjacent polymers, giving the chitin polymer matrix increased strength. (Campbell *et al.*; 1996).

Microbial chitinases attracted the attention as one of the potential enzyme for applications in agriculture, pharmaceutical, waste management, biotechnology and industry. Chitinases are reported to play a protective role against fungal pathogens (Boller, 1985). Use of chitinolytic microorganism take care of waste disposal. Chitinase play an important role in nutrition and parasitism (Flach *et al.*; 1992 ). Since the excessive use of chemical, pesticide has caused serious environmental problems. Chitinase is employed for their use as a biocontrol agent (Lorito *et al.*; 1993, Inbar and Chet *et al.*; 1991). Therefore, Chitinases as a biocontrol agents against insects pest, *Monochamus alternates*, a pine sawyer beetle which is one of the important factors causing pine tree destructive in Japan. Chitinases have many industrial and agriculture application. The ability chitinase to make chitin variable in the pest control and pollution amendment. They also have a critical role in fungal protoplast (Kelkar , 1990). Other applications of chitinase are bioconversions of chitin waste to single cell proteins, ethanol and fertilizers. Industrial applications of chitinase have been governed mainly by key factors such as cost production, shelf-life stabilities and improvement in enzyme properties by immobilization. In the present study , an industrial enzyme chitinase produced by *Bacillus subtilis* was produced, optimized and characterized.

## MATERIALS AND METHODS

### Sample collection :

Soil sample was collected from the agricultural field. The soil samples were taken from 2 to 3 cm depth with the help of sterile spatula and put in a sterile plastic bags and brought to laboratory for further processing.

### Isolation and identification of microorganism :

Pour plate techniques was used for the isolation of *Bacillus subtilis*. Well grown bacterial colonies were picked and further purified by streaking. The isolated strains were maintained on Nutrient Agar(NA) slants and stored at 4°C. Identification of the bacterial isolates were carried out by

routine bacteriological methods( i.e. colony morphology, preliminary tests like gram staining and endospore staining).

### Preparation of colloidal chitin

Flakes of chitin (10g) was added to the 150mL conc. HCl and kept over night at 4°C then 1000ml of ice cold ethanol was added to it and kept over night at room temperature (28°C). Next day it was centrifuged at 5000 rpm for 20 minutes. Pellets thus formed were washed with sodium phosphate buffer three times.

### Screening for chitin degradation :

Microorganisms isolated from the soil were inoculated on to Chitin Agar Plates (1% chitin) followed by incubation at 30°C for 7 days. Plates were examined for formation of clear zone (CZ). On the basis of CZ formation *Bacillus subtilis* was selected as chitinase producing bacteria.

### Analytical methods

#### Determination of chitinase activity :

DNS Method was used for determination of chitinase activity and N-acetylglucosamine (NAG) concentration. It was capable in detecting carbonyl group of reducing sugar (Miller, 1959). The reducing sugar can be detected by their colour intensities by using spectrophotometer at wavelength 540 nm .

#### Estimation of protein concentration :

Lowry Method was used for the determination of protein concentration (Lowry *et al.*; 2011).

#### Enzyme assay

Extracellular chitinase activity was determined by the method given by (Reissig *et al.*; 1995) using colloidal chitin as a substrate. Extracellular chitinase activity was determined by incubating 1 ml of culture suspension ( filtrate ) with 1 ml of 1 % colloidal chitin in 0.05 M phosphate buffer ( pH-7 ). The mixture was incubated at 35°C for 1 hour. Reaction mixture was centrifuged at 10000 rpm for 15 minutes. An aliquot of 1.5 ml of supernatant and 1.5 ml DiNitro Salicylic Acid (DNS) were mixed and heated at 100°C for 15 minutes. The absorbance of the reaction mixture was measured at 540 nm after cooling to room temperature against the blank prepared with distilled water in the absence of enzyme. One enzyme unit was defined as the amount of enzyme that produces 1  $\mu$ m reducing sugars/ under the reaction.

#### Optimization of physical parameters for chitinase production

The optimization of chitinase production by *B. subtilis* was carried out under the following physical parameters, they are (a) incubation time (24-120 h), (b) initial culture pH (4.0-8.0) and (c) incubation temperature (25-45°C). After incubation period, the culture broth was centrifuged at 10000 rpm for 20

min. at 4°C. Thus, obtained cell free extract was used as extracellular chitinase (crude enzyme extract) and subjected for the estimation of enzyme activity.

### Optimization of media ingredients for chitinase production

#### Effect of different carbon sources :

Carbon sources (chitin, glucose, fructose, maltose, sucrose and starch) were examined for optimum chitinase production in CYS media. All the flasks containing different carbon source (5 g/L) were incubated at 35±2°C for 0-3 days and analyzed every day as mentioned above. The CYS medium with chitin as carbon source was treated as control.

#### Effect of different nitrogen sources :

Nitrogen sources (yeast extract, NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NaNO<sub>3</sub>, KNO<sub>3</sub>) were examined for optimum chitinase production in CYS media. Similarly, various nitrogen sources (0.5 g/L) yeast extract, NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NaNO<sub>3</sub>, KNO<sub>3</sub> were also examined for optimum chitinase production. CYS medium with yeast extract as nitrogen sources was treated as control.

#### Substrate specificity for chitinase activity :

The chitinase was incubated with various substrates viz. colloidal chitin, CMC {Carboxymethyl Cellulose}, soluble starch (1% w/v) in 0.05M phosphate buffer, pH-7.0. The flasks were incubated at 35°C for 1h and chitinolytic activity was observed in spectrophotometer at A540 nm.

#### Effect of pH on enzyme activity

The optimal pHs for chitinase activity and stability of the chitinase were examined at different pH values using colloidal chitin as substrate. Buffers used were as follows (0.1M): acetate buffer (3.0-5.5), sodium citrate (6.0), phosphate buffer (7.0) and tris-HCl (7.5-9.0). The flasks were incubated at 35°C for 1h and chitinolytic activity was observed in spectrophotometer at A540 nm.

#### Determining the optimum temperature for enzyme activity

Enzyme temperature profiling was done to determine the temperature parameters under which the enzyme could function optimally. The temperature profile would serve to indicate the possible effect of temperature on the chitinase activity. For determining the temperature effect; chitinase enzyme, colloidal chitin as substrate and phosphate buffer were mixed properly. The reaction mixture was kept at different temperature i.e. 30°C, 35°C, 40°C, 50°C, 60°C. The remaining step of assay was followed by standard chitinase assay using DNS method.

#### Effect of different incubation time on enzyme activity:

Under optimized condition, chitinase production was

determined by incubating flasks under different incubation period (0 min., 20 min., 40 min., 60 min., 80 min. and 100 min.). Standard chitinase assay was carried out to observe chitinolytic activity in spectrophotometer at A540 nm.

## RESULTS AND DISCUSSION

### Isolation and Identification :

Preliminary morphological examinations were done in the laboratory. On the basis of morphological characters, the isolates identified were *Bacillus spp.* and *Pseudomonas spp.* These strain were further sent for confirmation to IMTECH, Chandigarh. Results sent by them confirm that the strains were *Bacillus subtilis* and *Pseudomonas aeruginosa*. The strain *Bacillus subtilis* was selected for further study to see the effect of different factors on chitinase production.

### Effect of Physical parameters on enzyme production :

The influence of pH, temperature and incubation period on chitinase production by the strain is depicted in Fig.-1, 2, 3, 4 and 5. The enzyme production varies as the pH of medium changes. A high level of chitinase activity (0.0063 nm/min./mL) was noticed in culture with pH-7, Temperature 35°C and at 72 hrs of incubation period. After 96 hrs of incubation period no significant difference was noticed in chitinolytic activity. The activity reduced when the pH of media adjusted below and above pH-7. At 45°C no enzyme activity was noticed. The activity achieved at 25°C and 30°C was lower than the optimum temperature. Temperature and pH affect biological process through several mechanisms including enzyme induction or suppression, protein denaturation and altering cell viability (Nampoothiri *et al*; 2004).

### Influence of Carbon sources on chitinase production :

The influence of additional carbon source on chitinase

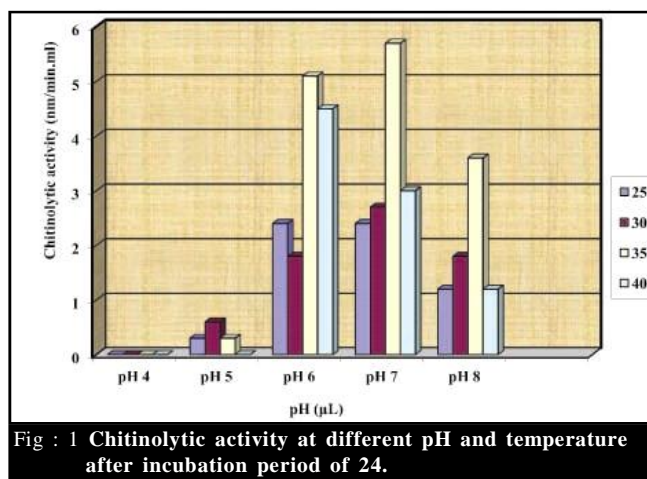


Fig : 1 Chitinolytic activity at different pH and temperature after incubation period of 24.

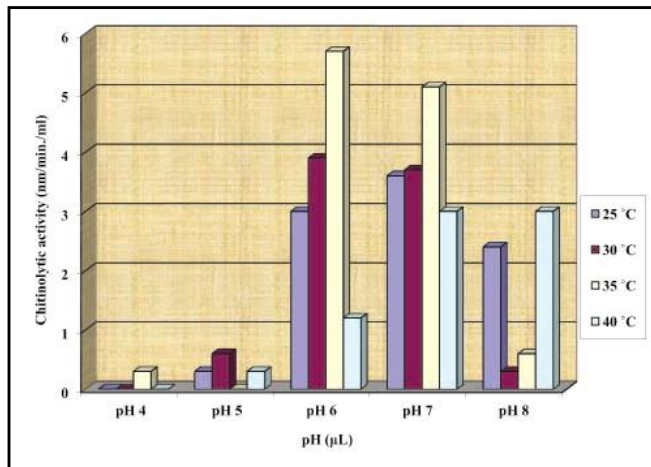


Fig 2 : Chitinolytic activity at different pH and temperature after incubation period of 48.

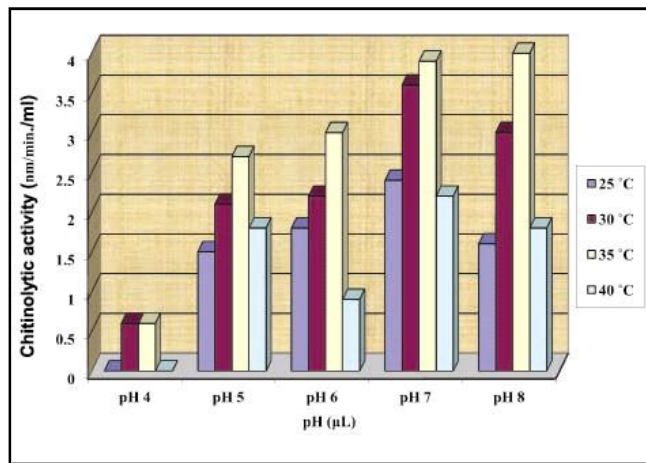


Fig 4 : Chitinolytic activity at different pH and temperature after incubation period of 96 h.

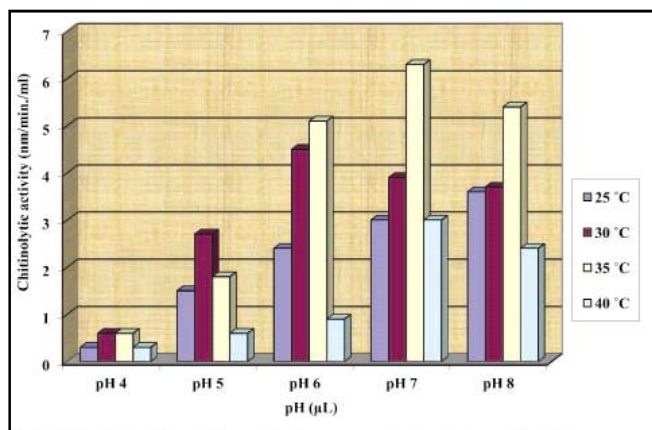


Fig 3 : Chitinolytic activity at different pH and temperature after incubation period of 72 h.

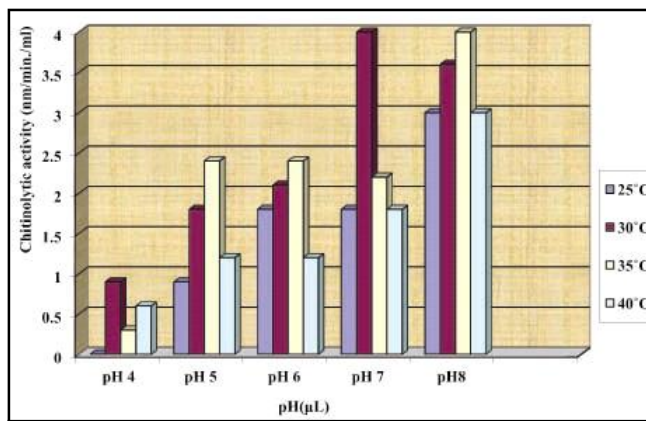


Fig 5 : Chitinolytic activity at different pH and temperature after incubation period of 120 h.

production was studied by supplying different carbon compound to CYS broth. The data on the effect on the several carbon source on enzyme production are presented in Fig.-6. Various carbon sources including (chitin, glucose, fructose, maltose, sucrose, starch) were examined at the concentration of 5g/L. The optimum conditions of pH, temperature and incubation period were maintained. The experiment was conducted in presence and absence of chitin. The experimental result implies that presence of chitin increase the production of chitinase where as absence of chitin does not improve the final reaction production. These experimental result were confirmed with the result produced by Vaidya *et al* (2001). From the result of different carbon sources it was noticed that chitin (5.7nm/min./mL) alone act as inducer and is the best carbon sources to improve the chitinase production. However, a very low activity (2.8 nm/min./mL) was noticed with culture grown in Glucose.

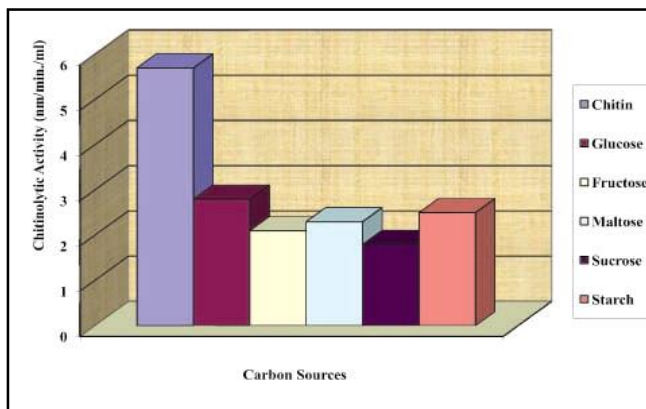


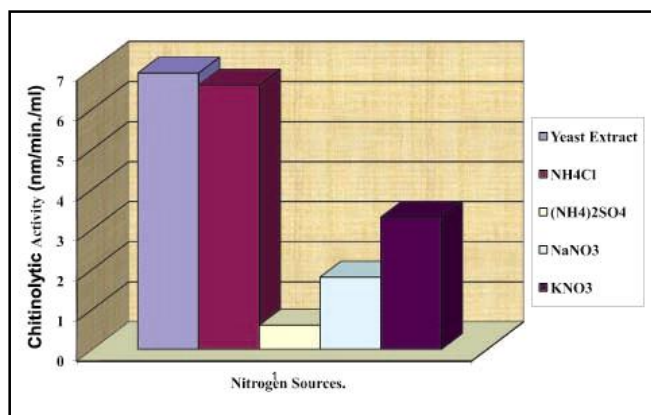
Fig 6 : Influence of various carbon sources on the production of chitinase enzyme

**Influence of Nitrogen sources on chitinase production :**

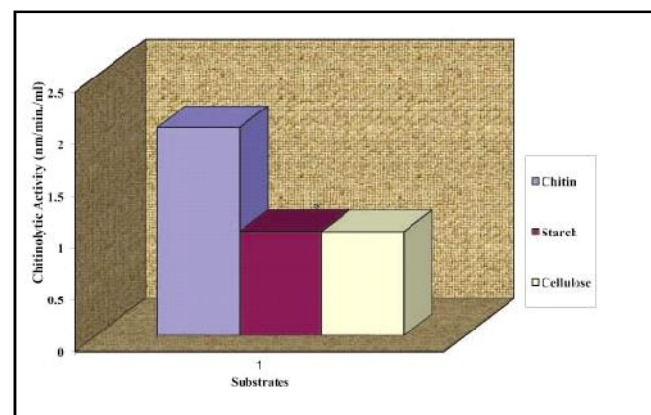
For nitrogen sources the medium was supplemented with yeast, NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NaNO<sub>3</sub> and KNO<sub>3</sub> in equivalent concentration (0.5 g/L) to the production media (CYS media). Among the different nitrogen sources involved in the reaction, the experimental result presented in Fig.-7, have shown that yeast extract (6.9 nm/min./mL) has the significant increase order than the other sources added to the medium. The optimum value of nitrogen sources of the media for chitinase production was 0.5g/L. Addition of yeast extract to media has been reported to enhance enzyme production in *Serratia marcescens* (Monreal Reese, 1996) and *Aspergillus carneus* (Sherief *et al*; 1991). The Ammonium chloride (6.6 nm/min./mL) and Potassium Nitrate (3.3 nm/min./mL) also give better response next to the above mentioned nitrogen sources.

**Effect of substrate specificity on the chitinase enzyme:**

The purified chitinase enzyme was incubated separately with different substrates using standard assay methods.



**Fig 7 : Influence of Nitrogen carbon sources on the production of chitinase enzyme**



**Fig 8 : Effect of Various Substrates on the production of chitinase enzyme**

Following incubation, the release of N-acetylglucosamine was measured. The relative activity was calculated using substrate blank as control. The influence of different substrates such as colloidal chitin, starch, carboxymethyl cellulose (CMC) on chitinase enzyme by the strain as depicted in Fig.-8, shows the results of hydrolysis of various substrates with the enzyme. The enzyme hydrolyze the colloidal chitin at high level but did not hydrolyze starch and cellulose upto the level, (Shon *et al*; 2001).

**Influence of different incubation time period on Chitinase enzymes :**

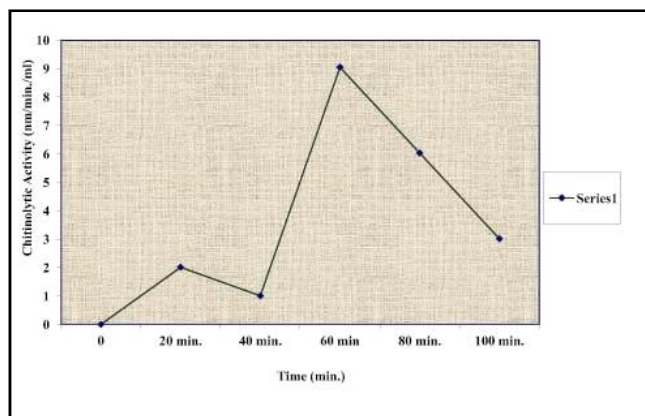
Fig.-9 shows that the highest chitinase activity (9.045 nm/min./mL) was observed at 60 min. incubation period. When the incubation period reduced (0 to 40 min.), the chitinase enzyme became inactive. Similarly, when the incubation period increased (80 to 100 min.) the chitinase enzyme exhibited the lower activity than that on 60 min. of incubation period.

**Effect of different temperature on Chitinase enzymes:**

The chitinase activity was most active at 35°C. Above 40°C, the activity decreased and was lost. The chitinase retained more than 75% activity at 50°C, and nearly 50% activity at 60°C after 1 hour, Fig.-10. A high level of chitinase activity (5.025 nm/min./mL) was observed at temperature 35°C.

**Effect of different pH activity on chitinase enzyme :**

The optimal pHs for chitinase activity was evaluated by incubating the enzyme solution for 1 hour at different pH values using colloidal chitin as substrate. Enzyme was most active between pH 7.0 - 8.0. ). The residual enzyme activity was determined under the standard assay condition. The activity of chitinase enzyme (8.04 nm/min./mL) was stable at pH 7.0 However, beyond these pH ranges, it lost its activity Fig.-11. Many chitinases including the present one, showed a pH optimum in



**Fig 9 : Effect of different incubation time period chitinase enzyme**

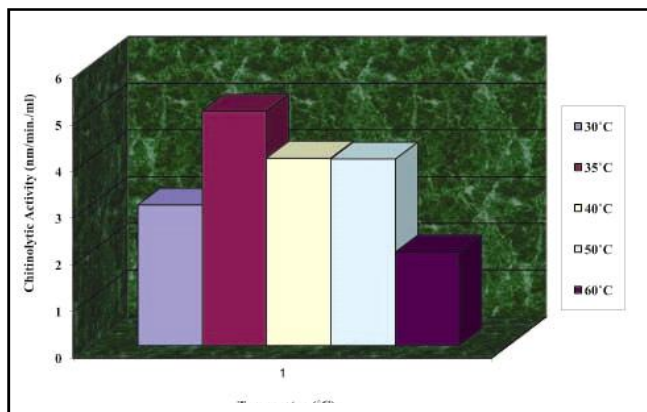


Fig 10 : Effect of different Temperature on chitinase enzyme

basic range. The *Bacillus spp.* LJ-25 was in the neutral range (Lee *et al.*, 2000).

## CONCLUSION

From the present investigation it is confirmed that strains isolated from soil such as *Bacillus* have chitinase activity and thus the enzyme extracted from these strains can be used as a catalyst for the degradation of chitin which is an abundant polysaccharide after cellulose.

The composition of medium plays a crucial role in the production of desired enzyme by the microorganisms under optimum conditions of pH, temperature and incubation period. Chitinase/chitinolytic enzymes have been widely used in various processes including the agricultural, biological and environmental fields. Thus there is a need to commercialize the production of chitinase/ chitinolytic enzyme from such microbial strains and production of chito-oligosaccharides which have beneficial aspects in pharmaceutical and food industries.

Characterization of biochemical properties suggested that the enzyme is thermo and pH-stable, suitable for various biotechnological applications, including bioconversion of colloidal chitin into NAG (N-acetyl-glucosamine).

## Acknowledgement

The authors thank Management committee, Principal and Department of Biosciences, D.A.V. College, Muzaffarnagar (U.P.) for providing lab facilities and constant encouragement for this research work.

## REFERENCES

- Anil Kumar Singh (2010)** Optimization of culture conditions for thermostable chitinase production by *Paenibacillus sp.* D1 **4**(21) : 2291-2298.
- Bhushan (2000)** Production and characterization of a thermostable chitinase from a new alkalophilic *Bacillus sp.* BG-11.

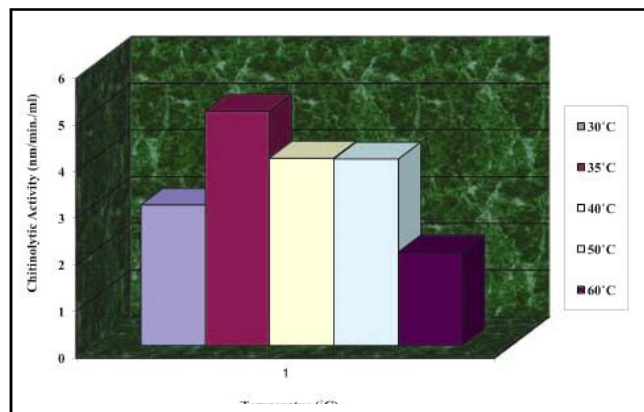


Fig 10 : Effect of different Temperature on chitinase enzyme

**Chet L (1987)** *Trichoderma* - application, mode of action and potential as a biocontrol agent of soil borne plant pathogenic fungi in innovative approaches to plant disease control (Ed.) Chet I John Wiley and Sons, New York 137-60.

**Christian Radauer, Farzaneh Adhami, Irene Fürtler (2010)** Latex-allergic patients sensitized to the major allergen hevein and hevein-like domains of class I chitinase show no increased frequency of latex-associated plant food allergy **48** : 600-609.

**Deshpande M V (1986)** Enzymatic degradation of chitin and its biological applications. **45** : 273-281.

**Flach Pilet P E and Jolles P (1992)** What's new in chitinase research? Experiment **48** : 701-716.

**Gooday G W (1990)** Physiology of microbial degradation of chitin and chitosan biodegradation **1**: 177-190.

**Gutowska M A, Drazen J C and Robinson B H (2004)** Digestive chitinolytic activity in marine fishes of Monterey Bay, California **139** (3) : 351-8.

**Inbar J and Chet I (1991)** Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. **23** : 973-978.

**Khan Minhaj and Ahmad Rafi Hamid (2010)** Optimization of culture media for enhanced chitinase production from a novel strain of *Stenotrophomonas maltophilia* using response surface methodology **20**(11): 1597-1602.

**Kyoung-Ja Kim and Yong-Joon Yang (2002)** Purification and Characterization of Chitinase from *Streptomyces sp.* M-20 **36**(2): 185-189.

**Lee E T and Kim S D (2000)** Selection and antifungal activity of antagonistic bacterium *Pseudomonas sp.* 2112 against red pepper rotting *Phytophthora capsici*. Korean J Appl. Micro. Biotech. **28**: 334-340.

**Lee J S, Joo D S, Cho S Y J H and Lee E H (2000)**. Purification and characterization of an extracellular chitinase produced by marine bacterium, *Bacillus sp.* LJ-25. *J. Microbiol. Biotech.* **10**, 307-311.

- Lorito, Harman M G E, Hayes C K and Broadway R M (1993)** Chitinolytic enzymes produced by *Trichoderma harzinum*: Antifungal activity of purified endochitinase and chitobiosidases. *Phytopathology* 83: 302-307.
- Lowry O H, Rosebrough N J, Farr A L and Randall R J (1951)** Total Protein Estimation by Lowry's Method. *J. Bio.Chem* 193 : 265.
- Miller G L (1959)** Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem.* 31, 426 - 428.
- Nampoothiri K M, Szakaacs G and Pandey A (2005)** Microbial synthesis of Chitinase in solid cultures and its potential as a biocontrol agent against phytopathogenic fungus *Colletotrichum gloeosporioides*. *Appl. Biochem. and Biotech.* 127: 1-15.
- Narayana K J P, Prabhakar P, Vijayalakshmi M, Vekateswarlu Y and Krishna P S J (2007)** Biological activity of phenylpropionic acid from a terrestrial *streptomyces*. *Polish J. Microbiol.*,56, 191-197.
- Nawani N N and Kapadnis B P (2001)** One step purification of chitinase from *Serratia marcescens* NK1, a soil isolate. *J. Appl. Microbiol.*, **90**, 803-808.
- Dahiya Neetu, Tewari Rupinder and Singh Hoondal Gurinder (2006)** Biotechnological aspects of chitinolytic enzymes: a review. *Applied Microbiol Biotechnology*, **71**:773-778.
- Reissig J L, Strminger J L and Lelair L F (1995)** A modified colometric method for the estimation for N-acetyl amino sugars. *J. BIOL. CHEM.*, **217**: 959-966.
- Kavi Karunya S, Reetha D, Saranraj P and John Milton D (2011)** Optimization and Purification of chitinase produced by *Bacillus subtilis* and its Antifungal activity against plant pathogen. *IJPBA* 2(6):1680-1685.
- Sherief AA, Sawah El M MA and Abd El-Naby MA (1991)** Some properties of chitinase produced by a potent *Aspergillus carneus* strain. *Applied Microbiology and Biotechnology* **35**: 228-280.
- Shon Y H, Ha Y M, Jeong T R, kim C H and Nam K S (2001)** Modifying Action of Chitosan Oligisaccharide on 2- Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MelQx)-induced Mutagenesis *J. Biochem. Mol. Biol.* **34**, 90-94.
- Tao Yong, Jin Hong, Long Zhangfu, Zhang Li, Ding Xiuqiong, Tao Ke, Ge Shaorong and Liu Shigui (2005)** Purification and characterization of an extracellular chitinase produced by bacterium C4. *Annals of Microbiology*, **55**(3) 213-218.
- Goel Vipul and Singh Anil (2005)** Bioprospecting and antifungal potential of chitinolytic microorganisms; 54-72.
- Wang San Lang and Bo-Shyun Lin (2010)** Purification and Characterization of chitinase from a new species strain, *Pseudomonas spp.* **20**(6): 1001-1005.

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