



## **Chitinase Production from Locally Isolated *Bacillus cereus* GS02 from Chitinous Waste Enriched Soil**

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### **Authors' contributions**

*This work was carried out in collaboration between both authors. Author GD carried the experimental work, performed literature searches and wrote the first draft of the manuscript. Author AK designed the study, managed the literature searches, corrected the entire manuscript and performed the analyses of the study. Both authors read and approved the final manuscript.*

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### **ABSTRACT**

**Background and Objective:** Chitin is world's second most abundant structural carbohydrate in nature and is found as a structural component in the cell wall of fungi and exoskeletons of invertebrates. During senescence, it is degraded by the enzyme, chitinase. In addition, chitinase has been exploited for various commercial applications such as control of insects and fungal pathogens in order to protect the crops, waste management, cosmetics and food industries. Chitinases have been found to be widely distributed in various organisms including viruses, animals, bacteria, fungi, higher plants and insects. In the present study, various soil samples enriched in chitinous waste were screened for the isolation of bacteria capable of producing chitinase.

**Methodology:** Chitinase producing bacteria were isolated using serial dilution plating technique onto different agar media. Primary and secondary screening were performed and the isolate producing maximum chitinase was selected for biochemical identification and 16s rRNA sequencing. The secretion of extracellular chitinase by this strain was optimized with respect to pH, temperature, incubation time, substrate concentration, carbon and nitrogen sources and inoculum size. All these components were optimized using OFAT (one factor at a time) approach.

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**Results:** A total of 29 bacterial isolates were found exhibiting secretion of extracellular chitinase as determined using zones of clearance. Based on the area of zone of clearance, six isolates were selected for secondary screening and the most potent isolate was identified as *Bacillus cereus*. The maximum chitinase production by this strain was obtained at 37°C and pH 7.0 after 48 hours of incubation. The maximum chitinase secretion was observed on addition of 1% colloidal chitin and 0.05% yeast extract in the medium.

**Keywords:** Chitinases; chitin; isolation; *Bacillus cereus*; 16S rRNA; media optimization.

## 1. INTRODUCTION

In the present scenario, crucial prospect of biotechnology is the production of microbial enzymes having pharmaceutical and industrial importance. Bacteria are the most preferred microbes for commercial production of enzymes because of their fast multiplication, ease to produce new enzymes by genetic modifications as compared to yeast and filamentous fungi [1].

Chitin is considered to be the world's second most abundant polysaccharide in nature [2,3]. It is an un-branched homo-polymer made up of  $\beta$ -1, 4 linked N-acetyl glucosaminyl moieties. Chitin is the main structural component of cell wall of fungus and invertebrates exoskeletons like insects and crustaceans [4]. With advancement, it has been shown that chitin and its derivatives are of pharmaceutical and other industrial importance [5]. Because of its various applications, it has got immense attention in the field of pharmacology, agriculture and biotechnology [6]. The most important chitin degrading enzyme is chitinase (EC. 3.2.1.14) which breaks  $\beta$ -1, 4 glycosyl linkages randomly in chitin and produces N-acetyl glucosamine and its various oligomers of different length.

The enzyme chitinase is widely distributed in various organisms including viruses, animals, fungi, higher plants, bacteria, and insects. It has different functions in these organisms. Chitinases have role in physiological, bioconversion processes, parasitism, nutrition, defense mechanism and recycling of chitin in nature [2,7,8]. In recent years, interest in chitinase grew exponentially due to its applications in medicine and therefore, there is need of highly purified enzyme [9]. Due to wide applications of chitinase in waste management, medicine, bio-control of phyto-pathogens and biotechnology, it is an interesting enzyme for study [10]. Many microorganisms found in various environments including soil, plant rhizosphere, water bodies and water sediments are capable of synthesizing chitinases [11]. The present research aimed the

isolation, screening and identification of chitinase producing bacteria from soil or water sediments and optimization of chitinase production from isolated strain.

## 2. MATERIALS AND METHODS

**Chemicals:** All the chemicals and media components used were of high quality analytical grade purchased from Hi-Media, Mumbai, India. Chitin powder and flakes from shrimp shells were purchased from Sigma- Aldrich, USA.

**Soil sample:** Soil samples were collected aseptically in clean zip-lock poly-bags from pond/river sediments, agricultural field soil, garbage dumping areas and fish market enriched in chitinous wastes from Mhow and Indore cities.

**Preparation of colloidal chitin:** Colloidal chitin was prepared according to the procedure described by Ferrari et al. [12] with some modifications. Chitin flakes (5 g) were treated with 60 ml of 12 N (37%, commercial) hydrochloric acid and stirred vigorously for 10 minutes. The suspension was kept in a refrigerator at 4 to 10°C for nearly 12 to 14 hours. Thereafter, two litres of chilled water was added to it and incubated for 24 hours at the room temperature. After that, suspension was centrifuged at 10,000 x g for 20 minutes at the room temperature to collect the pellet. The pellet was washed with water till its pH as tested by a pH strip became neutral. The neutral pellet of chitin was autoclaved at 15 lb pressure for 20 minutes and stored in a refrigerator for use as a substrate afterwards.

**Isolation and screening of chitinase producing bacteria:** Bacteria were isolated from the collected soil samples using serial dilution plating technique and upto 7 fold serial dilution were used for isolation purpose. The diluted soil samples were streaked onto chitin agar plates. The chitin agar plate medium was consisted of 6.0 gm of  $\text{Na}_2\text{HPO}_4$ , 3 gm of  $\text{KH}_2\text{PO}_4$ , 1 gm  $\text{NH}_4\text{Cl}$ , 0.5 gm NaCl, 0.05 gm yeast extract, 17

gm agar-agar and 10 gm colloidal chitin per litre, pH 7.0 [13]. The streaked plates were incubated at 37°C for 3 days. Bacterial colonies obtained were spot inoculated onto sterile colloidal chitin agar plates and incubated for 6 days at 37°C. The bacterial colonies showing larger zones of clearance were considered as potent chitinase producers and sub-cultured onto fresh plates of nutrient agar until pure isolates were obtained. Bacterial isolates selected after primary screening were further screened for maximum enzyme production in nutrient broth containing 1% colloidal chitin. The cultures were centrifuged at 10,000 x g for 15 minutes at 0 to 4°C and the supernatant was used for chitinase assay.

**Enzyme assay:** For chitinase enzyme assay, reaction mixture was consisted of 1 ml colloidal chitin (1.3% in 50 mM sodium phosphate buffer with pH 7.0), 0.1 ml enzyme and 0.9 ml of distilled water. The mixture was incubated at 37°C for 15 minutes. Thereafter, reaction was stopped by heating in a boiling water bath for 3 minutes. Subsequently, tubes were centrifuged at 10,000 x g for 20 minutes to settle the precipitate. In the supernatant, amount of reducing sugar was estimated using DNS method [14]. One unit of chitinase was taken as the amount of enzyme that produces 1 µmole of N-acetyl D-glucosamine equivalent reducing sugar per minute under the conditions of enzyme assay.

**Morphological, physiological and biochemical characterization:** Morphological and physiological characterization of the highest chitinase producing bacteria was performed according to Bergey's Manual of systematic bacteriology [15]. Besides, various biochemical tests were also performed.

**DNA isolation and identification of the strain:** The 16S rRNA gene sequencing was carried out at the National Chemical Laboratory (NCL), Pune. Chromosomal DNA was extracted by using spin column kit (Hi-Media, India or similar manufacturers). Bacterial 16S rRNA gene was amplified using polymerase chain reaction in a thermal cycler and were purified using Exonuclease I -Shrimp Alkaline Phosphatase (Exo-SAP) [16]. Purified amplicons were sequenced by Sanger method in ABI 3500xL genetic analyzer (Life Technologies, USA).

**Phylogenetic analysis:** Sequencing files (.ab1) were edited using CHROMASLITE (version 1.5) and further analyzed by Basic Local Alignment Search Tool (BLAST) with closest culture

sequence retrieved from the National Centre for Biotechnology Information (NCBI) database to find the regions of local similarity between sequences [17]. Further multiple sequence alignment and phylogenetic analysis were carried out for accurate species prediction and evolutionary relationship. The evolutionary history was inferred using Neighbor-Joining method. The percentage of replicate trees where associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method [18] and were in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1255 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [19].

**Accession no. of nucleotide sequence:** The 16S-rRNA partial gene sequence determined for the isolated bacterial strain was deposited in GenBank (Accession No. MN454860).

**Optimization of Culture conditions of the isolated strain:** The optimization of culture conditions (physical and nutritional) for chitinase production by the bacterial isolate was carried out by using one factor at a time (OFAT) approach. All the experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml LB broth as a basal medium supplemented with 1% colloidal chitin in an orbital shaker at 180 rpm. The optimum incubation time for chitinase production was determined by inoculating bacterial cultures into medium and incubating at 37°C for 6 days and supernatant was checked for enzyme activity every 24 hours. Effect of initial pH and incubation temperature was studied by growing cultures at different pH ranging from pH 3 to 10 and incubating at different temperatures ranging from 25 to 55°C. To determine the effect of different nitrogen sources, both organic and inorganic nitrogen sources at a concentration of 0.05% were used in the medium. Effect of different carbon sources (sucrose, glucose, maltose, fructose, starch, colloidal chitin and more) on production of chitinase by the isolated strain was also determined. The medium was supplemented with each carbon source (1%) separately and incubated using optimum conditions [20]. Effect of different concentration of colloidal chitin was also investigated by cultivation of the isolate in the medium with different concentrations (1, 2, 3,

4, 5, 10, 15, 20 g/l). Optimum inoculum concentration of the isolated strain was determined by inoculating the medium with different inoculum concentrations. All the experiments were performed in triplicates and the mean values were calculated. The data were analysed using analysis of variance (ANOVA) single factor and the P value,  $\leq 0.05$  was considered to be significant. Tukey post hoc test was also performed using PRISM 5.0 version of Graph Pad.

### 3. RESULTS AND DISCUSSION

#### 3.1 Isolation of the Bacteria

Different soil samples enriched in chitinous wastes were screened for chitinase producing bacteria using different growth media containing colloidal chitin as an inducer. A total of 29 isolates were obtained from different soil samples (Fig. 1) plated on minimal salt medium supplemented with 1% colloidal chitin on the basis of clear zones after six days of incubation at 37°C. These isolates were also investigated for production of chitinase under liquid culture conditions. Through these analyses, isolate S1, S2, S3, S6, S9, S11 and S15 were found to be potent chitinase producers (Table 1). When these isolates were grown in the liquid medium, chitinase activity ranging between 0.8 to 1.68 U/ml was found. Among these isolates, S1 strain showed maximum chitinase activity and hence was selected for further studies. Chitinase secretion by the present strain of *B. cereus* is comparable with the earlier reports. Wang et al [21] reported 11.2 U/50 ml by *B. cereus* strain TKU027. However, Meena et al [20] reported secretion of 213 U/ml chitinase activity by *Paenibacillus* sp. BISR-047. However, they took different units for enzyme activity, therefore, direct comparison can't be done.

**Table 1. Chitinase production from different isolates selected on the basis of zone of clearance and maximum enzyme activity.**

S.No.	Isolate name	Enzyme activity (U/ml)*
1.	S1	1.68
2.	S2	1.08
3.	S3	1.35
4.	S6	1.22
5.	S9	0.95
6.	S15	0.89

\*values are mean of three replicates.

#### 3.2 Identification of the Isolate

The isolate S1 was identified on the basis of morphological, biochemical and physiological characteristics (Table 2). The molecular identification of the selected strain was done using 16S rRNA sequencing. The bacterial 16S rRNA PCR amplicon was of nearly 1500 bp in size. The sequence was analysed using BLASTn with closest culture sequence retrieved from NCBI database. Multiple sequence alignment and phylogenetic analysis (Fig. 2) was also carried out to check the homology with other known bacterial strains. The clustering of isolate S1 with members of genus *Bacillus* showed nearly 99% sequence homology with *Bacillus cereus* strain ATCC14579. On the basis of this homology, the isolate was considered to be *Bacillus cereus* and was designated strain GS02. This nucleotide sequence submitted to GenBank was provided with Accession No. MN454860. Gram staining indicated it to be the Gram positive bacteria. On further studies, it was found to be the facultative anaerobe, non-motile and rod shaped bacteria. There are earlier reports also indicating *B. cereus* as chitinolytic enzyme producing bacteria and it has also been shown to have the ability to protect plants against fungal pathogens [22].

**Table 2. Morphological and biochemical characteristics of isolate *Bacillus cereus* GS02**

Characteristics	Observations
Colony shape	Circular
Color	Creamy white
Gram reaction	Gram positive
Cell shape	Rod shaped
Motility	Non motile
Catalase test	Positive
Starch hydrolysis	Positive
Methyl red	Positive
Voges-Proskauer reaction	Negative
Nitrate reduction	Negative
Urease activity	Positive
Gelatin hydrolysis	Positive
Citrate utilization	Negative
Oxidase	Negative

#### 3.3 Optimization of Culture conditions of Isolated Strain

Effect of incubation time on chitinase production by the isolate was observed up to six days and chitinase activity was determined after every 24 hours. Results indicated maximum production

after 48 hours (Fig. 3). Similar results have been reported where maximum chitinase secretion was found after 48 hours of incubation [23,24]. The isolate GS02 showed production of chitinase within first 24 hours and reached to its maximum level after 48 hours of incubation. The inoculum concentration was found to be important in production of chitinase and its effect was determined by varying inoculum amount from 0.5 to 3.0% (Fig. 4). It was observed that inoculum concentration of 1% gave better yield of chitinase. Effect of pH on production of chitinase revealed that enzyme activity increased with increase in pH and maximum

production was at pH 7 (Fig. 5). The results of enzyme production at different temperatures of incubation indicated maximum production at 37°C (Fig. 6). Bhattacharya et al [23] also showed maximum production of chitinase by *Bacillus* sp. between the temperature 35 to 37°C. However, Ong et al [25] reported maximum chitinase production by an *Enterobacter* sp. at 28°C and by a *Zymomonas* sp. at 30°C. They also mentioned that many other reports indicating wide variation in temperature for maximum chitinase production by different microbes are there.

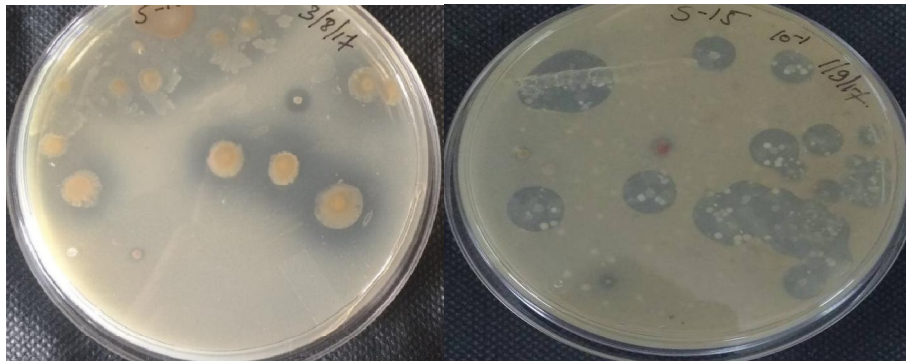


Fig. 1. Serial dilution results on colloidal chitin agar plates showing zone of clearance.

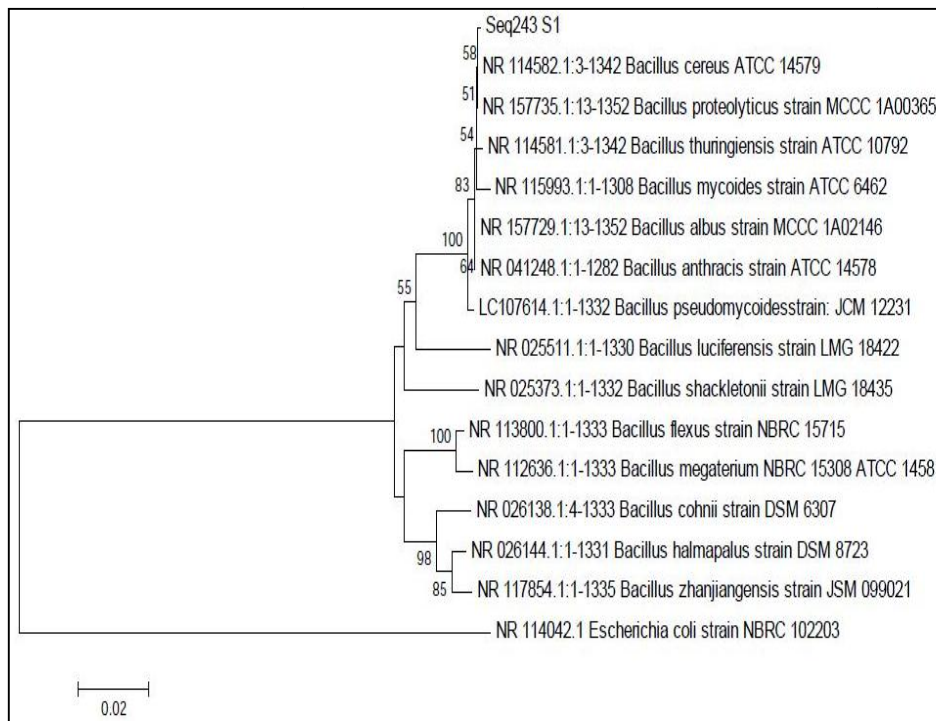
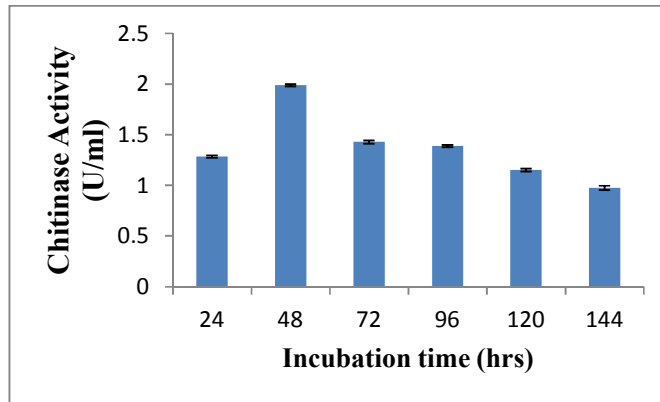
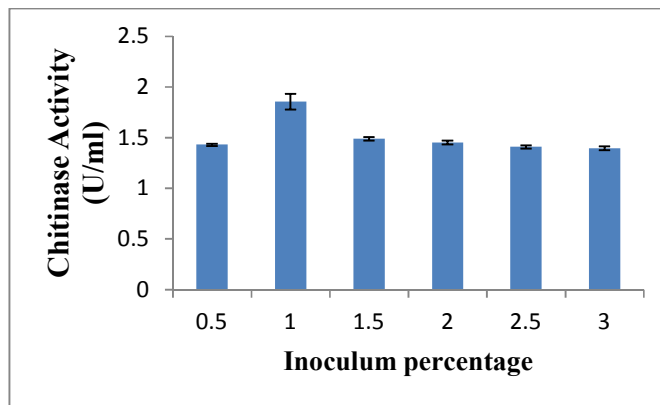


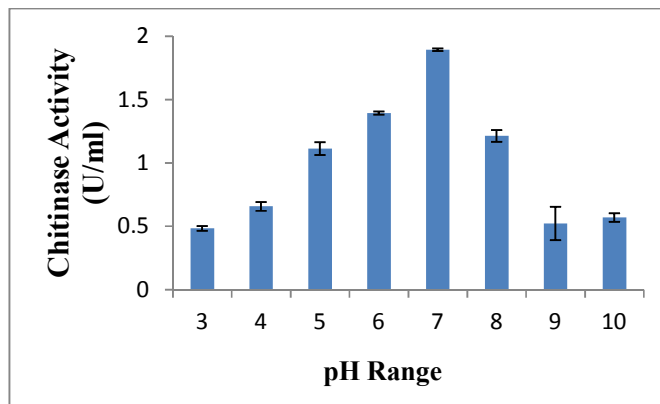
Fig. 2. Phylogenetic relationship based on 16S rRNA gene sequence of isolate GS02



**Fig. 3. Effect of incubation time on chitinase secretion by S1 isolate**  
Data represent mean  $\pm$  SD (n=3);  $P < 0.05$



**Fig. 4. Effect of inoculum percentage on chitinase secretion by S1 isolate**  
Data represent mean  $\pm$  SD (n=3);  $P < 0.05$

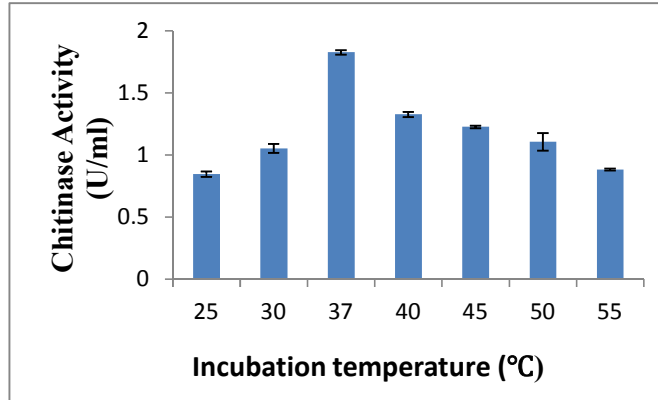


**Fig. 5. Effect of different pH on chitinase secretion by S1 isolate**  
Data represent mean  $\pm$  SD (n=3);  $P < 0.05$

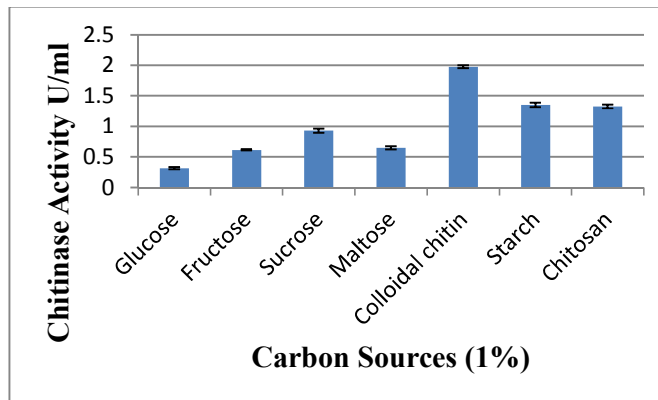
Among the carbon sources used for chitinase secretion, colloidal chitin proved to be the best carbon source. Being substrate of the enzyme, it is also the inducer for it (Fig. 7). On optimization

of concentration of colloidal chitin required for maximum chitinase production, results showed maximum production at 1% colloidal chitin (Fig. 8). Asif et al [26] also reported maximum

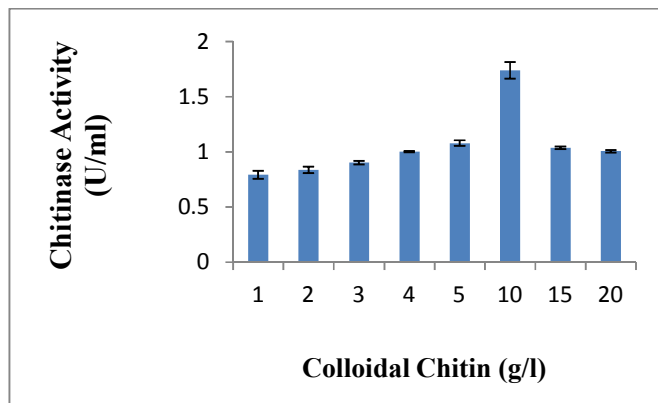
chitinase production by *Glutamicibacter uratoxydans* when 1% colloidal chitin was added in the growth medium. However, Ong et al [25] showed maximum chitinase production by *Enterobacter* sp. in the presence of 0.5% colloidal chitin.



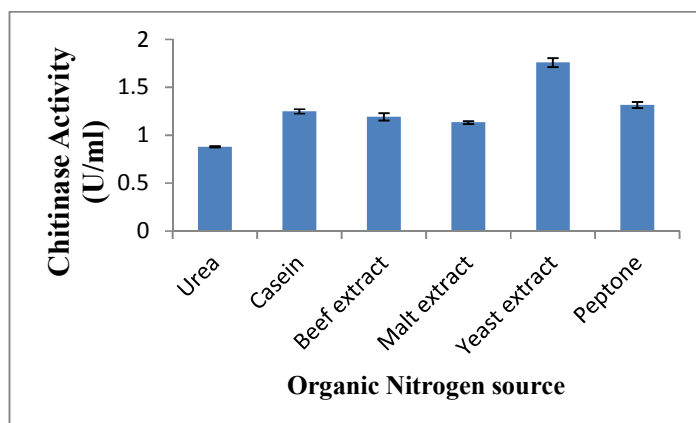
**Fig. 6. Effect of temperature on chitinase secretion by S1 isolate**  
Data represent mean  $\pm$  SD (n=3);  $P < 0.05$



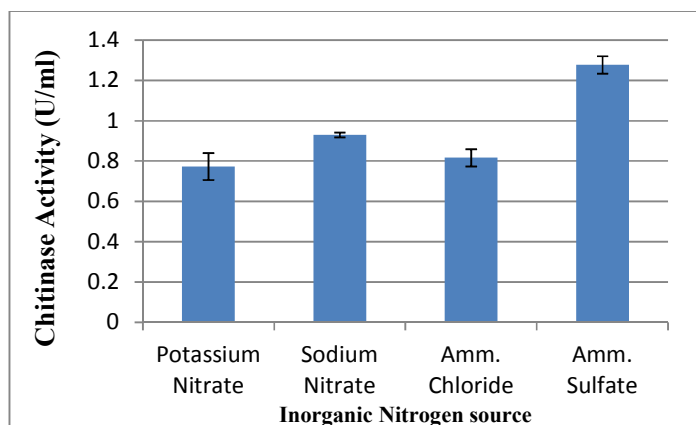
**Fig. 7. Effect of carbon sources on chitinase secretion by S1 isolate**  
Data represent mean  $\pm$  SD (n=3);  $P < 0.05$



**Fig. 8. Effect of substrate concentration on chitinase secretion by S1 isolate.**  
Data represent mean  $\pm$  SD (n=3);  $P < 0.05$



**Fig. 9. Effect of organic nitrogen supplements on chitinase secretion by S1 isolate**  
 Data represent mean  $\pm$  SD (n=3);  $P < 0.05$



**Fig. 10. Effect of inorganic nitrogen supplements on chitinase secretion by S1 isolate.**  
 Data represent mean  $\pm$  SD (n=3);  $P < 0.05$

Different nitrogen sources (both organic and inorganic) were supplemented in medium to determine their effect on chitinase production; here yeast extract and urea were the most and least effective nitrogenous additives, respectively (Figs. 9 and 10). Cheba et al. [27] studied effect of different nitrogen sources on *Bacillus* sp. R2 chitinase production and reported the same type of results as found in our present study. All the data were statistically analyzed by ANOVA single factor and Tukey post hoc test. The calculated critical value in Tukey test was found to be larger than the critical value from critical table which showed the two means are significantly different.

#### 4. CONCLUSION

In the past decades, a large number of studies are done on chitinases. Efforts are in progress to find novel chitinase having more efficiency and

production yield. There is much scope of chitinases in the field of medicine and waste management. The present study indicated that *B. cereus* GS02 is a mesophilic bacteria having the capability to secrete good amount of chitinase in reasonable period of time. Search has been done for cheaper carbon and nitrogen sources for economic production of the enzyme. The present enzyme may be useful for the management of chitinous wastes.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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