

EXPLORING AND OPTIMIZING THE POTENTIAL OF CHITINASE PRODUCTION BY ISOLATED *BACILLUS SPP*

RIDDHI J. JHOLAPARA^{1*}, RADHIKA S. MEHTA¹, ASHOK M. BHAGWAT² AND CHHAYA S. SAWANT²

¹School of Science, NMIMS (Deemed -to-be) University, ²Shri C. B. Patel Research Centre, Vile Parle (West), Mumbai 400056, India.
Email: riddhi.jholapara@nmims.edu.in

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ABSTRACT

Objective: The present study aims to isolate and identify chitinase producing organism. Further, it elucidates the effects of process parameters on chitinase production.

Method: The soil samples collected were evaluated for chitinase producing strain by carrying out enrichment in minimal salt medium containing colloidal chitin as sole carbon source. The chitinolytic activity of the strain was investigated by the ability of the isolate to produce zone of clearance on colloidal chitin agar. The shake flask method was employed to investigate the effect of process parameters on chitinase biosynthesis.

Results: Amongst the isolated strains, one chitinolytic strain was selected for the study. Biochemical identification tests indicated that the strain belonged to genus *Bacillus*. 16S rDNA sequencing test confirmed the identity of the isolate and identified up to species level. The strain was identified as *Bacillus cereus* strain. The optimum carbon source for chitinase production was colloidal chitin while peptone was optimum nitrogen source which resulted in increased chitinase biosynthesis. 0.06% of magnesium sulphate had positive effect on chitinase production whereas 0.75% of colloidal chitin as substrate augmented enzyme production. The favourable pH for chitinase production was when the initial pH of the medium was in the range of 6-7. 30-40°C was discerned to be the beneficial temperature range for enzyme production.

Conclusion: The optimization of process parameters played a focal role in augmenting the biosynthesis of chitinase enzyme. The potential of the isolated strain can be exploited for an assortment of applications such as production of pharmaceutically important chitinoligosaccharides, control of pathogenic fungi, preparation of single cell protein and management of chitinous waste etc.

Keywords: Chitin; Chitinases; Chitinolytic; Bacterium; *Bacillus*.

INTRODUCTION

Chitin is reputed to be the second most abundant polymer after cellulose. This polymer of N- Acetylglucosamine (GlcNAc) is widely distributed in nature in the form of exoskeletal material of crustaceans and insects. The marine environment presents the highest source of chitin and hence each year chitin tends to keep accumulating as seafood waste. Currently, seafood waste management involves usage of strong acids and bases in order to convert the waste into value added products. The usage of these acids and bases pose environmental hindrance to seafood processing countries. Hence, management of these wastes deserve special attention because in nature, chitin is tightly bound to lipids, proteins and calcium carbonate making it relatively difficult for conversion of chitinous wastes into low molecular weight chitooligomer mixture which has wide range of applications in the field of medicine, agriculture, food industry and wastewater industry[1].

Chitinases (E.C. 3.2.1.14) are a group of enzymes which are responsible for degradation of chitin. They play a pivotal role in recycling chitin in the nature. Chitinases are known to perform many biological functions and they occur in organisms such as bacteria, fungi, actinomycetes, insects and higher plants [2]. Microorganisms produce chitinase in order to utilize chitin as energy source whereas fungi and insect produce chitinases as they are involved in morphogenesis [3]. In plant, they are involved in defence against pathogens.

Since there are evidences of presence of chitinolytic microbes, it indicates that chitin may be a commonly available substrate in the soil. Thus presence of chitin degrading organisms in nature presents an economical and environmental friendly alternative in order to obtain chitinases. Over the decade, the exploration of microbes as the source of chitinases has increased due to its various applications. Chitinases obtained from microbial sources have been employed in various fields of medicine, biotechnology, food, wastewater and agricultural industries[4-6].

High production costs of chitinases necessitate the need for scientists to explore and understand the properties of microbial chitinases in order to formulate inexpensive and reliable chitinases

mixture. This will serve two purposes: reducing the environmental hazard and generating various value added compounds of industrial interest. The present study aims to isolate and identify chitinolytic organism from local fish market soil and coastal area; optimize process parameters in order to maximize microbial production of extracellular chitinase.

MATERIALS AND METHODS

Chemicals

Chitin powder was purchased from SD Fine Chemicals, Mumbai India. Nutrient Agar and Agar Powder were purchased from Himedia Laboratories, Mumbai, India.

Preparation of Colloidal Chitin

Colloidal chitin was prepared from commercial chitin as previously described [7] by modifying the method of Roberts and Selitrennikoff [8].

Sample Collection

Soil which were rich in chitin wastes were collected from local fish market and coastal area. The uppermost layer of soil samples were taken with the help of sterile spatula and put into sterile plastic bag which was transported back to laboratory for further processing.

Enrichment and Screening for Chitin Degraders

5 grams of soil samples were enriched in 100 ml of Minimal Salts Medium (MSM) containing chitin as sole source of carbon and nitrogen (Chitin powder 1g, KH₂PO₄ 0.03g, K₂HPO₄ 0.07g, MgSO₄ 0.05g, FeSO₄·7H₂O 0.001g, MnCl₂·4H₂O 0.001g ZnSO₄·7H₂O 0.001g in 100 ml of deionised water). The enrichment was carried out at room temperature with shaker conditions set at 150 rev/min. Regular viable counts was performed to assess for the type of microflora observed and select the colonies for further screening test.

The colonies which were persistently detected in viable count studies were further selected for screening of chitinase producing isolates. The screening was carried out using spot inoculation of each of the isolates at the centre of Colloidal Chitin Agar (CCA) media

plates containing (grams/100ml) K₂HPO₄ 0.03, KH₂PO₄ 0.07, (NH₄)₂SO₄ 0.025, MgSO₄ 0.05, Agar powder 1.5 and colloidal chitin 1. The plates were incubated at room temperature and were observed for a zone of clearance around the colony. The isolate which showed the maximum zone of clearance was considered as the potential chitinase producing strain and was selected for further studies.

Biochemical identification and 16SrDNA sequencing

The selected chitin degrading strain was identified by carrying out standard microscopic, cultural and biochemical tests according to the Bergey's Manual of Systematic Bacteriology [9]. For further confirmation by 16SrDNA sequencing, the genomic DNA was extracted and amplified by PCR (Polymerase Chain Reaction) using universal bacterial primer pair 8F/1391R (8F-5'AGAGTTTGATCCTGGCTCAG 3' and 1391R 5'GACGGGGCGGTACAA'). The amplified genomic DNA samples were sent for sequencing to GeneOmBio Research Laboratory, Pune. The sequencing data obtained was then analysed by NCBI BLAST tool. Alignment of 16SrDNA gene sequence with 20 closely related gene sequences was carried out using CLUSTALW2 and distance based phylogenetic tree was constructed using neighbour joining (NJ) algorithm.

Chitinase Assay

Chitinase was assayed with colloidal chitin as a substrate. One ml of the crude enzyme solution was added to 1% of substrate solution, containing 1% colloidal chitin in acetate buffer (20 mM, pH 4.6), and the mixture was incubated at 50°C for 30 minutes. After centrifugation, the amount of reducing sugar in the cell free supernatant was determined by the method suggested by Imoto and Yagishita using GlcNAc as standard [10]. One unit of enzyme activity was expressed as the amount of enzyme required to liberate one microgram of GlcNAc per minute under the assay conditions.

Optimization of culture conditions

In the optimization of cultural conditions, growth was carried out in 25ml of MSM incubated at 150 rev/min in rotary shaker. One ml of test organism with 0.1 Optical Density 600nm was inoculated as seed culture and enzyme activity was assessed fifth day post inoculation.

Investigation of the effect of additional Carbon Source was carried out using three simple carbon sources (1% w/v Glucose, Sucrose, Mannitol and GlcNAc) in addition to 1% colloidal chitin.

Investigation of the effect of additional Nitrogen Sources was determined using four different nitrogen sources such as 0.5% w/v: Ammonium Sulphate, Ammonium chloride, Tryptone and Peptone.

Optimization of MgSO₄ Concentration: The isolate was cultivated in the presence of different concentrations of MgSO₄ i.e. 0.04%, 0.05%, 0.06% and 0.07%, and the enzyme activity was assessed.

Optimization of Initial pH of Medium was carried out for maximum chitinase production by growing the isolate at different values of initial pH 5, 6, 7, 8 and 9 of the medium.

Optimization of Temperature was performed at different temperatures (25°C, 30°C, 35°C, 40°C and 45°C) in order to achieve maximum chitinase production.

To Optimize Substrate Concentration, enzyme activity was assessed by cultivating the isolate in the presence of different concentrations of substrate: 0.1%, 0.25%, 0.5%, 0.75%, 1%, 1.5% and 2% w/v of colloidal chitin.

Protein Determination

The protein concentration was estimated according to the method of Lowry *et al* using Bovine serum albumin as the standard [11].

Statistical Analysis

All the optimization parameters were conducted in triplicate and the data was analysed using single factor analysis of variance (ANOVA) and Tukey's test using GraphPad Prism 5. All the data are graphically presented as mean ± S.D. of triplicates. P ≤ 0.05 was considered statistically significant.

RESULTS

Isolation and screening of chitinase producing bacteria

A total of 40 isolates were obtained from soil samples which were enriched in MSM fortified with 1% chitin powder. Primary screening for isolates on colloidal chitin agar plates demonstrated the chitinolytic ability of the isolates. Amongst 20 isolates, one isolate which produced highest zone of clearance was selected for further studies.

Biochemical Identification and 16SrDNA Sequencing

The morphological and biochemical characterization was performed to identify the selected isolate. Morphological and biochemical characteristics of the isolate selected for the further studies have been summarized in Table-1 and Table-2 respectively. Biochemical tests as described in Bergey's Manual of Systematic Bacteriology demonstrated that the isolate belonged to the genus *Bacillus*.

Table 1: Morphological characteristics of the isolate

Gram's Nature	Motility	Spore
Gram Positive bacilli	Motile	Centrally positioned spore

Table 2: Biochemical characteristics of the isolate

Tests to distinguish between aerobic and anaerobic breakdown of carbohydrates	
O/F Aerobic	Positive (utilization of sugar)*
O/F Anaerobic	Positive (utilization of sugar)*
Tests to show degradation of range of carbohydrates and related compounds	
Glucose	Positive
Sucrose	Positive
Lactose	Negative
Maltose	Positive
Mannitol	Negative
Xylose	Negative
Tests for Specific Breakdown Products	
Methyl Red	Positive
VogesProskauer	Negative
Tests to show Ability to utilize particular Substrate	
Starch	Positive
Citrate	Negative
Tests for Metabolism of Proteins and Amino-acids	
Indole Production	Negative
Arginine dihydrolase	Negative
Gelatin hydrolysis	Positive

Tests for Enzymes

Catalase	Positive
Oxidase	Negative
Urease	Positive
Nitrate Reduction	Negative
Combined Tests	
Triple Sugar Iron(TSI)	A/A**

Key: *Utilization of carbohydrate resulted in yellow coloration in the medium, indicating aerobic as well as anaerobic fermentation. ** Acidic Slant, Acidic butt.

According to the 16SrDNA sequence comparison analysis of the isolate with the sequences of nearest type species retrieved by NCBI BLAST tool, this strain showed taxonomic affiliation with *Bacillus cereus* strain. The

partial 16SrDNA gene sequence of the selected isolate was deposited in the GenBank nucleotide sequence database under the accession number KF515695. The phylogenetic tree is represented in Fig.1.

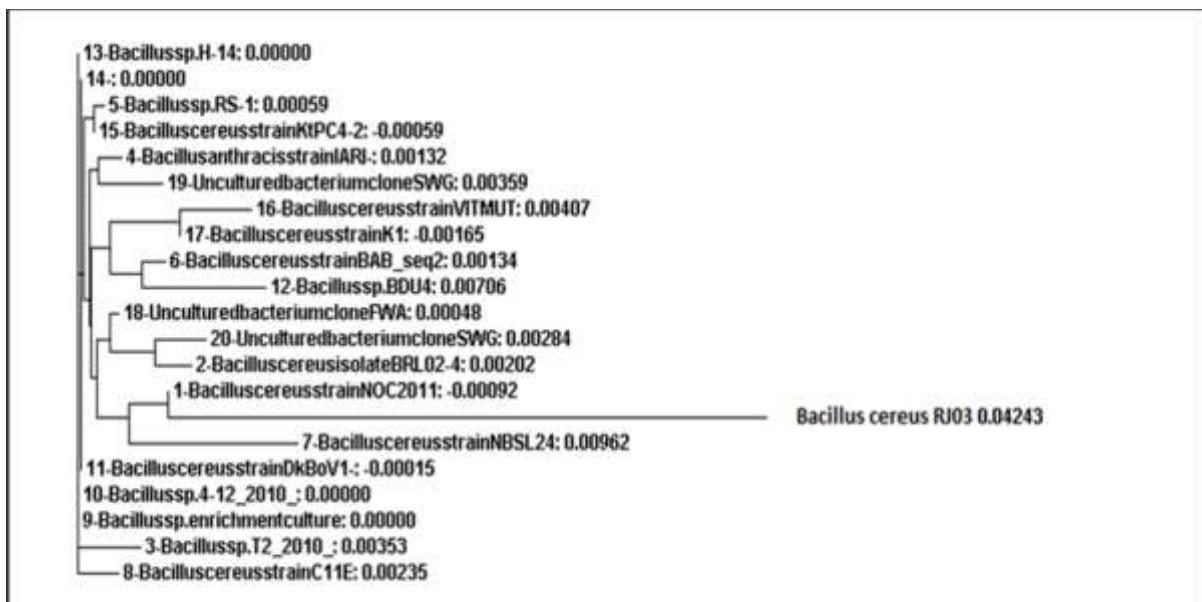


Fig. 1: Neighbour-joining phylogenetic tree of the strain *Bacillus cereus* RJ03 and its relatives based on 16S rDNA gene sequence comparisons.

Influence of culture conditions on chitinase production**Investigation of the effect of additional carbon source**

The growth and production of chitinase by the isolate was investigated using different carbon sources. Amongst all the

carbon sources used, colloidal chitin served as the best carbon source for the production of chitinase. Carbon sources like glucose, sucrose, mannitol and GlcNAc served to inhibit chitinase biosynthesis in spite of supporting prosperous cell growth of the isolate in the medium.

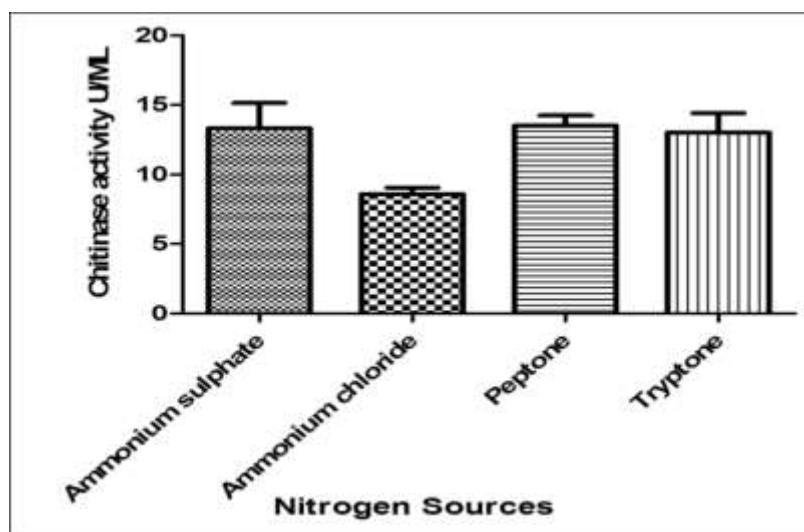


Fig. 2: Effect of different nitrogen sources on production of chitinase by *Bacillus cereus* RJ03

Investigation of the effect of additional nitrogen source

The effect of additional nitrogen sources on chitinase production is presented in the Figure - 2. The study was performed using two organic (peptone and tryptone) and two inorganic (ammonium sulphate and ammonium chloride) nitrogen sources, which were added in the medium that contained colloidal chitin as a sole source of carbon. Amongst the nitrogen sources investigated, peptone served as best nitrogen source for production of chitinase.

Optimization of MgSO₄ concentration

The optimization of MgSO₄ concentration on production medium is presented in the Figure - 3. It was observed that with the increase in the concentration of MgSO₄, the strain synthesized more chitinase. The maximum level of chitinase being 16.96 U/ml at 0.06% after which the level of chitinase remained constant (data not shown) in spite of increase in MgSO₄ concentration.

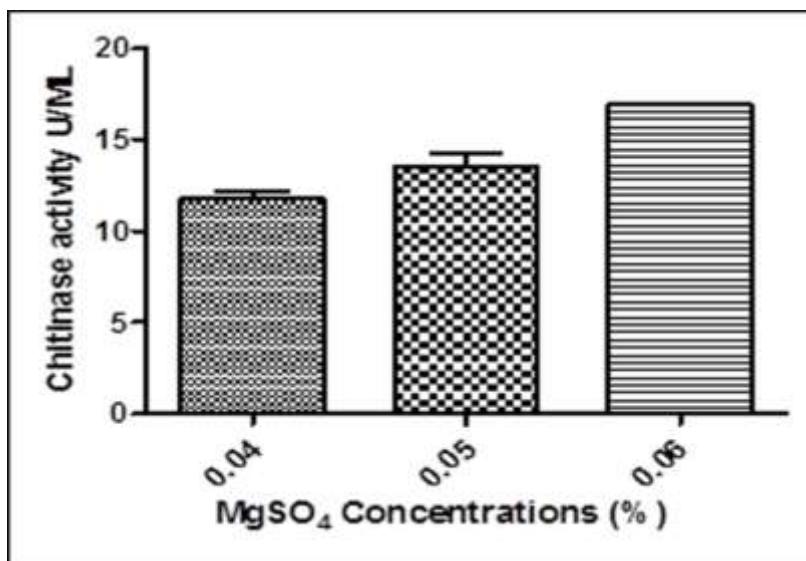


Fig. 3: Effect of different magnesium sulphate concentrations on production of chitinase by *Bacillus cereus* RJ03.

Optimization of Initial pH of the medium

Figure - 4 shows the influence of initial pH on the culture medium. Maximum chitinase production was observed over a range of pH 6-7. At pH higher and lower than the optimum pH range, enzyme production was repressed.

Optimum temperature for enzyme production

Figure - 5 depicts the influence of temperature on chitinase production. Maximum chitinase production was observed at range of

temperature 30-40°C. Increase in the temperature resulted in decrease in enzyme production. These results show the organism's mesophilic preference for enzyme production.

Optimization of substrate concentration

The optimization of substrate concentration is presented in the Figure - 6. Chitinase production was observed with respect to substrate concentration, the optimum level observed was in the range of 0.50-0.75%.

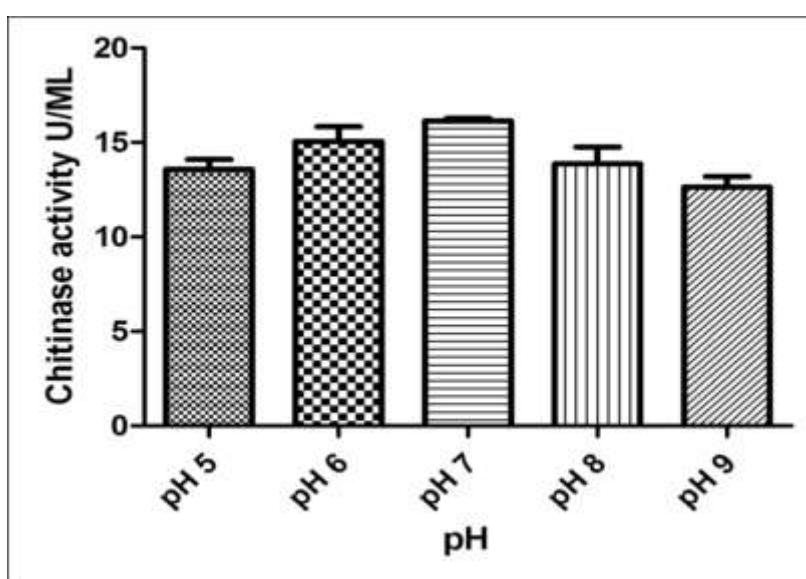


Fig. 4: Effect of different pH on production of chitinase by *Bacillus cereus* RJ03.

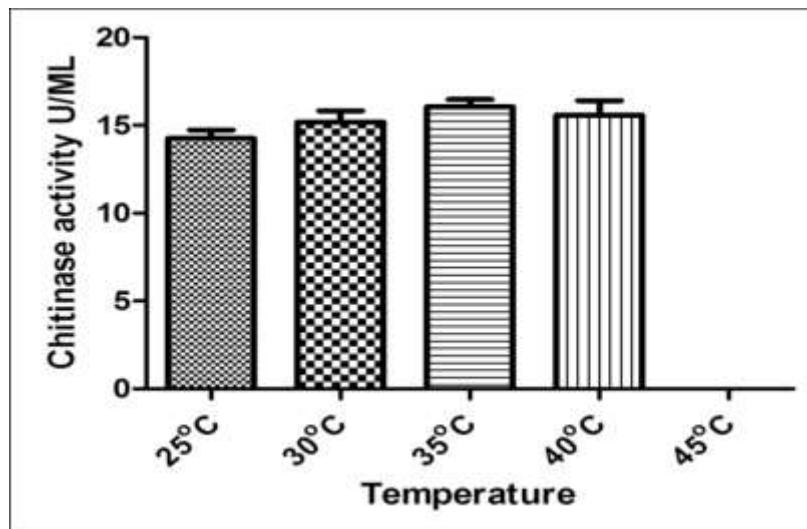


Fig. 5: Effect of different temperature on production of chitinase by *Bacillus cereus* RJ03.

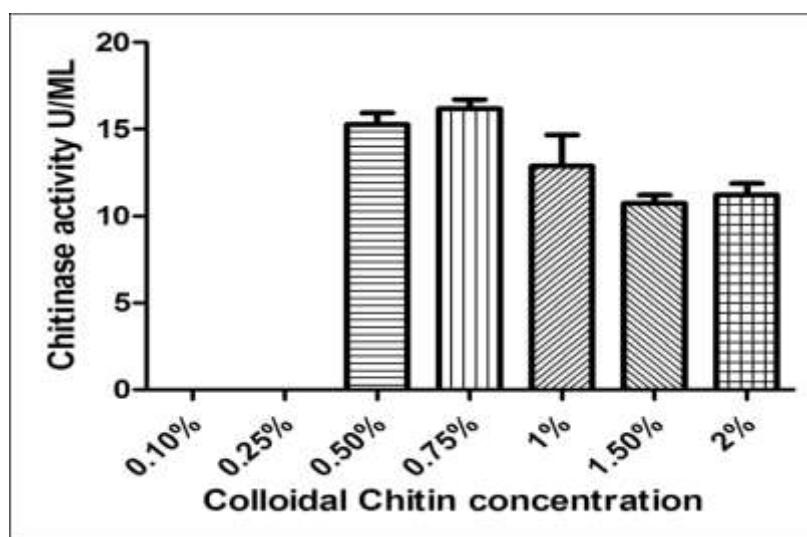


Fig. 6: Effect of different substrate (colloidal chitin) concentrations on production of chitinase by *Bacillus cereus* RJ03.

DISCUSSION

In the current study, soil samples were screened for chitinase producing organisms on selective medium containing colloidal chitin. The isolated organism from the above mentioned soil samples was selected for further studies due to its formation of largest zone of clearance on colloidal chitin agar plate. The morphological, biochemical and physiological characteristics of the isolate was investigated and it indicated that the isolate belonged to genus *Bacillus* species. The isolate under investigation was attributed to Genus *Bacillus* on the basis of 16SrDNA sequencing.

It is imperative to optimize the culture medium in order to ensure optimum growth of micro-organisms and production of metabolites. The study of effect of additional carbon source (1% w/v) on chitinase production demonstrated that no chitinase activity was observed in the medium which was supplemented with additional carbon sources such as glucose, sucrose, mannitol and GlcNAc in spite of prosperous cell growth of the isolate in the medium. This finding indicated that chitin or colloidal chitin was indispensable for chitinase production which coincides with previous reports that suggest employing colloidal chitin as a sole of carbon source for highest chitinase production [12, 13]. The study also supports the assumption that chitinase is subjected to stimulatory effect and catabolite repression by sugars. Previous report by Tweddellet *al*

suggests no chitinase production when glucose, sucrose or GlcNAc was used as carbon sources[14]. Similar findings report decrease in chitinase production by *M. timonae* and *Serratiamarcescens* when fructose, glucose, lactose, maltose and GlcNAc were used as carbon sources in the medium [15, 16].

The study of effect of additional nitrogen sources on chitinase production demonstrated that as compared to inorganic nitrogen sources, organic nitrogen sources served to increase chitinase production. Amongst the nitrogen sources, peptone was most effective as compared to ammonium sulphate, ammonium chloride and tryptone in increasing the production of chitinase. This maybe because peptone contains nitrogenous compounds, growth factors and oligomers of GlcNAc, so addition in low amount can have a stimulatory effect on cell growth [17]. This finding is in agreement with findings of S.M. Akhiret *al*which reported peptone as optimum nitrogen source which led to increase in chitinase production by *Bacillus licheniformis* strain[18]. Peptone has been reported to increase chitinase production in *Pantoea dispersa* and *Axyllosoxydans*[19, 20].

Chitinase production is also affected by presence of minerals in the production medium. The concentrations of Mg²⁺ ions play a crucial role in cell growth and enzyme production and stability. The study of optimization of MgSO₄ concentration demonstrated a positive effect

on chitinase production. This is in agreement with Gohelet *et al* and Han *et al* which reports positive influence of MgSO₄ on chitinase production by *P. dispersa* and *Streptomyces* respectively [21,22]. Similar report state that MgSO₄ concentration can increase chitinase production by *B. pumilus*[23].

The influence of environmental factors such as pH and temperature has an impact over biological processes such as enzyme production by controlling the availability of metabolic ions. Majority of bacteria are reported to produce maximum chitinase at neutral or slightly acidic pH [24]. This report coincided with the obtained results. In *Bacillus subtilis* pH 7 and 8 have been reported to be optimum for chitinase production [25]. Similarly, nearly neutral pH is reported as optimum pH for chitinase production by other strains of *Bacillus* and *Pseudomonas aeruginosa* K-187 [13,26, and 27]. Optimum temperature is crucial in production of chitinase since it influences cell growth. The observations as a result of the optimum temperature study is in complete agreement with the Das *et al* that reports 35°C as the optimum temperature for chitinase production by *B. amylolyticus* SM3 strain[28]. Previous report on *B. laterosporus* also state 35°C as the optimum temperature for chitinase production[2].

The optimum concentration of chitin as the substrate in the production medium is crucial for chitinase induction in order to produce maximum chitinase. Seven different colloidal chitin concentrations (0.1% - 2% w/v) were investigated for chitinase production and the observations coincides with the report by Taechowisanet *et al*. which states that chitinase produced by bacteria hydrolyzed colloidal chitin more rapidly than crude chitin or chitin from fungal cell walls [29].

CONCLUSION

The present study suggests, the potential use of the obtained isolate in production of chitinase using chitin or chitin derivatives as raw material. A detailed study is necessitated in order to harness the ability of the isolate to produce commercial value added products. In the course of the study it was established that culture medium and process parameters play a critical role in production of enzymes. The pilot scale study can be adopted in order to maximize the production of chitinase for further applications.

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Abbreviations

GlcNAc - N- Acetylglucosamine, MSM - Minimal Salts Medium, CCA - Colloidal Chitin Agar, PCR - Polymerase Chain Reaction, NJ - neighbour joining.

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