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OPTIMIZATION OF CULTURAL CONDITIONS FOR CHITINASE PRODUCTION FROM CHITINOLYTIC BACTERIUM ISOLATED FROM SOIL SAMPLE.

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ABSTRACT

Chitinases are group of enzymes which play a significant role in degrading chitin. The present study explores the natural ability of the bacteria to utilize chitin as a source of energy. Soil samples were screened for chitinolytic organisms and strain which produced highest chitinolytic activity was selected. Biochemical analysis revealed that the isolate belongs to the genus *Bacillus*. It was identified upto the species level as *Bacillus licheniformis,* using 16S rRNA based identification system. The cultural conditions for production of chitinase was optimized which resulted in a significant increase in the enzyme activity. The study demonstrates the potential of the isolate for industrial application such as production of bioactive chitin-oligosachharides.

KEYWORDS: **Chitinolytic, *Bacillus licheniformis*, Chitin, Chitinase.**

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INTRODUCTION

Chitin is the structural constituent of insect exoskeleton, crustaceans, certain green algae and fungal cell walls[1]. This polymer of N-acetylglucosamine (GlcNAc) units is the second most abundant biopolymer in nature after cellulose. Chitin in nature accumulates as waste products from seafood production and processing industries. These accumulations of wastes result in environmental hazard to various seafood processing countries. Traditional methods involve converting chitinous wastes to useful chitin and chitin-oligomers using strong acids and bases. Due to its crystalline nature, hydrolysis of chitin is an expensive and ecologically harmful process thus necessitating the search for an alternative eco-friendly approach to this problem. Vast diversity and the beneficial applications of micro-organisms need to be understood as they serve as an enormous source of a variety of enzymes. Chitinases are a group of enzymes that catalyze the hydrolysis of chitin directly to low-molecular weight products. They are reported to be found in organisms like bacteria, fungi, insects, higher plants and some vertebrates[2, 3, 4, 5, 6, 7, 8]. Organisms use chitin as an energy source thereby allowing in recycling of carbon and nitrogen back into the ecosystem[9]. Over the years, chitinases are gaining importance since they serve as an eco-friendly and cost effective alternative in the fields of medicine, biotechnology, agriculture, industrial applications and waste water management[10, 11, 12]. The present study involves screening for chitinolytic organisms from soil samples collected from various environments. The chitinolytic strains were screened and amongst the isolates, a bacterial isolate which later identified as *Bacillus licheniformis* was found to produce the maximum chitinase. Chitinases from different *Bacillus* species have been reported. However, not many reports are available for production of chitinase by *B.licheniformis*. Hence, the study was undertaken to evaluate the process parameters for maximum chitinase production.

MATERIALS AND METHODS

Chemicals

Agar powder and Nutrient Agar were purchased from Himedia Laboratories, Mumbai, India. Chitin powder was purchased from SD Fine Chemicals. All the other chemicals were purchased from Qualigens Fine Chemicals., Mumbai, India.

Collection of Soil Samples and Screening for chitin degraders

Soil samples were collected from chitinous waste rich soil from the local fish market and coastal area. Both the soil samples were collected from 2 to 3 cm depth in plastic bags and were transported to the research center. The soil samples were enriched in a minimal medium containing chitin as a sole source of carbon, nitrogen and energy. 5 grams of each of these soil samples were enriched in 100 ml of Minimal Salts Medium (Chitin powder 1g, KH_2PO_4 0.03g, K_2HPO_4 0.07g, $MgSO_4$ 0.05g, $FeSO_4.7H_2O$ 0.001g, $MnCl_2.4H_20$ 0.001g $ZnSO_4.7H_20$ 0.001g in 100 ml of deionised water). The enrichment was carried out at room temperature with shaker conditions set at 150 RPM. Regular viable counts was performed to assess for the type of microflora observed and select the colonies for further screening test.

Preparation of Colloidal Chitin

Colloidal chitin was prepared from commercial chitin by the method of Roberts and Selitrennikoff[13] with few modifications adopted as follows: To five grams of powdered chitin 50 ml of concentrated HCl was added slowly. The mixture was left standing for 6 hours. The mixture then was added to 250 ml of ice-cold ethanol with continuous stirring. The pH of the solution was adjusted to 7 and the precipitate was collected by centrifugation at 8000 RPM for 30 minutes at 4^{o}C. The colloidal chitin collected was dried at room temperature and later stored at 4^{o}C for further use.

Screening of chitin – degraders

The colonies which were persistently detected in viable count studies were selected for further screening. The screening of chitinase producing isolates was performed by spot inoculating each of the isolates at the center of Colloidal Chitin Agar (CCA) media plates containing colloidal chitin. The plates were incubated at room temperature and were observed for a zone of clearance around the colony. The isolate which showed the highest clear zone was considered as the potential chitinase producing strain and was selected for further studies.

Identification of the isolate

The selected chitin degrading strain was subjected to morphological and biochemical identification as per Bergey's Manual of Systematic Bacteriology[14].

DNA Extraction and Polymerase Chain Reaction (PCR) Analysis

The identity of the strain was further confirmed by sequencing the gene encoding for 16S rRNA at GeneOmbio Research laboratories (Pune, Maharashtra, India). DNA was extracted according to the standard protocol for extraction of Bacterial Genomic DNA (gDNA)[15]. The extracted gDNA was qualitatively analyzed using Agarose Gel Electrophoresis (AGE). The entire 16S rRNA fragment was amplified by PCR, using a universal primer pair 8F/1391R (8F - 5'AGAGTTTGATCCTGGCTCAG3'and 1391R -5'GACGGGCGGTGTACA3') for bacterial strains. The amplified PCR products were purified and sequenced at GeneOmbio Research Laboratories, Pune.

Chitinase Assay

Chitinase was assayed with colloidal chitin as a substrate. The strain was grown in chitin broth with continuous shaking at 150 RPM at 30^{o}C for five days. The cell suspension was centrifuged at 8,000 RPM for 20 minutes at 4^{o}C to obtain cell free supernatant which was used for chitinase assay. One ml of the crude enzyme solution was added to 1% of substrate solution in acetate buffer (20 mm, pH 4.6), and the solution was incubated at 50^{o}C for 30 minutes. After centrifugation, the amount of reducing sugar in the cell free supernatant was determined by the method of Imoto and Yagishita[16]. The activity was calculated from the standard curve plotted using known concentrations of N-Acetylglucosamine (GlcNAc). One unit of enzyme activity was expressed as the amount of enzyme required to liberate one microgram of GlcNAc per ml per minute under the assay conditions.

Optimization of culture conditions for maximum enzyme production

For optimization of cultural conditions, One ml of test organism with 0.1 OD_{600nm} was inoculated in 25 ml of Minimal Salts Medium (MSM) and incubated at 150 RPM in a rotary shaker for 5 days at room temperature (except for temperature experiments) after which the culture medium was centrifuged at 8000 RPM at 4^{o}C for 20 minutes. Cell free supernatant was obtained and used as an enzyme source for assessing for chitinase activity. The protein content of the supernatant was estimated according to Folin-Lowry[17] method. These proteins were used to correlate the activity of the enzyme vis-a-vis the culture of the test organism. All the experiments were conducted in triplicates.

- Investigation of the effect of additional Carbon sources: The addition of three different carbon sources (1% w/v Glucose, Sucrose and Mannitol) was investigated by supplementing the MSM with each compound separately.
- Investigation of the effect of additional Nitrogen sources: The addition of four different nitrogen sources (0.5% w/v: Ammonium Sulphate, Ammonium chloride, Tryptone and Peptone) was investigated.
- Optimization of initial pH of the growth medium: The parameter of initial pH of the growth medium was optimized 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 growth temperature: - The cultivation of the isolate was carried out at different temperatures: 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C in order to optimize chitinase production.

- Optimization of Substrate (colloidal chitin) concentration: The isolate was cultivated in the presence of different substrate concentrations: 0.1%, 0.25%, 0.5%, 0.75%, 1%, 1.5% and 2% w/v of colloidal chitin.

RESULTS AND DISCUSSION

Isolation and Screening of Chitinolytic Bacteria

A total of 40 isolates were isolated both the soil samples which were enriched in MSM supplemented with 1% chitin powder. On the basis of screening on colloidal chitin agar plates, five isolates demonstrated chitinolytic potential. Amongst five isolates, one isolate which produced a highest zone of clearance was selected for further studies. Morphological and biochemical characteristics of the selected isolate are recorded in Table –1 and Table –2 respectively. Biochemical tests described in the Bergey's Manual of Systematic Bacteriology revealed the isolate belonged to Genus *Bacillus*. The identity of the isolate was further confirmed by performing 16S rRNA technique which showed 98% homology of the isolate to *Bacillus licheniformis*. The morphological and biochemical characteristics of the rest of the isolates are not included in this paper.

Table 1
Morphological Characteristics

Gram's Nature	Motility	Spore
Gram positive	Motile	Sub Terminal spore

Table 2
Biochemical Characteristics

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	Positive
Maltose	Positive
Mannitol	Positive
Xylose	Negative
Tests for Specific Breakdown Products	
Methyl Red	Positive
Voges Proskauer	Negative
Tests to show Ability to utilize particular Substrate	
Starch	Negative
Citrate	Negative
Tests for Metabolism of Proteins and Amino-acids	
Indole Production	Negative
Arginine dihydrolyase	Negative
Gelatin hydrolysis	Positive
Tests for Enzymes	
Catalase	Positive
Oxidase	Negative
Urease	Positive
Nitrate Reduction	Positive
Combined Tests	
Triple Sugar Iron(TSI)	A/A**

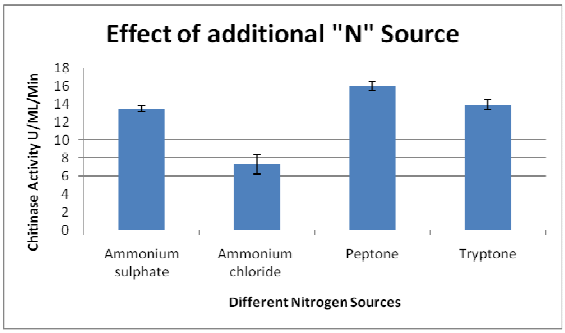
Key: *Utilization of carbohydrate resulted in yellow coloration in the medium, indicating aerobic as well as anaerobic fermentation. ** Alkaline Slant, Acidic butt.

Effect of cultural conditions on chitinase production

Culture medium is responsible for growth of microorganisms and production of an assortment of metabolites. The effect of additional carbon sources (1% w/v) on chitinase production was investigated and although addition of glucose, sucrose, mannitol and Glc-N-Ac to the culture medium enhanced the cell growth of the isolate, it

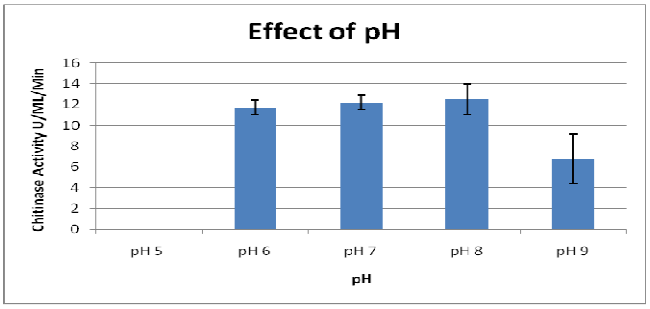
suppressed chitinase production at 1% w/v as compared to use of colloidal chitin as a sole source of carbon (14.38U/ml/min). This report supports the assumption that chitinase production is subjected to stimulatory effect; also the presence of easily available carbon sources suppresses chitinase activity. Overall, the data show that *B.licheniformis* chitinase is inducible and is subjected to catabolite repression by sugars. These finding coincides with previous studies that suggest employing colloidal chitin as sole carbon source for highest chitinase production[18, 19]. Previous report by St Leger *et al*. (1986) suggests the repression of chitinolytic enzymes by addition of additional carbon source such as GlcNAc[20]. The effect of additional nitrogen source (0.5% w/v) on chitinase production is shown in the Graph- 1. Addition of peptone was most effective in stimulating high chitinase production which has been previously reported by S.M Akhir *et al*. (2009) as compared to ammonium sulphate, ammonium chloride and tryptone[21]. Peptone has been reported to be an effective nitrogen source for chitinase production in organisms like *A.xylosoxydans*[22]. A previous report by J-H Huang *et al*. (1996) suggests that organic nitrogen sources are most effective compared to inorganic sources for chitinase production[23]. Similarly, other nitrogen sources such as tryptone, urea and yeast extract have been shown to enhance the chitinase production in organisms belonging to genus *Aeromonas*, *Serratia, Myrothecium* and *Beauveria* [23,24,25,26].

Graph 1
Effect of additional Nitrogen Sources on chitinase production.
Data represent mean ± S.D.

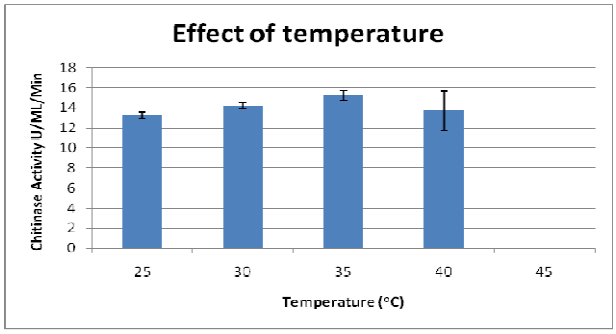


Environmental factors such as temperature and pH play a key role in either enhancing or diminishing enzyme production. The effect of pH on chitinase production is shown in Figure – 4. Neutral or slightly acidic pH has been reported to enhance chitinase production in *Bacillus* species[19]. According to current findings, chitinase production was observed at slightly acidic pH but optimum pH for chitinase production was found to be 8. Similar optimum pH of 8 has been reported for chitinase production in *B. pabuli K1*[27]. The effect of temperature on chitinase production was investigated using different temperatures from 25°C, 30°C, 35°C, 40°C and 45°C. Amongst the temperature range, 35°C was suitable for high chitinase production (Figure - 5). Similar findings have been reported which suggests 35°C to be the optimum temperature for high chitinase production by *Bacillus* spp. Seven different concentrations of colloidal chitin were investigated for chitinase production (0.1% to 2% w/v); low concentrations of colloidal chitin (0.1% and 0.25%) showed negligible chitinase production whereas 1.5% of colloidal chitin recorded highest chitinase production. In contrast to this finding, 0.3% of colloidal chitin is reported as optimum concentration for maximum chitinase production by *B.licheniformis MB-2* [28].

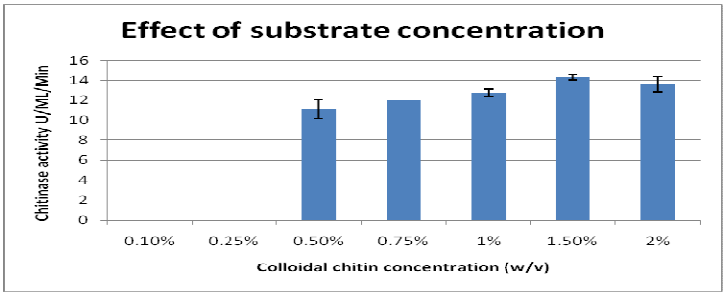
Graph 2
Effect of Initial pH on chitinase production.
Data represent mean ± S.D.



Graph 3
Effect of temperature on chitinase production. Data represent mean ± S.D.



Graph 4
Effect of colloidal chitin concentration on chitinase production.
Data represent mean ± S.D.



CONCLUSION

Chitinolytic organisms have been isolated from various environments. It is still imperative to screen new sources for chitinolytic organisms having industrial applications. In the present study, it was observed that cultural medium and process parameters play a pivotal role in production of chitinase. *Bacillus licheniformis* isolated from local fish market soil has remarkable potential of producing chitinase on an industrial scale. Micro-organisms producing complex set of mycolytic enzymes such as chitinases, glucanases are known to be ideal candidates for biocontrol agents. In these lines, further studies on the strain isolated are in progress to purify and characterize the enzyme, in order to investigate its capability of being biocontrol agent.

ABBREVIATIONS

GlcNAc - *N*-acetyl-*β*-D-Glucosamine, MSM –Minimal Salts Media.

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