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Research Article

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Identification and optimization of cultural conditions for chitinase production by *Bacillus amyloliquefaciens* SM3

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ABSTRACT

A total of 8 chitinolytic bacteria were isolated from 18 marine samples collected from different beaches of Tamil Nadu, India. Among them, a strain designated as Bacillus amyloliquefaciens SM3 which was produced highest chitinolytic activity in primary and secondary screening in colloidal chitin agar. The optimization of colloidal chitin in fermentation medium, temperature, pH, incubation period for the production of chitinase enzyme by this strain was done. A high level of chitinase activity was observed in the nutrient broth with 0.5% colloidal chitin at pH 7.5 and at 35°C after 3 days of incubation. Under optimized conditions, the chitinase produced with was 33.5 Uml^{-1} which was enhanced three folds than unoptimized conditions.

Keywords: Chitinolytic bacteria; *Bacillus amyloliquefaciens*; chitinase; optimization.

INTRODUCTION

Chitin, a insoluble linear β-1,4-linked homopolymer of N- acetylglucosamine, is one of the most abundant natural renewable compounds. It is a nitrogen containing polysaccharide, related chemically to the cellulose [1]. It is a major cell wall constituent of higher fungi belonging of chitidiomycets, ascomycetes, basdiomycetes and deuteromycetes, insect exoskeletons and crustacean shells [2, 3]. Chitinase enzyme (EC 3.2.1.14) has responsible for catalyzing the biological hydrolysis of chitin to its monomer N-acetyl-D-glucosamine and has been found to be produced by various types of microorganisms such as bacteria, fungi, yeast etc. As chitin is degraded by chitinase enzyme, this extracellular hydrolytic enzyme is most promptly used in the biological research as a controlling agent for the generation of fungal protoplasts due to its degrading nature of cell wall [4]. Therefore this enzyme is used as nontoxic alternative to chemical fungicides.

Bacteria produce chitinase to digest chitin and utilize it as carbon and energy source [5]. In the present study, a number of chitin degrading bacteria were isolated from various coastal area of Tamil Nadu, India. Among them, a bacterial isolate which later identified as *Bacillus amyloliquefaciens* SM3 was found to produce high amount of chitinase. Although it has been already reported that *Bacillus cereus* [6], *Bacillus circulans* [7], *Bacillus licheniformis* [8], *Bacillus megaterium* [9], *Bacillus subtilis* [10], *Bacillus stearothermophilus* [11] have the chitinase producing ability.

Studies on medium optimization for chitinase production are a worthwhile technique for multifactor experiments because it is less time consuming and capable of detecting the true optimum level of the factor [12]. Because different medium constituents impact highly on the product of extracellular chitinase from the microorganisms. Therefore in the present work, a chitinolytic bacterial strain (later identified as *Bacillus amyloliquefaciens* SM3) has isolated and chosen for the production of chitinase and various physical and nutritive parameters were screened to ensure the maximum production of chitinase.

EXPERIMENTAL SECTION

Preparation of colloidal chitin

Colloidal chitin was prepared from chitin flakes (Hi-Media) [13]. The chitin flakes were ground to powder and added slowly conc. HCl and kept overnight at 4°C with vigorous stirring. The suspension was added to ice cold 50% ethanol and kept overnight at room temperature with rapid stirring. The precipitate was collected by centrifugation at 8000rpm for 20 min and wash with strile distilled water until the colloidal chitin became neutral (pH 7.0). It was lyophilized and stored in a dark place at 4°C for further application.

Isolation of chitinase producing bacteria

Chitinase producing bacteria were isolated from various coastal area of Tamil Nadu. A total of 18 soil samples were collected and serial dilution (10^{-5} to 10^{-7}) were plated for isolation on 0.5% colloidal chitin agar (CCA) plates which were incubated at 30°C for 48 h. After the incubation period, isolates capable of degrading chitin were screened on the bases of clearance zone: colony size (cz/cs) ratio on CCA plates. So the desired colonies were picked and streaked for culturing pure colonies.

Primary and secondary screening of chitin degrading bacteria

Primary screening was performed by spot inoculating all the chitin degrading bacterial isolates on CCA using toothpick heads of 2 mm diameter and incubated at room temperature. The zone of clearance due to chitin hydrolysis was recorded up to 5 days. The bacterial isolates producing clear zones over 0.5 cm alone were selected and subjected to secondary screening. Secondary screening was performed with the culture filtrates of the 8 selected bacterial isolates using well diffusion method. All the 8 isolates were grown in Nutrient broth (NB) containing 0.1% colloidal chitin. One ml of each test bacterial inoculum with 0.5 OD was inoculated to 100 ml of medium and incubated at 100 rpm in a rotary shaker at room temperature. After two days of incubation, the cultures were harvested, centrifuged at 10000 rpm for 15 min at 4°C and the supernatant was collected. Colloidal chitin (0.5%) agar plates were prepared and wells were made using 9 mm sterile cork borer. Culture filtrate of each isolate was placed at 100 µl in each well and incubated at 37°C. After 12 h, the development of clear zone around the well was observed [14].

Identification of chitin degrading bacterium

To identify the bacterium, a polymerase chain reaction (PCR) was performed to amplify the 16S rRNA gene from the genomic DNA of the strain using universal primers fP1 (5'-GAGTTTGATCCTGGCTCA-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') as described earlier [15]. The nucleotide sequence of the 16S rRNA gene was determined by an ABI Prism DNA sequencer (PE Applied Biosystems, Foster City, CA, U.S.A) and compared with published 16S rRNA sequences using Blast search at GenBank data base of NCBI.

Optimization of culture conditions

Effect of substrate concentration on chitinase production

Bacillus amyloliquefaciens SM3 was grown at different concentration of colloidal chitin to determine the optimum concentration (0.2% - 1.0%) of substrate for chitinase production. Samples were collected at interval of 24h to determine the chitinase activity.

Effect of temperature and pH on chitinase production

To determine the optimum temperature and pH for chitinase production, the bacterium was inoculated in CCA medium at different temperature (20°C- 40°C) and pH (5-10) for 120h. After 5 days of growth, the cultures were harvested, centrifuged and supernatant was used for chitinase assay.

Incubation period

Bacillus amyloliquefaciens SM3 was grown in nutrient broth with optimized growth conditions (0.5% colloidal chitin, pH 7.5 and temperature 35°C) with different incubation period ranges from 1 to 5 days. At each day, the production of chitinase in the culture filtrate was assayed.

Chitinase assay

The reaction mixture contained 1 ml of 0.1% colloidal chitin in sodium acetate buffer (0.05 M, pH 5.2) and 1 ml culture filtrate was incubated at 37ºC for 2 h in a water bath with constant shaking. Suitable substrate and enzyme blanks were included. Chitinase activity was assayed by the colorimetric method of [16]. The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetraborate, pH 9.2 to 0.5 ml of reaction mixture and then boiled in a water bath for 3 min. Then 3 ml of diluted p-dimethylaminobenzaldehyde (p-DMAB, Sigma Chemicals Company, USA) reagent was added and again incubated at 37ºC for 15 min. The released product in the reaction mixture was read at 585 nm in a spectrophotometer (Hitachi, Japan). Chitinase activity was determined using N-acetylglucosamine (Sigma Chemicals Company, USA) as the standard [12]. One unit of chitinase activity was defined as the amount of enzyme, which produces 1 µ mole of N-acetylglucosamine in 1 ml of reaction mixture under standard assay condition [17].

RESULTS AND DISCUSSION

Isolation and identification of chitinase producing bacteria

A total of 8 chitinolytic bacteria isolated from various coastal area of Tamil Nadu were screened on the bases of cz/cs ratio. The clear zones were due to hydrolysis of colloidal chitin by culture filtrates. After the secondary screening, the isolate SM3 was found to be the best chitin degrader and was further analyzed for the rest of the studies. Result of Table 1 revealed that the isolate SM3 is a gram-positive, endospore forming, rod shaped bacterium with Catalase, Voges-Proskauer positive reactions. Based on the physiochemical, morphological characteristics and 16s rRNA sequence analysis, the isolate was identified as *Bacillus amyloliquefaciens* SM3 and the NCBI GenBank Accession No was given JQ999961 for this bacterium.

Table. 1 Morphological, physiological and biochemical characteristics of the isolated strain SM3.

Characters	Results
Morphology	Rod shaped, gram +ve, aerobic and with endospore
Motility	+ve
Catalase	+ve
Urease, oxidase, Dnase	+ve
Voges- Proskauer	+ve
Acid production from glucose, glycerol, sucrose, ribose, starch, lactose	+ve
Hydrolysis of starch	+ve
Hydrolysis of casein	+ve
Degradation of tyrosine	-ve
Hydrolysis of urea	-ve
Formation of Indole	-ve
Nitrate reduction	+ve

Optimization of culture conditions

Optimization of cultural condition for the production of chitinase was carried out using five different concentrations, among these concentrations tested, colloidal chitin at 0.5% considerably enhanced the chitinase activity (15.8U/ml). Fig.1 shows that beyond 0.5%, the substrate concentrations decreased the enzyme activity.

The pH of the culture medium is playing an important role in chitinase production. Majority of bacteria reported to produce maximum level of chitinase at neutral or slightly acidic pH and whereas fungi mostly secret it in acidic conditions [17]. Here among the different pH tested, pH 7.5 favoured the chitinase production at the maximum of 17.9U/ml (Fig.2).

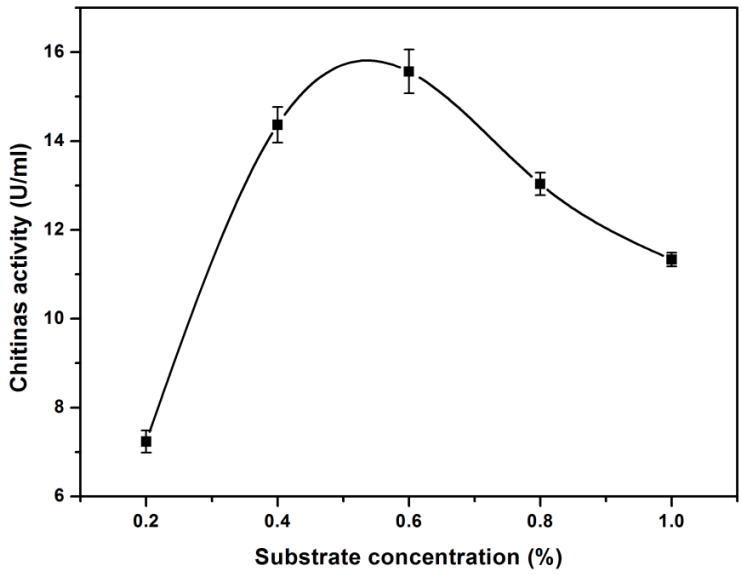

Fig.1 Optimization of substrate concentrations

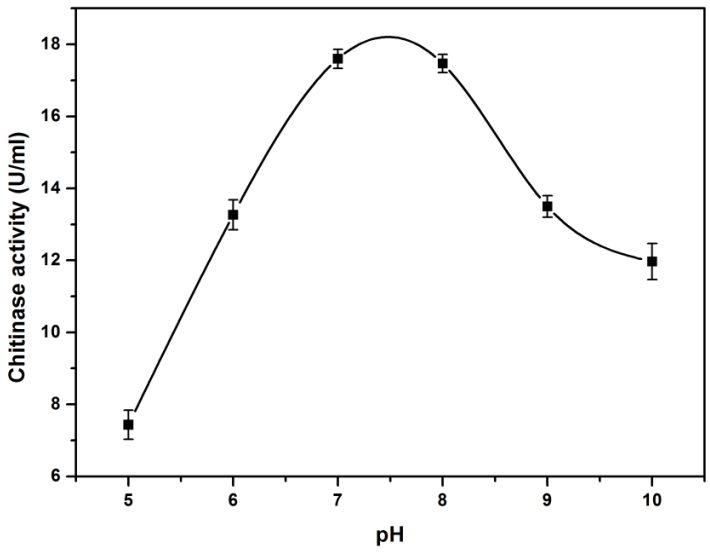

Fig.2 Optimization of pH

Among the different temperature tested, *B. amyloliquefaciens* SM3 was showed maximum chitinase activity of 19.8U/ml at 35°C. It has been observed that the chitinase activity was decreased in both the lower and higher temperatures (Fig.3).

Further it was observed that the production of chitinase enzyme was maximum on 72 h with all the other optimized conditions such as 0.5% colloidal chitin substrate in nutrient broth, pH 7.5 and temperature 35°C. The chitinase activity was declined in subsequent ages (Fig.4).

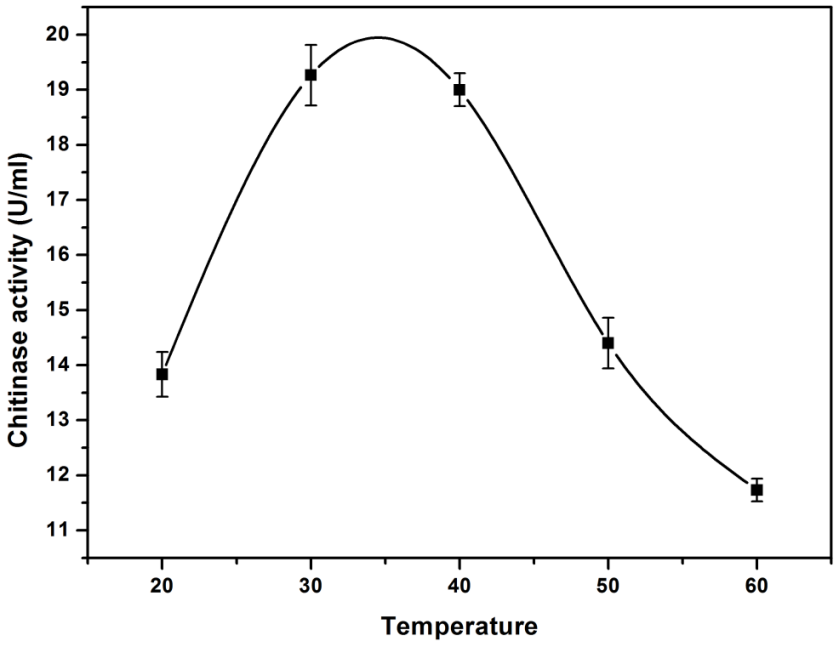

Fig.3 Optimization of temperature

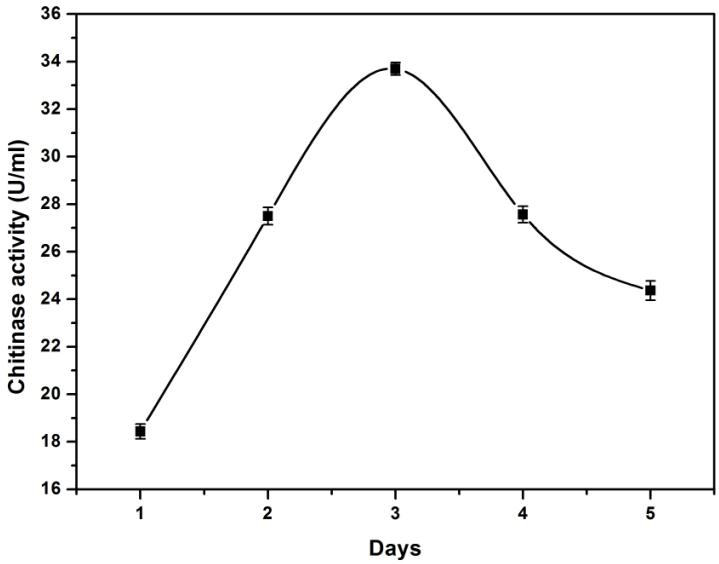

Fig.4 Optimization of incubation period

By optimizing the above cultural conditions, the production of chitinase has been increased to three fold to 33.5 U/ml at the final stage. Analysis of the result has revealed that the potential of any microbial culture could be increased for the production of different metabolites by adapting suitable cultural conditions.

CONCLUSION

From these studies it has been found that the composition of medium plays a crucial role in the production of fermented products and optimization of the culture medium and conditions. The best *Bacillus amyloliquefaciens* SM3 were nutrient broth with 0.5% colloidal chitin, pH 7.5, temperature 35°C with 72 h of incubation. Chitinase is a potent biocontrol agent [4,5,17,19,20]. Although fungal species are the producer of this enzyme but the production

time is larger and the initial cost is also very high, but in case of bacterial strain, the time consuming for production is low and easily can grow and produce huge amount of enzyme. In this line future studies are in progress to purify and characterize the chitinase in order to study its role in the control of phytopathogens.

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