ORIGINAL ARTICLE

# Optimization of Chitinase Production from Lake Sediment Inhabitant *Bacillus thuringiensis* Strain LS1 and *Bacillus cereus* Strain LS2

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## Abstract

**Objective:** Chitinase production from freshwater lake sediment-derived bacteria by submerged fermentation and media optimization. **Methodology:** Isolation of bacteria from freshwater lake sediment by serial dilution followed by spread plate technique on colloidal chitin agar (CCA). The potential bacterial isolates were detected by qualitative cup plate assay and were identified by 16S rRNA sequencing. Optimization of the chitinase production was done by varying different physicochemical factors one at a time keeping the other factors constant. **Results:** Two isolates were selected for chitinase production based on the zone of clearance on CCA and were identified as *Bacillus thuringiensis* strain LS1 (MG948147) and *Bacillus cereus* strain LS2 (MG948148) based on 16S rRNA sequencing. The enhanced production of chitinase by *B. thuringiensis* strain LS1 was observed in minimal medium amended with 1% colloidal chitin, glucose as a carbon source, and malt extract as a nitrogen source, in pH 7.0, at 35°C in 72 h of incubation. The optimal condition for chitinase production by *B. cereus* strain LS2 was minimal medium amended with 1% colloidal chitin, sucrose as carbon source, and yeast extract as nitrogen source with pH 7.0 at 35°C in 96 h of incubation. **Conclusion:** Lake sediment bacterial community was screened for chitinase production and the potential strains were identified and its 16S rRNA sequences were submitted in GENE BANK.

**Key words:** *Bacillus cereus*, *Bacillus thuringiensis*, microbial chitinase, optimization, production

## INTRODUCTION

Chitin is a polymer of N-acetylglucosamine, linked with β-1,4-glycosidic bonds. It is a key portion of cell walls of fungi, exoskeleton of insects, and crustacean shells. Even though its abundance, chitin does not accumulate in the environment due to the presence of chitinolytic enzymes known as "chitinases." Several organisms, including bacteria, fungi, insects, plants, and animals, produce chitinases.[1] Microorganisms, particularly bacteria, form one of the major sources of chitinase.[2]

Chitinase-producing microorganisms occur widely in nature and are preferred source of chitinase production because of their low production cost and easy availability of raw materials in comparison to plant, human, and insect's chitinase. Bacteria such as *Xanthomonas maltophilia*, *Serratia marcescens*, *Stenotrophomonas maltophilia,* and *Paenibacillus illinoisensis* have been proved as potent chitinolytic bacterial agents.[3]

Chitinases are produced by different microorganisms which generally present a wide multiplicity of enzymes that are mainly extracellular. They have established increased attention due to their wide range of biotechnological applications, especially in the production of chitooligosaccharides and N-acetyl D-glucosamine,[4] biocontrol of pathogenic fungi,[5] synthesis of spheroplasts and protoplasts from fungal and yeast species,[6] and bioconversion of chitin waste to single-cell protein.[7]

Microbial chitinases fascinated the consideration as one of the potential enzymes for applications in agriculture, pharmaceutical, waste management, biotechnology, and industry.[8] Their high demand and wide potential use have led to the discovery of new strains of microorganisms that

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are capable to produce enzymes with novel properties and the development of low-cost industrial media formulations.[9] Our study focused on isolation of chitinase-producing bacteria from lake sediment a less explored ecosystem and media optimization for maximum chitinase production.

## METHODOLOGY

### Sample collection

Sediment sample (10g) was collected from the Kolavai freshwater lake at Chengalpattu, Tamil Nadu, India. The sample was collected in a sterile plastic bag and transferred to the laboratory and stored at 4ºC until use for the isolation procedure.

### Isolation of chitin degrading bacteria

Sediment sample was collected from the Kolavai freshwater lake at Chengalpattu, Tamil Nadu, India. The isolation of chitinase-producing bacteria was done by serial dilutions and spread plate technique. A 1 ml of each dilution was plated in triplicates on nutrient agar medium supplemented with 1% colloidal chitin and incubated at room temperature (27°C) for 3 days, and the bacterial growth was observed from the 3rd day onward. The chitinase producers were selected based on the morphology, color, and zone of chitin hydrolysis in the colloidal chitin-incorporated medium.[10]

### Colloidal chitin preparation

Colloidal chitin was prepared from the chitin powder (HiMedia, India) by the method of Mathivanan.[11] Five grams of chitin powder were added slowly to 60 ml of concentrated HCl (10 N HCl) and kept overnight at 40°C with vigorous stirring. The mixture was added to 500 ml of ice-cold 50% ethanol with vigorous stirring at 25°C and kept in the rotary shaker at 200 rpm overnight. The precipitate was collected by centrifugation at 10,000 rpm for 20 min and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). It was stored at 40°C until further use.

### Screening for potential isolate using crude chitinase enzyme

All the selected isolates were grown in nutrient broth containing 1% colloidal chitin and incubated at 100 rpm in a rotary shaker at room temperature for 4 days. After 96 h of incubation, the culture broth was centrifuged at 10,000 rpm for 15 min and the supernatant was collected for screening studies. The screening was performed with the crude enzyme of all the bacterial isolates on 1% of colloidal chitin containing minimal salt medium by well diffusion method. Wells were made on 1% colloidal chitins agar plates using 6 mm sterile cork borer. A 100 µL culture filtrate of each isolate was placed in each well and incubated at 37°C. After 24 h, the development of clear zone around the well was observed (Abirami *et al*.).[12]

### Identification of selected isolates by 16S rRNA sequencing

The sequence of the 16S rRNA gene has been widely used as a phylogenetic marker to study genetic relationships between different strains of bacteria. The analysis of this gene can therefore be considered as a standard method for the identification of bacteria at the family, genus, and species levels[13,14] and has been included in the latest edition of Bergey's manual of systematic bacteriology.[15] Genomic DNA was isolated from the pure culture pellet and approximately 1.4 kb fragments corresponding to 16S rRNA were amplified using universal primers, high-fidelity polymerase chain reaction (PCR). The PCR product was sequenced bidirectionally using the forward and reverse primer. This sequence was compared with the 16S rDNA sequence data from strains available at the public databases (GENBANK, EMBL, and DDBJ) using BLAST sequence match routines.[16] The sequences are aligned using CLUSTALW2 program employing the neighbor-joining algorithm to establish the phylogeny.

### Chitinase enzyme production

From the selected potential isolates, the chitinase enzyme was produced and confirmed by specific enzyme assay for chitinase. The colloidal chitin broth (100 ml) in 250 ml Erlenmeyer flasks was inoculated with 1.0 ml chitinase positive cultures separately and incubated at 37°C for 4 days. The culture broths were centrifuged at 8000× *g* for 20 min and cell-free supernatant was collected. The clear culture filtrates saturated with ammonium sulfate to 60–70% and kept at 40°C overnight to extract the enzymes. The precipitates were collected by centrifugation at 10,000 rpm at 40°C and dissolved in 50 mM phosphate buffer, pH 7.0.[17]

### Measurement of chitinase activity

Enzymatic assay was performed with colloidal chitin as the substrate. Enzyme solution (0.5 ml) was added to 1.0 ml of substrate solution, which contained 0.5% suspension of the colloidal chitin prepared in a phosphate buffer (50 mM, pH 7.0) and the mixture was incubated at 37°C for 15 min. After centrifugation, the quantity of reducing sugars produced in the supernatant was determined by the dinitrosalicylic acid method for the estimation of reducing sugars using N-acetylglucosamine as a standard.[18] One unit of chitinase activity was defined as the amount of the enzyme that produced 1 µmol of reducing sugar per minute.[19]

### Optimization of chitinase production by one variable at a time

Optimization of the chitinase production was done by varying different physicochemical factors one at a time keeping the other factors constant.

### Effect of different culture media on chitinase enzyme production

Three different media, namely, nutrient broth, Luria-Bertani broth, and minimal medium amended with 0.5% colloidal chitin were used to determine the growth and chitinase production of the selected bacterial isolates. One milliliter of strain LS1 inoculum and LS2 inoculum was inoculated with 100 ml of different media and incubated at room temperature in a rotary shaker of 100 rpm. After 3 days of incubation, the culture medium was centrifuged at 1000 rpm for 15 min, the supernatant was used for chitinase assay.[20]

### Effect of different concentrations of colloidal chitin on chitinase production

Strains LS1 and LS2 were grown on different concentrations (0.1, 0.3, 0.5, 0.8, and 1%) of colloidal chitin amended with minimal medium to determine the optimum concentration of substrate (colloidal chitin) for chitinase production.

### Effect of different pH on chitinase production

The effect of pH on chitinase production was determined by strains LS1 and LS2 that were grown at different pH range of 3–10. Acetate buffer (50 mM) was used for pH 3–6; phosphate buffer (50 mM) was used for pH 7; glycine-NaOH (50 mM) for pH 10–11 in minimal medium containing 1% colloidal chitin to determine the optimum pH for chitinase production.

### Effect of temperature on chitinase enzyme production

The effect of temperature on chitinase production was determined by incubating strain LS1 and LS2 on different temperatures of 25°C, 30°C, 35°C, 40°C, and 45°C in minimal medium containing 1% colloidal chitin. The method was used to determine the optimum temperature of chitinase production.

### Effect of incubation period on chitinase production

Strains LS1 and LS2 were grown in minimal medium with optimized growth conditions (1% colloidal chitin, pH 7, and temperature 40°C up to 4 days). Every day, growth and the production of chitinase was assayed in the culture filtrate.[21]

### Effect of different substrate on chitinase production

Strains LS1 and LS2 were grown in minimal media amended with 1% fish shell powder, colloidal chitin, chitin powder under optimized growth condition (pH 7 and temperature 40°C up to 4 days) used to determine the optimum substrate of chitinase production.[21]

### Effect of carbon source on chitinase production

Strains LS1 and LS2 were grown in minimal media amended with 1% colloidal chitin under optimized growth condition (pH 7 and temperature 40°C up to 4 days) inoculated with 1% glucose, fructose, sucrose, and lactose. Simultaneously, media without any carbon source were used as control.

### Effect of nitrogen source on chitinase production

Strains LS1 and LS2 were grown in minimal media amended with 1% colloidal chitin under optimized growth condition (pH 7 and temperature 40°C up to 4 days) inoculated with 1% yeast extract, malt extract, peptone, casein, and ammonium sulfate. Simultaneously, media without any nitrogen source were used as control.

### Partial purification of chitinase

Colloidal chitin broth (100 ml) in 250 ml Erlenmeyer flasks was inoculated with 1.0 ml culture of and incubated at 37°C for 3 days. The culture broths were centrifuged at 8000× *g* for 20 min and cell-free supernatant was collected. The clear culture filtrates were kept at 35°C overnight to extract the enzymes. The precipitates were collected by centrifugation at 10,000 rpm at 40°C and dissolved in 50 mM phosphate buffer, pH 7.0.[17]

## RESULTS AND DISCUSSION

### Isolation of chitinolytic bacteria

The sediment sample was collected from Kolavai freshwater lake, at Chengalpattu, from the serially diluted sediment samples; many morphologically different bacterial strains were obtained. A total of 20 isolates showed clear hydrolysis zone on colloidal chitin agar (CCA) medium [Figure 1]. The heterotrophic bacterial genera, include *Aeromonas, Enterobacter, Chromobacterium, Arthrobacter, Flavobacterium, Serratia, Bacillus, Erwinia,* and *Vibrio, were primarily involved in chitin decomposition in the aquatic environments*.[22,23] Brzezinska *et al*.,[24] 15% of bacteria decomposed chitin in eutrophic lake Chełmżyńskie, but, in the bottom sediments of this lake, a much lower number of chitinolytic microorganisms were identified.

### Screening of chitin degrading bacteria

The culture filtrates of all the selected 20 isolates were checked for chitinase production by well diffusion method. Among that, four bacterial isolates showed zone of clearance over 5 mm in CCA medium. Only two isolates (LS1 and LS2) produced prominent and maximum clear zone of 14 mm and 11 mm, respectively, and they were selected for further optimization studies [Figure 2]. Similar observations were also reported by Abirami *et al*.;[12] the culture filtrates of 2-day-old chitinolytic bacterial types were tested for the presence of chitinase enzyme by well diffusion method. Only two isolates (SSCL10 and SSCL 14) produced prominent and maximum clear zone of 14 mm and 11 mm, respectively, and the remaining 14 isolates ranged between 7 mm and 9 mm zone of clearance in CCA medium.

### Molecular characterization of the potential bacteria

The selected bacteria were identified as *Bacillus thuringiensis* strain LS1 and *Bacillus cereus* strain LS2 by 16S rDNA analysis. The bacterial DNA was isolated and the 16S rDNA sequence was amplified and sequenced. The 16S rDNA sequence of the selected bacteria was obtained and compared with the non-redundant BLAST database to obtain the sequences that displayed maximum similarity. All the sequences reported by BLAST revealed that the bacterial species LS1 and LS2 showed a very high percentage of similarity (98.87%) with the sequences of *B. thuringiensis and B. cereus,* respectively, with a reasonably high score and *E*-value being 0. The sequences showing the maximum similarity were used for alignment using CLUSTAL W2 to derive the phylogenetic relationship [Figure 3].

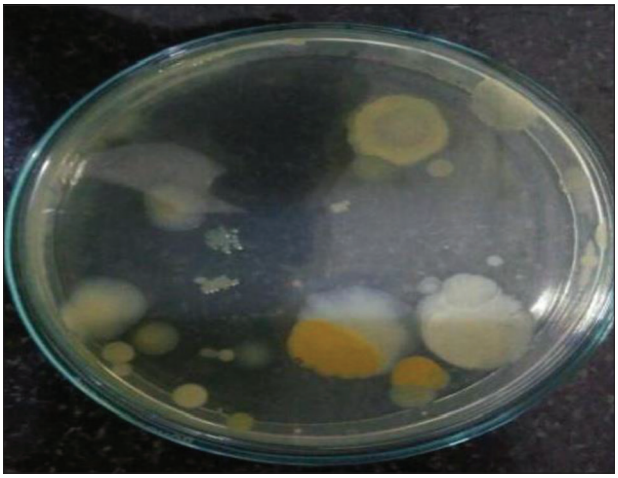

**Figure 1:** Bacterial colonies showing clear hydrolysis zone on colloidal chitin agar


**Figure 2:** Crude enzyme of isolate LS1, LS2, and LS3 showing clear hydrolysis zone on colloidal chitin agar

There exists a clear evolutionary relationship between all the 16S rDNA sequences as this represents a highly conserved sequence. All the taxa under comparison belong to the genera Bacillus and species cereus except for a few species.

The sequence of the bacterium LS1 was shown to be related to *B. thuringiensis* to form a clade with KT714039 and they exhibit a very high similarity (99.87%) and very low *E*-value indicating its closest resemblance to the sister group. The sequence of the bacterium LS2 was shown to be related to *B. cereus* to form a clade with MH210881 and they exhibit a very high similarity (99.86%) and very low *E*-value indicating its closest resemblance to the sister group.

### Effect of different culture media on chitinase enzyme production

Based on the different culture media tested, minimal media inoculated with 1% colloidal chitin showed the highest enzyme production for both the strains LS1 and LS2. The enzyme activity was highly recorded as 1.3 U/ml for LS1 strain and 1.2 U/ml for LS2 strain [Figure 4]. Similarly, the maximal production of extracellular chitinase in minimal media with 0.5% colloidal chitin supported high chitinase production (1.8 Units/ml) as compared to Luria-Bertani (0.8 units/ml) and nutrient broth medium (0.2 units/ml).[12]

### Effect of different concentrations of colloidal chitin on chitinase production

Strains LS1 and LS2 were grown on different concentrations (0.1, 0.3, 0.5, 0.8, and 1%) of colloidal chitin amended with minimal medium. The optimum concentration of substrate (colloidal chitin) for chitinase production was found on 1% colloidal chitin for both the strains LS1 and LS2, and the enzyme activity was recorded as 2.8 U/ml and 2.4 U/ml, respectively [Figure 4]. Our results were also supported by the findings of Souza *et al*.[25] and Karunya *et al*.[26] who reported the maximum chitinase production at 0.3% colloidal chitin.

### Effect of different pH on chitinase production

The effect of pH on chitinase production was determined by strains LS1 and LS2 that were grown at different pH

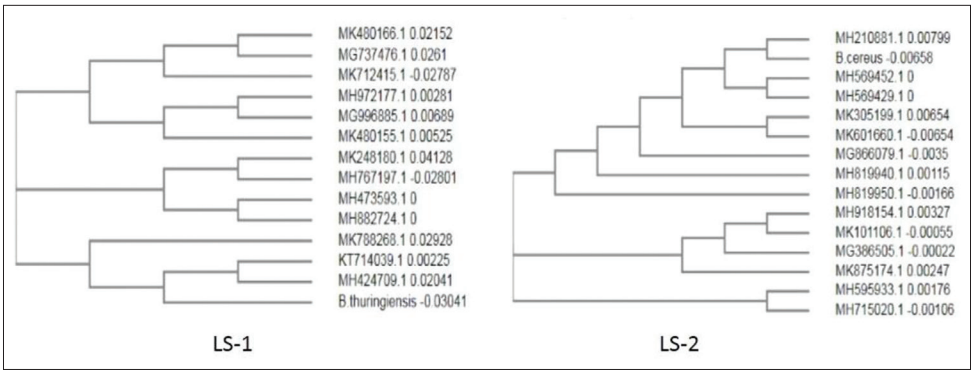


**Figure 3:** Phylogenetic relationship between the isolated bacterial 16S rDNA and the BLAST related sequences derived using CLUSTALW

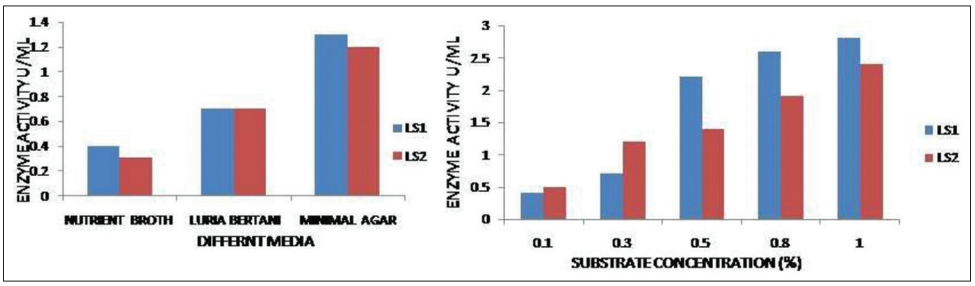


**Figure 4:** Effect of different media and substrate concentration on chitinase production

range of 3–10. The optimum pH for chitinase production was found to be at pH 7 and the enzyme activity was noted as 1.2 U/ml for CL 1 and 1.6 U/ml for CL 3 strain. After pH 7, increase in pH resulted in decreased enzyme activity [Figure 5]. The pH of the culture medium is playing an important role in chitinase production. Majority of the bacteria reported to produce maximum level of chitinase at neutral or slightly acidic pH, whereas fungi mostly secret it in acidic conditions.[27]

### Effect of temperature on chitinase enzyme production

The optimum temperature of chitinase production was determined as 40°C for LS1 with enzyme activity of 1.8 U/ml and 35°C for LS2 with enzyme activity of 2.4 U/ml. After 40°C, the growth was found to decrease gradually [Figure 5]. Our results were close with the chitinase producer *B. thuringiensis* sp. *kurstaki* HD-1(G) as reported by other researchers[28,29] with maximum chitinase production at 37°C.

### Effect of incubation period on chitinase production

Strains LS1 and LS2 were grown in minimal medium with optimized growth conditions (1% colloidal chitin, pH 7, 40°C for LS1 and 35°C for LS2 up to 4 days). Every day, the production of chitinase was assayed in the culture filtrate. Maximum enzyme production was found on 72 h for strain LS1 with an enzyme activity of 1.6 U/ml and 96 h for strain LS2 with an enzyme activity of 2.0 U/ml [Figure 5], which was within the range of the previous report by the bacterium *Chitiolyticbacter meiyuanensis* SYBC-H. Initially, chitinase yield was very low, and after a lag phase of near 48 h, chitinase yield gradually increased. After about 84 h of incubation, chitinase reached the maximum yield.[30]

### Effect of incubation of different substrates on chitinase production

Strains LS1 and LS2 were grown in minimal media amended with 1% of colloidal chitin, chitin powder, and fish shell powder under optimized growth condition (pH 7 and temperature 40°C up to 4 days). The optimum substrate for chitinase production was found to be colloidal chitin for both the strains LS1 and LS2 with an enzyme activity of 1.4 U/ml and 1.0 U/ml, respectively [Figure 6]. Similar observation has also been reported by Kuddus and Ahmead;[21] colloidal chitin was found to be the best substrate for chitinase production by both the strains 40.74 and 49.6 U/ml by *Aeromonas hydrophila* HS4 and *Aplysia punctata* HS6, respectively. It was assumed that chitin and its degradation products played a role as an inducer system in stimulating the production of chitinase, as reported.[31]

### Effect of carbon source on chitinase production

Strains LS1 and LS2 were grown in minimal media amended with 1% colloidal chitin under optimized growth condition (pH 7 and temperature 40°C up to 4 days) inoculated with

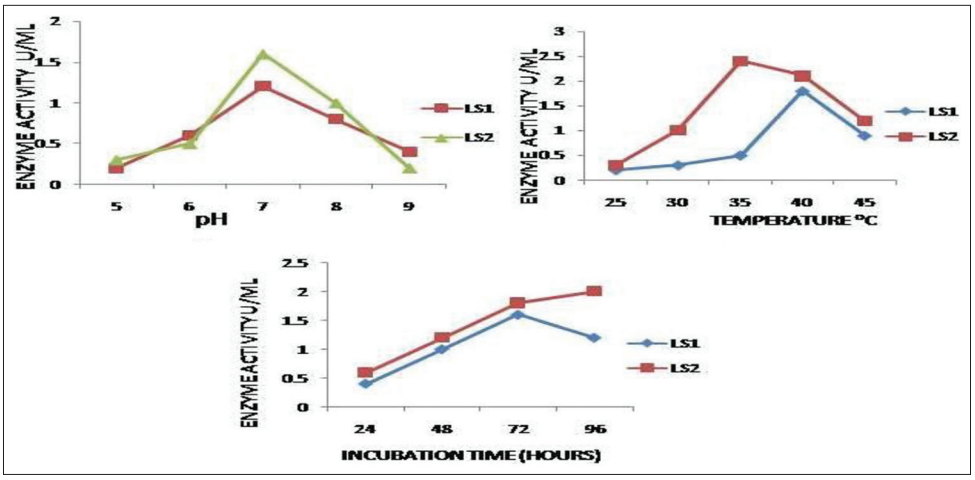


**Figure 5:** Effect of different pH, temperature, and incubation time on chitinase production

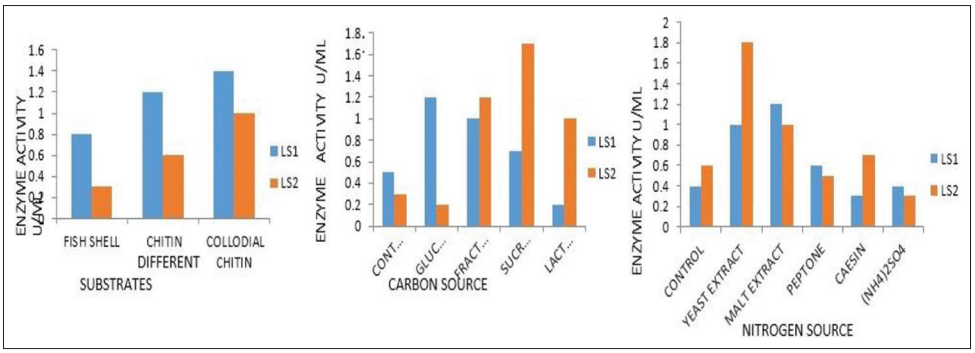


**Figure 6:** Effect of different substrates, carbon source, and nitrogen source on chitinase production

1% glucose, fructose, sucrose, and lactose. Every day, growth and the production of chitinase were assayed in the culture filtrate. Maximum enzyme production was found on glucose for strain LS1 with an enzyme activity of 1.2 U/ml and sucrose for strain LS2 with an enzyme activity of 1.7 U/ml [Figure 6]. Like that in a previous study, sucrose enhanced the chitinase production in *Bacillus* spp.[32] Wanga *et al*.[33] reported that the addition of simple sugars such as glucose and fructose enhanced the chitinase activity, by *S. marcescens* strain JPP1.

### Effect of nitrogen source on chitinase production

Strains LS1 and LS2 were grown in minimal media amended with 1% colloidal chitin under optimized growth condition (pH 7 and temperature 40°C up to 4 days) inoculated with 1% yeast extract, malt extract, peptone, casein, and ammonium sulfate. Every day, growth and the production of chitinase were assayed in the culture filtrate. Maximum enzyme production was found on malt extract for strain LS1 with an enzyme activity of 1.2 U/ml and yeast extract for strain LS2 with an enzyme activity of 1.8 U/ml [Figure 6]. Similarly, malt extract enhanced the chitinase production in *A. hydrophila* HS4 (86.01 U/ml) and yeast extract in *A. punctate* HS6 (82.64 U/ml).[21]

## CONCLUSION

From this work, we have isolated the fresh water lake bacteria *B. thuringiensis* strain LS1 and *B. cereus* stain LS2 for chitinase production. The optimization suggests the optimal value for each variable of isolate LS1 and LS2 for the enhanced production of chitinase. Our attempt makes us to discover two bacterial isolates capable to produce chitinase enzymes. The characterization and novel properties of the enzyme have to be studied for better application.

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## AUTHOR'S CONTRIBUTIONS

The authors Dr. Sudha have supervised this research work, whereas the coauthor Priyanka Sharon, Revathy Yadav, and Sherly Priyanka have been materially participated in this work. Besides, all authors are contributed in preparation of the manuscript, experimental, and interpretation of data.

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