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. Microorganisms, particularly bacteria, form one of the major sources of chitinase.

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.

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, biocontrol of pathogenic fungi, synthesis of spheroplasts and protoplasts from fungal and yeast species, and bioconversion of chitin waste to single-cell protein.

Microbial chitinases fascinated the consideration as one of the potential enzymes for applications in agriculture, pharmaceutical, waste management, biotechnology, and industry. Their high demand and wide potential use have led to the discovery of new strains of microorganisms that...
are capable to produce enzymes with novel properties and the development of low-cost industrial media formulations. 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 4°C 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.

Colloidal chitin preparation

Colloidal chitin was prepared from the chitin powder (HiMedia, India) by the method of Mathivanan. 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 supernantant 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.

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 and has been included in the latest edition of Bergey’s manual of systematic bacteriology. 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. 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.

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-acetylgalcosamine as a standard. One unit of chitinase activity was defined as the amount of the enzyme that produced 1 µmol of reducing sugar per minute.
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% yeast extract, malt extract, peptone, casein, and ammonium sulfate. 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 Chelmżyńskie, 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 SSCL14) 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].

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.

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
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.\cite{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\cite{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.\cite{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 Ahmed;\cite{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.\cite{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
Sudha, et al.: Optimization of chitinase production from Bacillus spp.

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 1.7 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.

ACKNOWLEDGMENTS

The authors are grateful to the management and staff of Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu, India.

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.
## REFERENCES

1. Adrangi S, Faramarzi MA. From *Bacteria* to human: A journey into the world of chitinases. Biotechnol Adv 2013;31:1786-95.

2. Bhattacharya D, Nagpure A, Gupta RK. Bacterial chitinases: Properties and potential. Crit Rev Biotechnol 2007;27:21-8.

3. Jung WJ, An KN, Jin YL. Biological control of damping off caused by *Rhizoctonia solani* using chitinase-producing *Paeonibacillus illinoisensis* KJA-424. Soil Biol Biochem 2003;35:1261-4.

4. Pichyangkura R, Kudan S, Kutiyyawong K, Sukwattanasint M, Alba S. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. Carbohydr Res 2002;337:557-9.

5. Chernin LS, De la Fuente L, Sobolev V, Haran S, Vorgias CE, Oppenheim AB, *et al*. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. Appl Environ Microbiol 1997;63:834-9.

6. Balasubramanian N, Juliet GA, Srikalaivani P, Lalithakumari D. Release and regeneration of protoplasts from the fungus *Trichothecium roseum*. Can J Microbiol 2003;49:263-8.

7. Dahiya N, Tewari R, Tiwari RP, Hoondal GS. Chitinase production in solid-state fermentation by *Enterobacter* sp. NRG4 using statistical experimental design. Curr Microbiol 2005;51:222-8.

8. Gupta R, Saxena RK, Chaturvedi P, Virdi JS. Chitinase production by *Streptomyces* indicus: Its potential in fungal cell wall lysis. J Appl Microbiol 1995;78:378-83.

9. Saito S, Suzuki S, Takayanagi T. Nested PCR-RFLP is a high-speed method to detect fungicide-resistant *Botrytis cinerea* at an early growth stage of grapes. Pest Manag Sci 2009;65:197-204.

10. Anand P, Chellaram C, Shanthini F. Isolation, and screening of marine *Bacteria* producing antibiotics against human pathogens. Int J Pharma Biosci Technol 2011;2:1-15.

11. Mathivanan N. Studies on Extracellular Chitinase and Secondary Metabolites Produced by *Fusarium Chlamydosporum*, An Antagonist to *Puccinia Arachidis*, the Rust Pathogen of Ground Nut. India: University of Madras, Ph. D. Thesis; 1995.

12. Abirami S, Yogalsakshmi K, Pushpa AS, Kananan M. Screening and identification of chitin degrading *Bacteria* from shrimp shell waste dumping soil environment and its media optimization for chitinase enzyme production. World J Pharm Pharm Sci 2016;5:743-57.

13. Woese CR. Bacterial evolution. Microbiol Rev 1987;51:221-71.

14. Garrity G. AAVV: Bergey's Manual of Systemic Bacteriology. London: Springer; 2005.

15. Procópio RE, Araújo WL, Maccheroni W Jr., Azevedo JL. Characterization of an endophytic bacterial community associated with *Eucalyptus* spp. Genet Mol Res 2009;8:1408-22.

16. Sette LD, Passarini MR, Delarmelina C, Salati F, Duarte MC. Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. World J Microbiol Biotechnol 2006;22:1185-95.

17. Bansode VB, Bajekal SS. Characterization of chitinases from microorganism isolated from Lonar Lake. Indian J Biotechnol 2006;5:357-63.

18. Imoto T, Yagishita K. A simple activity measurement of lysozyme. Agric Biol Chem 1971;35:1154-6.

19. Mathur A, Rawat A, Bhatt G, Baweva S, Ahmad F, Grover A, *et al*. Isolation of *Bacillus* producing chitinase from soil: Production and purification of chito-oligosaccharides from chitin extracted from fresh water crustaceans and antimicrobial activity of chitinase. Recent Res Sci Technol 2011;3:1-6.

20. Toharisman A, Suhartono MT, Spindler-Barth M, Hwang JK, Pyun YR. Purification and characterization of a thermostable chitinase from *Bacillus licheniformis* Mb-2. World J Microbiol Biotechnol 2005;21:733-8.

21. Kuddus SM, Ahmed RI. Isolation of novel chitinolytic *Bacteria* and production optimization of extracellular chitinase. J Genet Eng Biotechnol 2013;11:39-46.

22. Donderski W, Trzebiatowska M. Influence of physical and chemical factors on the activity of chitinases produced by planktonic *Bacteria* isolated from Jeziorkar Lake. Pol J Environ Stud 2000;9:77-82.

23. Brzezinska MS, Donderski W. Chitinolytic bacteria in two lakes of different trophic status. Pol J Ecol 2006;54:295-301.

24. Brzezinska MS, Lalke-Porczyk E, Donderski W, Walczak M. Occurrence and activity of microorganisms in shrimp waste. Curr Microbiol 2008;57:580-7.

25. Souza RF, Soares RM, Nascimento RP, Coelho RR, Gomes RC. Effect of different carbon sources on endochitinase production by *Colletotrichum gloeosporioides*. Curr Microbiol 2006;51:16-21.

26. Karunya SK, Reetha D, Saramraj P, Milton DJ. Optimization and purification of chitinase produced by *Bacillus subtilis* and its antifungal activity against plant pathogens. Int J Pharm Biol Arch 2011;2:1680-5.

27. Sharaf EF. A potent chitinolytic activity of *Alternaria alternata* isolated from Egyptian black sand. Pol J Microbiol 2005;54:145-51.

28. Wiwat C, Thaithanun S, Pantowatana S, Bhumiratana A. Chitinolytic activities in *Bacteria* isolated from Egyptian black sand. Pol J Microbiol Biotech 2006;22:1185-95.

29. Liu M, Cai QX, Liu HZ, Zhang BH, Yan JP, Yuan ZM. Chitinolytic activities in *Bacillus thuringiensis* and their synergistic effects on larvicidal activity. J Appl Microbiol 2002;93:374-9.

30. Hao Z, Cai Y, Liao X, Zhang X, Fang Z, Zhang D. Optimization of nutrition factors on chitinase production from a newly isolated *Chitinolyticbacter meiyuanensis* SYBC-H1. Braz J Microb 2012;43:177-86.

31. Felse PA, Panda T. Production of microbial chitinases:
A revisit. Bioprocess Eng 2000;23:127-34.
32. Prakash B, Perumal P, Gowrishankar J, Sivasankari P, Ashokkumar L, Tamilmani P. Optimization of cultural conditions for production of chitinase by Bacillus sp. Isolated from agriculture soil using substrate as marine crab shell waste. Int J Curr Microbiol Appl Sci 2015;4:192-8.
33. Wang K, Yan PS, Cao LX. Chitinase from a novel strain of Serratia marcescens JPP1 for biocontrol of aflatoxin: Molecular characterization and production optimization using response surface methodology. Biomed Res Int 2014;2014:482623.

Source of Support: Nil. Conflicts of Interest: None declared.