



PRODUCTION, PURIFICATION AND CHARACTERIZATION OF A THERMOPHILIC ALKALINE PROTEASE FROM *BACILLUS LICHENIFORMIS* MS12

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A thermophilic bacterial isolate MS12, producing extracellular thermostable alkaline protease was isolated from Manikaran hot water spring of Himachal Pradesh (India). This thermophilic bacterial isolate MS12 was studied morphologically and biochemically, followed by sequencing of its 16S rRNA gene. BLASTn search analysis of the sequence showed maximum identity with *Bacillus licheniformis* BCRC 11702 and the G+C content was found to be 55.6%. *Bacillus licheniformis* MS12 bacterial isolate was quantitatively screened for protease activity and it was found to show maximum thermostable protease activity of 4.7 U/ml. The optimum yield and the maximum protease activity was achieved at 24 hours of incubation period, with the pH 9.0 at 60°C. The desired protein was precipitated and purified from the crude extract by using ammonium sulfate (20-80%), Sephadex G-100 column and Anion exchange chromatography. The procedure yielded 3.63 mg protein with 29.04 fold purification with a percent yield 15.48% and molecular mass of the enzyme was determined to be 29 kDa by SDS-PAGE.

With the advent of new frontiers in biotechnology, the spectrum of protease applications has expanded into many new fields that execute a wide variety of functions and are thus employed in many important biotechnological applications¹. Proteases are protein hydrolases, also known as proteinases or proteolytic enzymes which catalyze peptide bond hydrolysis in protein or peptide substrate. These are the single class of enzymes which occupy a great position with respect to their application in both physiological and commercial fields. Proteases are used in various industrial processes as detergents, pharmaceuticals, leather, meat tenderizers, protein hydrolyzates, food products and even in the waste processing. Various *Bacillus* spp., the most widely exploited alkaline proteases producer, are often commercially used in bioremediation mixes, or as probiotic agent in aquaculture. A large number of thermostable enzymes from many genera have been reported such as *Chromohalobacter* spp.², *Streptomyces*³, *Geobacillus* spp.⁴⁻⁵, *Bacillus* spp.⁶⁻¹². However, *Bacillus* spp. has remained as major sources of enzymes of industrial and commercial value. In the present investigation, we hereby report about the thermostable alkaline protease producing bacterium *Bacillus licheniformis* MS12 from Manikaran hot water spring, India.

MATERIALS AND METHODS

Isolation and screening of thermostable protease producing thermophilic bacteria: Samples from different

sites of the hot water spring of Manikaran were collected in sterilized screw capped vials and jars and were brought to the laboratory and kept at 4°C in refrigerator till further processing. One gram of soil sample/one ml of water sample was added to 50 ml of skim milk broth, containing glucose (1g), yeast extract (2.5g), casein (5g), skim milk (1 g) and incubated at 60°C for overnight under shaking. One ml of enriched sample was serially diluted to 10⁻³ to 10⁻⁶ times with 0.85% (w/v) physiological saline. Each of the diluted culture was uniformly spread on the surface of solidified skim milk agar medium (2% agar) and these were incubated in covered water bath incubator for 24 hours in autoclavable polythene bags to prevent drying out. After 24 hours, morphologically distinct colonies were sub cultured on respective agar plates to get pure isolated colonies. A clear zone of casein hydrolysis gave an indication of thermostable protease producing organisms. One particular isolate with high zone of clearance was purified by subculturing on fresh plates. The pure culture was maintained on slants and stored at 4°C for further use.

Characterization of bacterial isolate: Selected thermophilic bacterial isolate was studied for various morphological characteristics viz., colour, gram reaction, shape, spore formation and motility¹³. Various biochemical tests were carried out for the biochemical characterization of selected bacterial isolate viz., catalase test, urease test, oxidase test, indole test, citrate test, MR-VP test and fermentation of sugars¹⁴⁻¹⁵.

All the biochemical test reagents were procured from Himedia, Mumbai (India).

Genomic DNA extraction: Thermophilic bacterial culture was inoculated into 20 ml skim milk broth and incubated at 60°C overnight. Culture was centrifuged at 14000 rpm for 5 min, cell pellet was washed two times with distilled water, then used for DNA isolation using Genomic DNA extraction Mini-Kit (Real Genomics) according to manufacturer's instructions.

PCR amplification of the 16S rRNA gene: The PCR amplification of the 16S rRNA gene from purified genomic DNA was carried out in 0.2 ml PCR tubes with 20 µl reaction volume by using universal primers viz., B27F 5'-AGAGTTTGATCCTGGCTCAG-3' and U1492R 5'-GGTTACCTTGTTACGACTT-3' and all the amplifications was performed using thermal cycler (MultiGene PCR system, Labnet) and with a temperature profile standardized for 16S rRNA gene amplification.

Sequencing analysis: The PCR product obtained through amplification with universal primers targeting 16S rRNA gene was sequenced, using same upstream and downstream primers, by a commercial sequencing facility (Xcleris lab). The sequence of the bacterial isolate after sequencing was blasted using online NCBI BLAST program¹⁶. Phylogenetic analysis were used for comparative genomics to show evolutionary relationships. The analysis began with aligning of sequences using tools like Clustal W and after alignment, phylogenetic tree was constructed with MEGA version 6.0 using the neighbor-joining method. The partial 16S rRNA gene sequence of the isolate was submitted to GenBank database.

Optimization of culture conditions: The thermophilic bacteria was cultured in different medium, different pH, incubation temperature and incubation time to enhance the growth of thermophilic bacterial isolate which in turn leads to increase in enzyme production. Culture media, tried for the optimization of thermostable protease producing bacterial isolates were nutrient agar (NA) containing 1% skim milk, skimmed milk medium¹⁷ and minimal synthetic medium with skim milk¹⁸. The pH range was optimized at 60°C for 24 hrs using standardized medium adjusted from 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 separately and temperature range for incubation investigated varied from 40, 50, 60 and 70°C whereas, effect of different incubation times for the growth of

these thermophilic bacteria was studied for 24, 48, 72, 96 and 120 hrs. In all cases optical density was monitored at 540 nm on a double beam UV/VIS scanning spectrophotometer.

Production of thermostable protease enzyme: 1% concentration of inoculum (overnight culture) would be inoculated into the skim milk broth of pH 9.0, for thermostable protease production followed by incubation at 60°C for 24 hrs at 150 rpm. The cells were separated by centrifugation at 10,000 rpm, 4°C for 10 mins and the culture supernatant obtained by centrifugation was used as crude extracellular enzyme. Protein concentration estimated in crude enzyme extract by standard Lowry's method¹⁹.

Protease assay: Protease assay of the enzyme with casein as the substrate, was determined by the modified method of Kunitz²⁰. 200 ml of the enzyme solution was added to 800 ml of the substrate solution (0.5 % casein in 0.1 M Tris-HCl buffer) and the mixture was incubated at 60°C for 15 min. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid (TCA) and the sample was incubated at room temperature for 15 min. Followed by centrifugation at 12,000 rpm for 10 min, the absorbance of the supernatant was measured at 280 nm. A control was run simultaneously, in which TCA was added prior to the addition of enzyme solution.

Ammonium sulphate precipitation: Precipitation of thermostable protease enzyme was performed at a temperature of 4°C using 0.1 M Tris-HCl buffer, pH 9.0 and the technique used was ammonium sulfate precipitation. The cell free extract was subjected to various concentration of ammonium sulfate (0-80%). Ammonium sulfate was added with continuous stirring and stored at 4°C for overnight. The precipitated proteins were recovered by centrifugation at 20,000 rpm for 20 mins and the pellet was dissolved in 0.1 M Tris-HCl buffer (pH 9.0). The dialysis was carried out for 24 hrs against three successive changes of dialysis buffer (Tris-HCL buffer) in dialysis bag.

Sephadex G-100 Column Chromatography: The protein pellet obtained after saturation with 0-80% ammonium sulfate and dissolved in 0.1 M Tris-HCl buffer, pH 9.0 was passed through a Sephadex G-100 Column (1.5cm x 27 cm) which had been previously equilibrated with 0.1 M sodium phosphate buffer, pH 9.0. The column was eluted at a flow rate of 0.5 ml min⁻¹ using same buffer. Fractions (3 ml) were collected, and

active fractions were pooled.

Anion-exchange chromatography: The DEAE-cellulose anion exchange column (1x8 cm) was washed five times with 1.0 M NaOH followed by washing with distilled water. Then the column was equilibrated with low ionic strength sodium phosphate buffer (0.1 M, pH: 9.0). The column was loaded with pooled sample from previous step. The unbound molecules were washed off with sodium phosphate buffer (0.1 M), pH 9.0. The bound proteins were eluted by using high ionic strength buffer containing 0.5 M NaCl and 1.0 M NaCl. The flow rate of the eluent was adjusted to 0.5 ml/min and fractions of 3 ml each were collected. Fractions with high protease activity were pooled and used to determine molecular weight of extracellular protease of thermophilic bacterial isolate MS12, electrophoretically.

SDS polyacrylamide gel electrophoresis: The molecular mass of the partially purified protease from MS12 isolate was determined by SDS-PAGE as described by Laemmli²¹ using 12.5% acrylamide gel. Gels were stained with Coomassie

brilliant blue G-250 and destained with 10% methanol and 10% acetic acid. The standard protein was used as protein molecular weight marker for SDS-PAGE for estimation of molecular size of polypeptides of protease of the selected isolate.

RESULTS AND DISCUSSION

Isolation and screening of alkaliphilic proteolytic bacteria: For the isolation of extracellular thermostable protease producing thermophilic bacteria, samples were collected from Manikaran hot water spring, India and isolations were performed using skim milk agar media. Skim milk medium has been shown to be the best medium for the isolation of thermostable protease producing bacterial isolate *Bacillus licheniformis* MS12, using which putative thermostable protease producing bacterial isolates could be distinguished by the formation of a zone of clearance, as casein which on hydrolysis by proteolytic microorganisms leads to the formation zone of clearance. Clear zone forming colonies were sub-cultured for the purification of the isolate (Plate-1a). By visual observation, bigger clear zone forming one thermophilic bacterial isolate was selected (Plate-

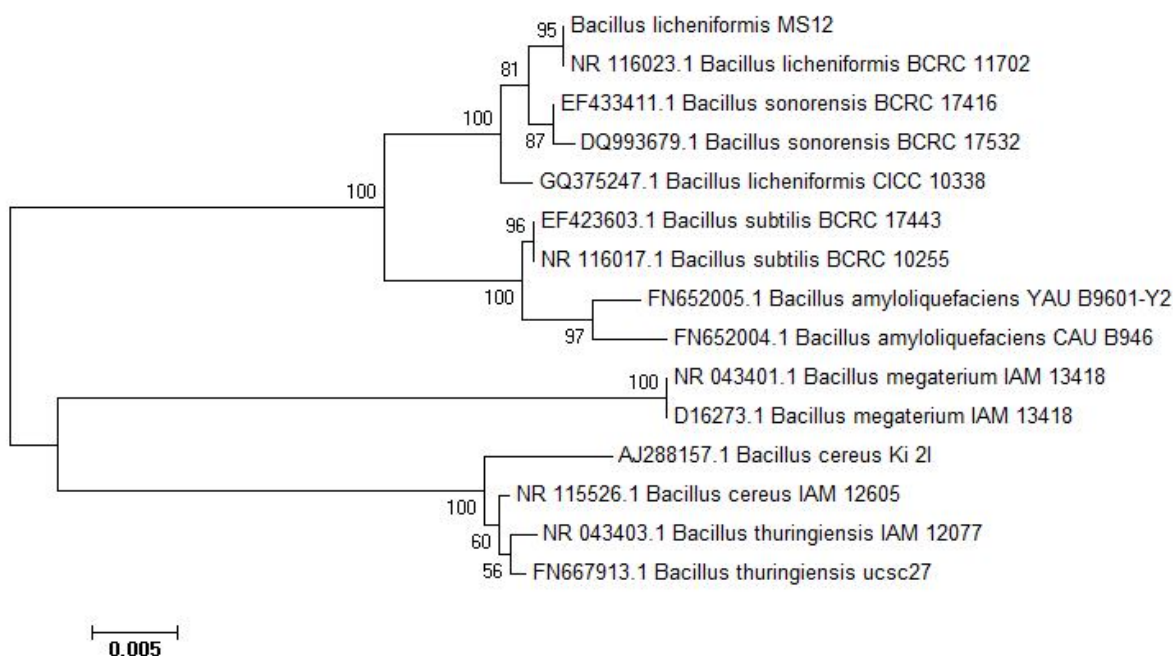


Figure-1. Phylogenetic tree of *Bacillus licheniformis* strain MS12 16S rRNA gene. The GenBank accession numbers of the analyzed sequences are shown and a total of 100 bootstrap replicates were performed, and the bootstrap values are indicated at the branching points.

Table-1. Partial purification of thermostable protease from *Bacillus licheniformis* strain MS12.

Steps	Total Enzyme Activity (U)	Total soluble protein (mg)	Specific activity (U/mg protein)	Fold purification	Percent yield
Crude extract	2642.42	680	3.88	1.0	100
Ammonium sulfate precipitation	1692	253	6.69	1.72	64.03
Gel filtration chromatography	583.2	11	53.01	13.66	22.07
Ion exchange chromatography	408.96	3.63	112.66	29.04	15.48

1b). Clear zone was formed because of the hydrolysis of casein by protease produced from the isolate. Since the fat content of the whole milk inhibits the growth of bacteria, skim milk was used throughout the present study. The widest zone of diameter 12 mm was produced by MS12 bacterial isolate. Similar reports of skim milk medium for successful isolation of thermostable protease producing bacteria have been communicated²²⁻²³. However nutrient with skim milk medium and minimal synthetic medium supplemented with skim milk have also been used for the isolation of thermostable protease producing thermophilic bacteria¹⁸. There are many reports on isolation of protease producing microorganisms from soil²⁴⁻²⁶, tannery waste¹, industrial effluent²⁷⁻²⁸. Adinarayana *et al.*²⁹ reported quantitative screening of alkalophilic *Bacilli* for protease production by zone of clearance or precipitation of casein around the colonies.

Characterization of thermostable extracellular protease producing bacteria : In this study, morphological characteristics and various biochemical tests were performed for the characterization of this thermostable extracellular protease producing isolate. Colony characteristics of this MS12 isolate were observed after growing on skim milk agar plate for 24 hours at 60°C. The isolate was white in colour, rhizoid shape with curled edge. The texture of this isolate was rough with flat elevation. The microscopic characters of the isolate MS12 were also studied and found to have rod shaped cells staining Gram positive and sporulating. The bacterial isolate MS12 produced free oxygen as gas bubbles in catalase reaction

and showed oxidase positive reaction by forming indophenols blue colour. The isolate showed positive results in methyl red and negative for voges-proskauer, indole formation and urease tests. The isolate was able to grow on citrate agar. The isolate also showed positive results in fermentation of sugars viz., glucose, sucrose and lactose.

Identification of bacteria and 16S rRNA gene analysis:

The PCR amplification and sequencing of the 16S rRNA gene of strain MS12 was carried out. The product of PCR amplification was approximately 1500 bp. 16S rRNA gene sequence of strain MS12 when compared with 16S rRNA gene sequences of other isolates, the selected bacterial isolate MS12 was found to belong to genera *Bacillus* (<http://www.ncbi.nlm.gov/BLAST/>). Multiple sequence alignment of MS12 nucleotide sequence was found to possess 99% homology with *Bacillus licheniformis* strain BCRC 11702, 16S ribosomal RNA, partial sequence (NR_116023.1). Finally the phylogenetic relationship of the test isolate with other sequences retrieved from NCBI database using MEGA 6.0 software generated a Neighbor-Joining tree which was divided into many branches and 16S rRNA gene of MS12 bacterial isolate was supported by a bootstrap value of 95 (total of 100 replicates) (Fig.-1). The data of 16S rRNA gene sequence has been submitted to GenBank (GenBank accession no. KF 131538) and the strain has been designated as *Bacillus licheniformis* MS12.

Optimization of the growth conditions of the selected bacteria: There are many *Bacillus* *sps.* in nature, which

produce proteases. There is a little quantitative data on the optimization of production process for extracellular proteases obtained at large scale²⁶. In order to obtain high and commercially viable yields of a microbial protease, it is essential that a suitable over-producing microbial strain is isolated, identified and the factors controlling its growth and protease production are optimized. The production of proteases is influenced in a complex manner by various factors; therefore some important parameters were optimized for extracellular protease production by *Bacillus licheniformis* MS12. Skim milk medium was found to be best for both, growth and thermostable protease enzyme activity as maximum growth O.D. of 1.48 at wavelength of 540 nm and thermostable protease activity of 4.2 U/ml was observed by using this medium. Similar reports of skim milk medium for thermostable protease production have been communicated²²⁻²³. Maximum growth (1.52 growth O.D. at 540nm) was observed after 48 hrs of incubation while maximum thermostable protease activity (4.5 U/ml) was observed after 24 hrs of incubation. In the reports on thermostable alkaline protease production by *Bacillus spp.*, an incubation period of 24 hr to 36hr has been observed to be optimum for enzyme production^{12,30}. Maximum growth (1.46 growth O.D. at 540 nm) and thermostable protease activity of 4.4 U/ml was observed at pH 9.0. Different *Bacillus* spp. have been reported to produce alkaline protease in alkaline range, e.g. *Bacillus subtilis* at pH 7.412, *Bacillus aryabhatai* K3 at pH 8.011. Incubation temperature of 60°C was found optimum for growth as well as maximum thermostable protease activity as maximum growth OD of 1.55 at 540 nm wavelength and maximum thermostable protease enzyme activity of 4.7 U/ml was observed at 60°C temperature. Optimum temperature for different bacterial proteases has been reported to be 50°C-75°C^{12,31}. The selected optimal parameters were used for production of thermostable protease enzyme.

Protease assay: Extracellular thermostable protease activity of the crude enzyme preparation of selected bacterial isolate was determined quantitatively by modified protease assay²⁰. Casein is a milk protein which acts as a high source of aminoacids for growth of bacteria to high cell densities and proteases/thermoproteases degrade this substrate into peptides followed by amino acids. Casein degradation is initiated by extracellular serine protease such as subtilisins of different species of *Bacillus* also has reported similar results of

thermostable protease enzyme activity assay using casein as the substrate³²⁻³³. However, azocasein has also been reported to be used for determining thermostable protease activity²³⁻³⁴. Thermostable protease activity was determined after 24 hrs of incubation for the test bacterial isolate. It was found out that MS12 bacterial isolate showed maximum extracellular thermostable protease activity of 4.5 U/ml after 24 hrs of incubation.

Production and purification of thermostable protease enzyme: Thermostable protease production was carried out for extracellular preparation of the enzyme using all the standardized and selected optimum conditions of medium, temperature, time and pH. 1% primary inoculum size was used to inoculate skim milk broth at pH: 9.0, at 60°C for 24 hrs. Crude extract was used to precipitate thermostable protease enzyme using ammonium sulphate (0-80%) saturation and it was found out that maximum thermostable protease enzyme activity was detected at 20-80% level of saturation. It was purified protease to 1.72 fold with 64.03% protein recovery from *Bacillus licheniformis* strain MS12 obtained from this study (Table-1). Bajaj and Jamwal¹⁰ partially purified thermostable alkaline protease from *Bacillus pumilus* D-6 by ammonium sulphate precipitation at 40-60% level of saturation. Sharma *et al.*¹¹ reported approximately 6.6 fold purification of protease from *Bacillus aryabhatai* K3 by ammonium sulphate precipitation (80%) with a recovery of 54.62% enzyme. The enzyme preparation at this stage was treated as ammonium sulphate fraction (ASF), which then applied to Sephadex G-100 column chromatography. It further purified protease to 13.66 fold with 22.07% protein recovery from *Bacillus licheniformis* MS12 strain obtained from this study (Table-1). Moharam *et al.*³⁵ purified protease from two strains of *Bacillus sephaericus* by using ammonium sulphate fractionation and Sephadex G-100 column chromatography while, Nilegaonkar *et al.*³⁶ recorded 10 folds increase in specific activity of protease when purified using Sephadex G-100 column chromatography. The most active fractions from Sephadex G-100 column chromatography were pooled and dialyzed. The dialyzate was loaded on the DEAE column and the bound sample was eluted with sodium phosphate buffer (0.1 M, pH 9.0) containing 0.1 M NaCl at a flow rate of 0.5 ml/min and fractions of 3 ml each were collected. It further purified protease to 29.04 fold with 15.48 % protein recovery from *Bacillus licheniformis* MS12

strain obtained from this study (Table-1). SDS-PAGE analysis of protease exhibited a single band with molecular mass estimated to be 29 kDa (Plate-2). A varieties of molecular mass for proteases from other *Bacillus* spp. had been reported: 30.9 kDa, 37, 34 kDa, 38, 38 kDa, 39, 49 kDa, 40 and 75 kDa.

CONCLUSION

In the present study, a thermostable alkaline protease producing thermophilic bacteria has been isolated from Manikaran thermal spring, India. This bacteria has been identified as *Bacillus licheniformis* strain MS12 after morphological, biochemical and molecular characterization by 16S rRNA gene technology. The sequence has been submitted to NCBI database under accession number KF938887. The desired protein has been purified by using ammonium sulfate (20-80%), Sephadex G-100 column and Anion exchange chromatography. The procedure yielded 3.63 mg protein with 29.04 fold purification with a percent yield 15.48% and molecular mass of the enzyme has been determined to be 29 kDa by SDS-PAGE.

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