

Thermostable Lipase Production by *Aneurinibacillus thermoaerophilus* MBW2 Strain Isolated from Indian Hot Water Spring

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Four hot water springs viz., Manikaran, Vashisht, Khirganga and Tattapani were purposely selected for isolation of thermostable lipase producing bacterial isolates. The pH and temperature of the four thermal springs were ranged from 4.0-6.0 and 51-105°C respectively. Isolated forty two thermophilic bacterial isolates, were described as putative thermostable lipase producers on the basis of their ability to form zone of clearance on tributyrin agar medium. Quantitative screening led to the selection of MBW2 bacterial isolate showing maximum thermostable lipase activity of 4.83U/ml after 24 hrs of incubation time at 60° C temperature, was selected for morphological, biochemical and molecular characterization. Genomic DNA isolated from the selected MBW2 bacterial isolate was subjected to PCR amplification followed by sequencing using universal primers for 16S *rrna* gene. *In silico* molecular analysis identified MBW2 bacterial isolate as *Aneurinibacillus thermoaerophilus* strain MBW2. Optimum culture conditions for growth and thermostable lipase activity of selected isolate were used for the enzyme production which was purified to 3.08 fold with percent yield of 13.73% using ammonium sulphate precipitation technique, gel filtration chromatography and ion exchange chromatography technique. The molecular weight of the purified enzyme was found to be 42.5 kDa using SDS-PAGE.

Keywords: Thermostable lipase, thermophiles, hot water spring.

Lipase (triacylglycerol hydrolase, E.C. 3.1.1.3) are the enzymes of serine hydrolase family which can catalyze the hydrolysis and synthesis of esters from glycerol and fatty acids. These reactions usually proceed with high chemo-, regio- and/or enantio selectivity at the interface between the insoluble substrate and water¹. The products of lipase-catalyzed reactions have higher quality and their energy consumption is lower compared to the conventional high-temperature and high-pressure-steam splitting methods². Lipases are used widely in different industries such as food

and dairy, detergent, cosmetic, leather, paper and pulp, biodiesel and pharmaceutical³. The major requirement for commercial lipases is thermal stability which would allow enzymatic reaction to be performed at higher temperatures and would be helpful to increase conversion rates, substrate solubility and the viscosity of the reaction medium⁴. Further, thermophilic lipases have special characteristics which are more in interest in industrial processes such as higher stability and more activity in higher temperature and in the presence of chemicals^{5,6,7}. This has drawn the interest towards thermostable lipases in both research and industries.

The enzyme lipase can be isolated from bacteria, fungi, plant, algae and animals⁸. But, microbial enzymes are known to be superior

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enzymes obtained from different microorganisms, particularly for applications in industries on commercial scales⁹. Special characteristics of microbial enzymes include their capability and appreciable activity under abnormal conditions, mainly of temperature. Microorganisms with systems of thermostable enzymes that can function at higher than normal reaction temperatures would decrease the possibility of microbial contamination in large scale industrial reactions of prolonged durations¹⁰. Further, the quality of thermostability in enzymes promotes the breakdown and digestion of raw materials¹¹. Thermophiles growing at the temperature range of 60–100°C have complete thermal equilibrium with the microenvironments and secrete enzymes that are stable at this temperature to support the physiological processes¹². Therefore, the thermophiles can act as reliable source of the thermostable enzymes.

A hot spring is a spring produced by the emergence of geothermally heated ground water that rises from the earth's crust. Hot springs are a store house of a no. of such thermophilic microorganisms Himachal Pradesh, situated in the lap of Himalayas, also have a no. of thermal springs. These springs are habitat for a no of thermophilic microorganisms which can be explored for the synthesis of no of thermostable enzymes at the industrial level. Pursuant to the above, screening of thermophilic microorganisms for lipolytic activities could facilitate the discovery of novel lipases that are stable and function optimally at high temperatures. Therefore, the objective of current study was to isolate, identify and characterize thermophilic bacteria from hot springs of Himachal Pradesh (India) for production of thermostable lipase enzyme along with the purification of the thermostable lipase.

MATERIALS AND METHODS

Sampling

Samples in the form of water, soil, pebbles and rock matings from different sites of Manikaran, Vashisht, Khirganga and Tattapani thermal springs located in the districts of Kullu and Mandi of Himachal Pradesh (India), were collected in sterilized screw capped vials and jars. All these samples were kept at 4°C in refrigerator in laboratory till further experimentation. Parameters viz., pH and

temperature were studied for all the selected sites (Fig.1)

Isolation of the lipase producing thermophilic bacterial isolates

Three different isolation medium viz., tributyrin medium [Peptone (5g/L); Yeast extract (3g/L); Tributyrin (10 ml/L) and Agar (20 g/L) at pH 7], rhodamine medium [Nutrient agar 28g/l, NaCl 4g/l, Rhodamine B (10 mg/l), olive oil (31ml/l) at pH 7] and olive oil medium [Peptone 5g/l, Beef extract (3g/l), NaCl (2g/l), olive oil (60ml/l), twin 80 (10ml/l), Agar (20g/l) at the pH 8] were investigated for the isolation of thermostable lipase producing bacteria. The medium showing maximum growth O.D. at 540 nm was selected for the isolation of thermostable lipase producing thermophilic bacteria. Tributyrin medium was found best for the isolation of the thermostable lipase producing thermophilic bacteria.

Incubation conditions

The different samples were incubated in tributyrin broth with pH 7 at 60°C for 24 hrs in water bath shaker incubator at 150 rpm. The cultures showing growth turbidity were streaked on plates of solidified tributyrin agar medium. Individual colonies were restreaked repeatedly on the same tributyrin medium to get the axenic cultures. The thermostable lipase producing bacterial isolates were screened by the presence of zone of clearance around the colonies.

Quantitative screening of bacterial isolates

Quantitative screening was performed to select the isolate showing maximum thermostable lipase activity after 24 and 48 hrs of incubation.

Determination of thermostable lipase activity

Thermostable lipase activity was determined spectrophotometrically at 420 nm with pNP-laurate as a substrate¹³. The reaction mixture contained 0.1 ml crude enzyme extract (suitable diluted), 0.8 ml of 0.05 M phosphate buffer (pH 8.0) and 0.1 ml of 0.01 M pNP-laurate. The reaction was held at 60°C for 30 mins followed by addition of 0.25 ml of 0.1 M sodium carbonate to stop the reaction. One unit of thermostable lipase activity was defined as the amount of enzyme which liberates 1 µg p-nitrophenol from pNP-laurate as substrate in 30 minutes under standard assay conditions.

Characterization of selected bacterial isolate

The selected isolate was then studied for

various morphological, biochemical and molecular characters.

Morphological and biochemical characterization

Various morphological characters viz., cell shape, size, texture, nature of multicell aggregates, formation of spores, and reaction to the Gram stain were examined for selected bacterial isolate. To carry out biochemical characterization of selected isolate various biochemical tests viz., catalase test, urease test, oxidase test, MR-VP test and fermentation of sugars were performed.

Molecular characterization

The selected thermostable lipase producing bacterial isolate was subjected to molecular characterization using 16S ribosomal DNA (16S rRNA gene) technology.

Extraction of genomic DNA

Thermophilic bacterial culture was inoculated into 20ml tributyrin broth and incubated at 60°C for 24 hrs. Culture was centrifuged at 13000 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

The DNA sample extracted from selected isolate was selectively amplified using PCR technology. Universal primers for 16S rRNA gene was used for the experiment. 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., forward primer (5'-GGTCAGCGGCGGACGGGTGAGTAAC-3') and the reverse primer (5'-GACGGGCGGTGTGTACAGAGGCCCG-3') and all the amplifications were performed using thermal cycler (MultiGene PCR system, Labnet).

Sequence analysis

The PCR product obtained through amplification with universal 16S rRNA gene primers was sequenced, using same upstream and downstream primers, by a commercial sequencing facility (Eurofins lab). Similarity of nucleotide sequence was determined using online available bioinformatic tool, BLAST. The phylogenetic analysis was performed using Clustal W tool¹⁴. These sequence data has been submitted to the GenBank database.

Optimization of growth conditions

Optimization of various culture conditions such as different media, incubation time, pH of the medium and incubation temperature were performed for maximum thermostable lipase enzyme activity as well as for best growth of the selected bacterial isolate. Three different media viz., tributyrin medium (TB), rhodamine medium (RB) and olive oil medium (OB) were investigated for the optimum growth and maximum lipase activity of the selected bacterial isolate. The effect of different incubation times for the growth and maximum lipase activity of the selected thermophilic bacteria was studied for 24, 48, 72, 96 and 120 hrs. The pH range was optimized using optimum medium adjusted to a pH range from 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 separately where as incubation temperature range investigated varied from 40, 50, 60 and 70°C for maximum growth and thermostable lipase enzyme activity. In all cases optical density was monitored on a double beam UV/VIS spectrophotometer.

Production of extracellular thermostable lipase enzyme

1% inoculum (overnight culture) was inoculated into the standardized medium for thermostable lipase production followed by incubation at optimum temperature for an optimum period of time at 150 rpm. The cells were collected by centrifugation at 10,000 x g, 4°C for 10 mins. The resulting cell free supernatant (*i.e.* cell free extract, CFE) was used as crude enzyme for subsequent thermostable lipase purification. Thermostable lipase enzyme activity as well as protein content of crude extract was calculated. The protein content was determined by using Lowry's method¹⁵.

Partial Purification of thermostable lipase enzyme from selected bacterial isolate

The step of purification was performed at a temperature of 4°C using 0.05 M sodium phosphate buffer of pH 8.

Ammonium sulphate precipitation

The cell free culture supernatant was precipitated by using solid ammonium sulphate to 70% saturation. The pellet obtained after centrifugation was dissolved in 0.05ml sodium phosphate buffer (pH 8). The lipase activity and protein content was determined. Then dialysis was carried out for 24 hrs against three successive

changes of dialysis using 0.05 M sodium phosphate buffer in dialysis bag.

Gel filtration column chromatography

The reconstituted fraction was dialysed against same buffer and then loaded on the Sephadex G-100 column. This column was eluted with phosphate buffer and 3 ml fractions were collected. The collected fractions were analyzed for protein content and for enzyme assay. The most active fractions were pooled and stored at 4°C till further purification steps.

Ion exchange chromatography

The pooled enzyme preparation from Sephadex G-100 column was applied to DEAE Sephadex equilibrated with 0.05M phosphate buffer (pH 8). The column was first eluted with phosphate buffer (pH 8) to wash out unbound proteins. The bound proteins were eluted with linear salt gradient using four bed volumes of 0.1M NaCl, 0.2M NaCl, 0.3M NaCl, 0.4M NaCl, 0.5M NaCl, 0.6M NaCl, 0.7M NaCl, 0.8M NaCl, 0.9M NaCl, 1.0M NaCl, 1.1M NaCl, 1.2M NaCl, 1.3M NaCl, 1.4M NaCl, 1.5M NaCl in phosphate buffer (pH 8). The lipase was eluted with 1M NaCl. The fractions were collected and assayed for lipase activity. The specific activity of purified enzyme was compared with that of crude enzyme and purification factor was calculated. The active fraction was pooled, concentrated and analyzed for purity by SDS-PAGE.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis of partially purified thermostable lipase enzyme was performed by using Mini Dual Gel Electrophoresis System (Atto Corporation, Japan) in 10% polyacrylamide gel at 100 V. Gels were stained with Coomassie brilliant blue G-250 and destained with 10% methanol and 10% acetic acid. The standard protein molecular weight marker was used as for estimation of molecular size of polypeptides of

thermostable lipase of the selected bacterial isolate.

Thermostability characterization

Thermophilic characterizations were analyzed at two levels. First, isolated organisms were cultured in high temperature (60°C) and the thermophilic property of this isolated strain and its enzymes, was established. The thermophilic activity of lipase from isolated strain was assayed in the second level by monitoring the lipase activity at different temperatures.

RESULTS

In the present study, the occurrence of thermotolerant bacteria producing thermostable lipase were investigated in the four hot water springs of the state of Himachal Pradesh, India. A total of forty six samples from four hot water springs were collected. The pH and temperature of the four thermal springs were found to range from 4.0-6.0 and 51°C-105°C respectively.

Isolation of thermophilic bacterial isolates

Tributylin medium was selected for the isolation of bacterial isolates as maximum growth O.D. of 1.2 was observed using this medium. Forty two thermophilic bacterial isolates were isolated by using tributyrin medium at 60°C for 24 hours from water and soil samples whereas no isolate was isolated from pebble and rock matting samples. The 42 thermophilic bacterial isolates were identified by their ability of formation of zone of clearance on the tributyrin medium [Fig.2]

Quantitative screening of thermostable lipase activity

Quantitative screening study showed that thermostable activity of 42 isolates were varied from 0.082 U/ml to 4.83 U/ml after 24 hrs and 0.020 to 1.56 U/ml after 48 hrs of incubation. Bacterial isolate MBW2 was found to show maximum

Table 1. Partial purification of thermostable lipase from *Aneurinibacillus thermoaerophilus* strain MBW2

| Steps | Total enzyme activity (U) | Total soluble protein (mg) | Specific activity (U/mg protein) | Fold purification | Percent yield |
|---------------------------------|---------------------------|----------------------------|----------------------------------|-------------------|---------------|
| Crude extract | 546 | 717 | 0.76 | 1.0 | 100 |
| Ammonium sulphate precipitation | 289.5 | 325.4 | 0.89 | 1.17 | 53.02 |
| Gel filtration chromatography | 145.06 | 120.88 | 1.2 | 1.57 | 26.56 |
| Ion exchange chromatography | 75 | 32 | 2.34 | 3.07 | 13.73 |

extracellular thermostable lipase activity of 4.83 U/ml after 24 hrs of incubation and 1.56 U/ml after 48 hrs. Therefore, MBW2 was selected [Fig.3] further for morphological, biochemical and molecular characterization.

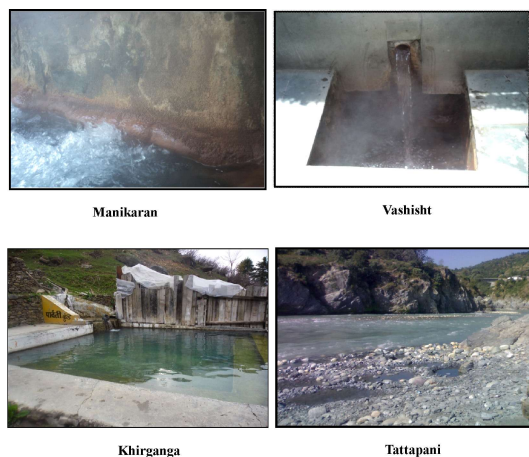


Fig.1. Selected four hot springs

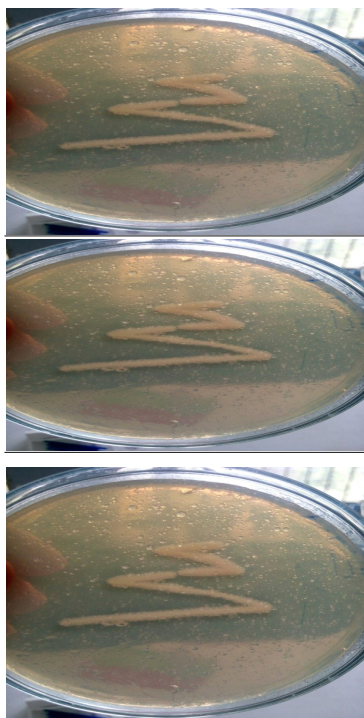


Fig. 2. Zone of clearance around colonies on tributyrin agar medium

Morphological and biochemical characterization

The MBW2 bacterial isolate was found to be creamish in colour, 2.20 mm in colony size and irregular in texture. Microscopic study revealed it a gram positive, single, rod shaped bacteria with spore formation ability. The strain was found to give negative results in reactions with oxidase, urease, indole, VP and lactose fermentation. Positive results were recorded for catalase, methyl-red, citrate and fermentation of glucose and sucrose.

Molecular characterization

16S *rrna* gene amplification

The DNA sample extracted from selected isolate was selectively amplified using PCR technology. After amplification, an amplicon of a size i.e. of 1250 bp was obtained. This amplified DNA was eluted and sequenced.

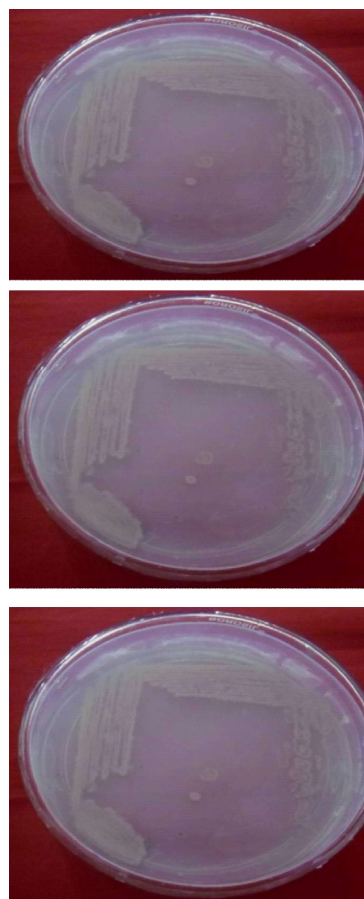


Fig. 3. Selected MBW2 bacterial isolate

In silico analysis

BLAST results of 16S rRNA gene sequence of MBW2 was found to show maximum homology (99%) with *Aneurinibacillus thermoaerophilus* strain L420-91, ribosomal RNA, partial sequence with accession number [NR_029303.1](#). Phylogenetic tree also declared the MBW2 bacterial isolate as *Aneurinibacillus thermoaerophilus* as the isolate MBW2 clustered closely with *Aneurinibacillus*

thermoaerophilus strain L420-91 (NR_029303.1) with boot strap value of 100 [Fig. 4]. Thus the bacterial isolate MBW2 was identified as *Aneurinibacillus thermoaerophilus* strain MBW2. This isolate was isolated from Manikaran hot spring. The sequence has been submitted to NCBI and has been assigned with the accession no. of KF93886.

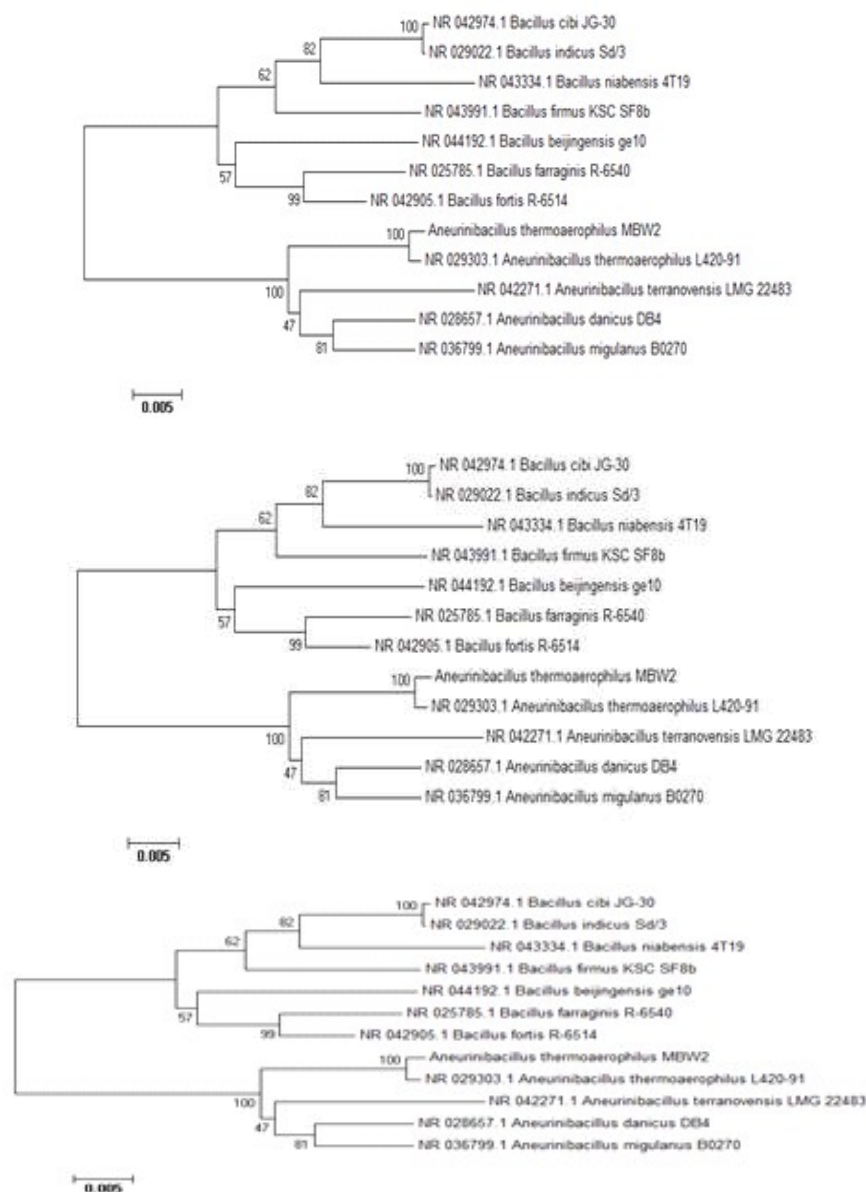


Fig. 4. Phylogenetic analysis of 16S rDNA gene sequences of isolate from Manikaran hot spring

Optimization of culture conditions

The selected bacteria was found to show a maximum growth OD of 2.46 at a wavelength of 540 nm and maximum enzyme activity of 2.44 U/ml using tributyrin broth (Fig.5). It has been observed that bacterial growth increased exponentially depicting an OD of 0.136 at a wavelength of 540 nm after first 24 hrs and then it enhanced up to 48 hrs, thereafter it declined up to 120 hrs (Fig.6). Maximum enzyme activity of 4.83 U/ml was

produced after 24 hrs of incubation followed by a sharp decline up to 120 hrs. A pH of 7 was observed optimum for both, best growth as well as maximum lipase activity (Fig.7).

The growth was found to increase with increase in temperature up to 60°C and then a steady decrease was observed till 70°C however maximum thermostable lipase activity was observed to increase with increasing temperature from 40°-60°C showing maximum enzyme activity

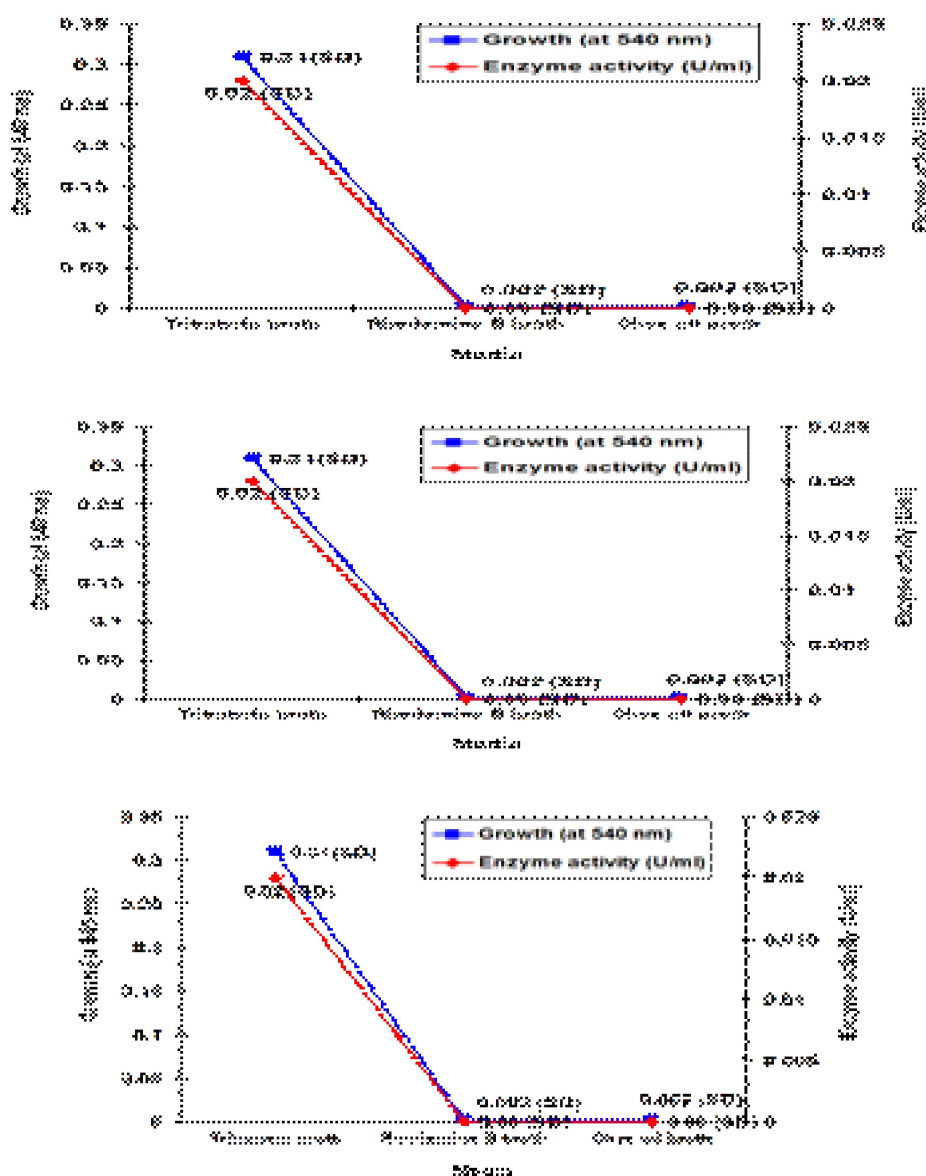


Fig. 5. Effect of medium on growth and enzyme activity

of 4.81 U/ml at 60°C temperature and then after enzyme activity (Fig.7) was decreased at 70°C. Thus the optimum incubation temperature selected for maximum growth and thermostable lipase activity was 60°C.

Production and partial purification

1 % primary inoculum size was used to inoculate tributyrin broth at pH: 7.0 at 60°C for 24

hrs of incubation time. This extracellular thermostable lipase was purified to homogeneity by ammonium sulphate precipitation, sephadex G-100 column chromatography and ion exchange chromatography with a total yield of 13.73% and 3.07 fold purification (Table-1). The pool of the thermostable lipase of last purification step produced a single protein band in SDS-PAGE with

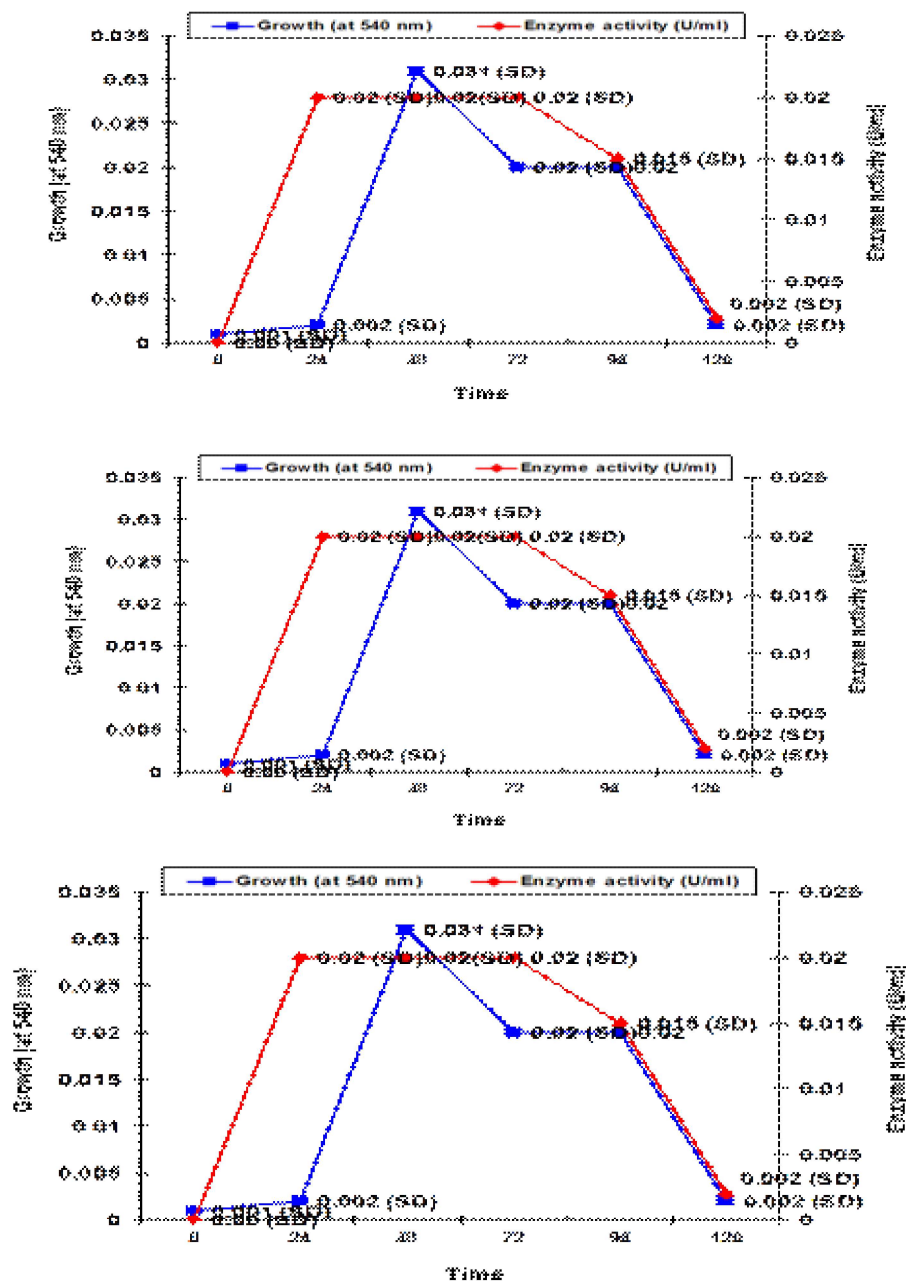


Fig. 6. Effect of incubation time on growth and enzyme activity

a relative molecular mass of 42.5 KDa (Fig. 9).

Thermostability characterization

Thermostability of the partially purified enzyme was studied at different temperature ranging from 40°C to 100°C. The enzyme activity

was found to increase from 40°C up to 60°C. At 60°C the activity was maximum. After that the activity was found to decrease and becomes zero at 90°C (Fig.10)

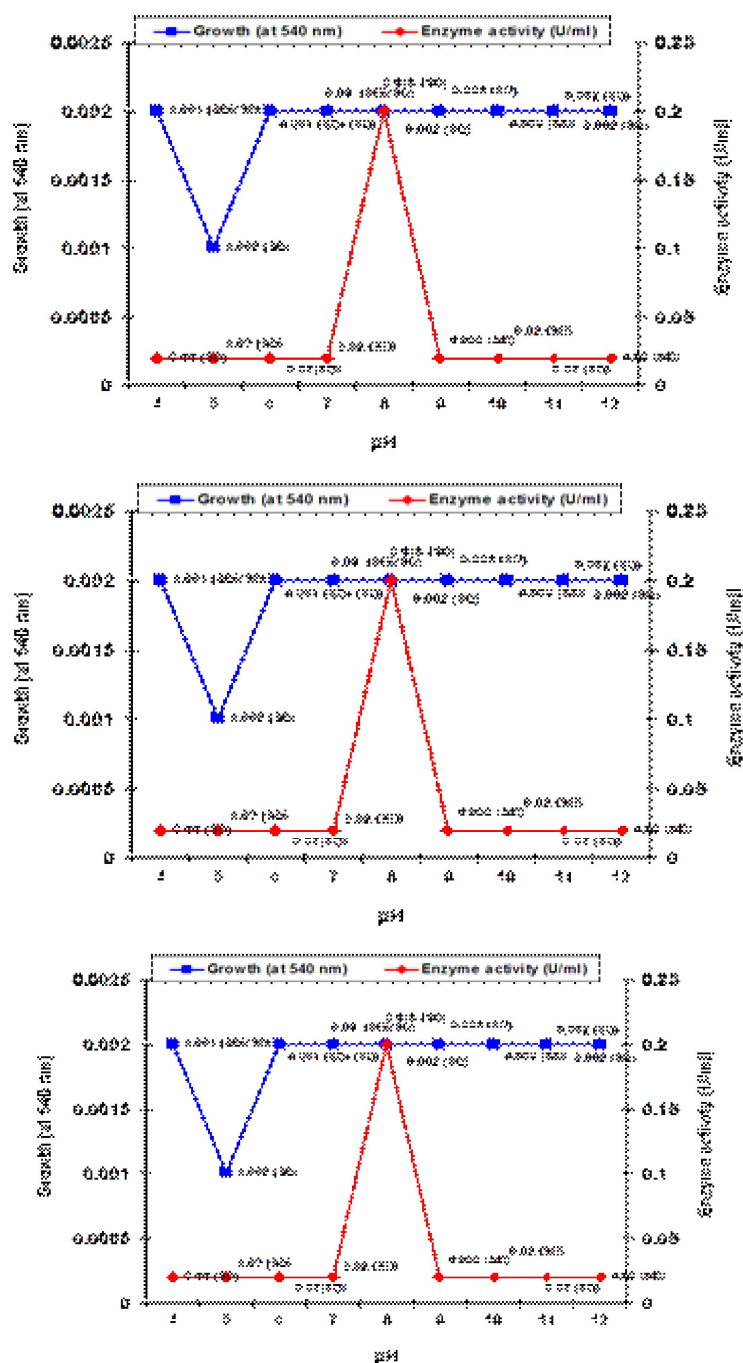


Fig.7. Effect of pH of medium on growth and enzyme activity

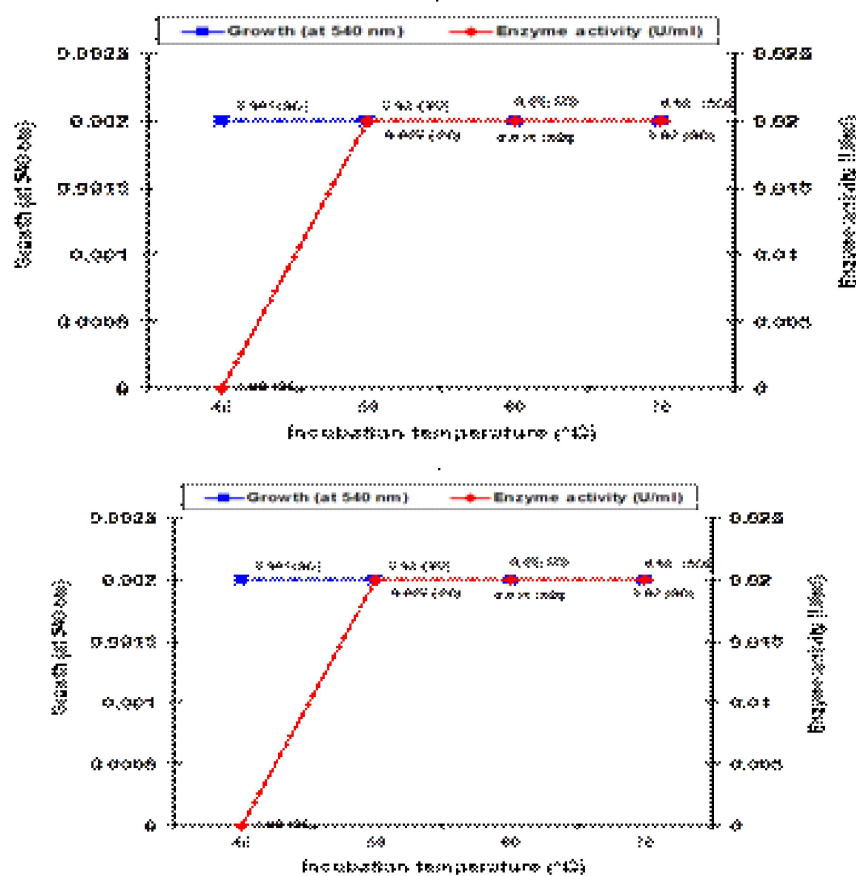
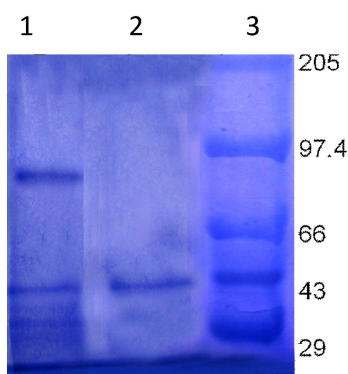


Fig. 8. Effect of incubation temperature on growth and enzyme activity



Lane 1: Crude extract, Lane 2: Purified sample, Lane 3: Protein marker

Fig. 9. SDS-PAGE of thermostable lipase from the *Aneurinibacillus thermoaerophilus* strain MBW2

DISCUSSION

The stability of biocatalysts is an important criterion when dealing with bioprocesses at high temperature for sustainable operation. Enzyme stability is dictated by its three dimensional configuration, which in turn is determined by genetic and environmental factors¹⁶. Therefore, thermophilic microorganisms unequivocally represent a valuable source of highly thermostable enzymes, with numerous advantages towards biotechnological applications due to their overall inherent stability and high reaction rates at elevated temperatures^{17,18}. Among them, lipases, the enzymes that catalyze both the synthesis and hydrolysis of long chain fatty acid esters (depending on water availability), constitute one of the most versatile and widely used biocatalytical group¹⁹. The importance of thermostable lipases

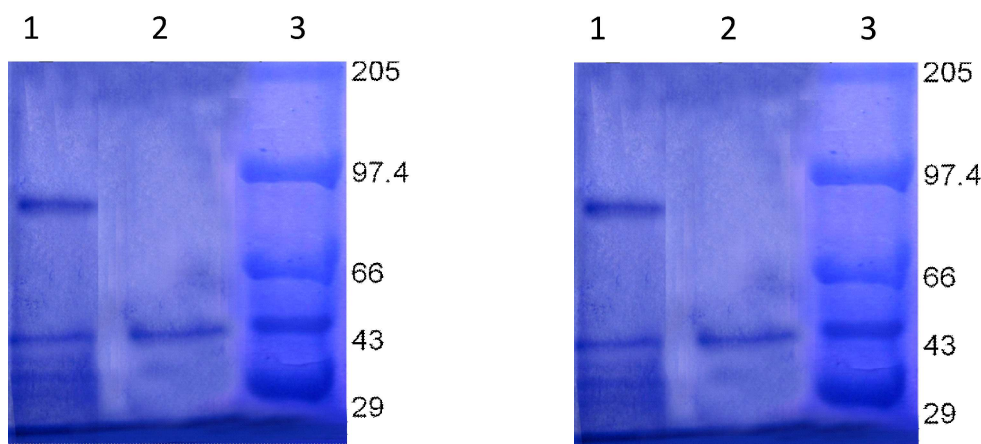
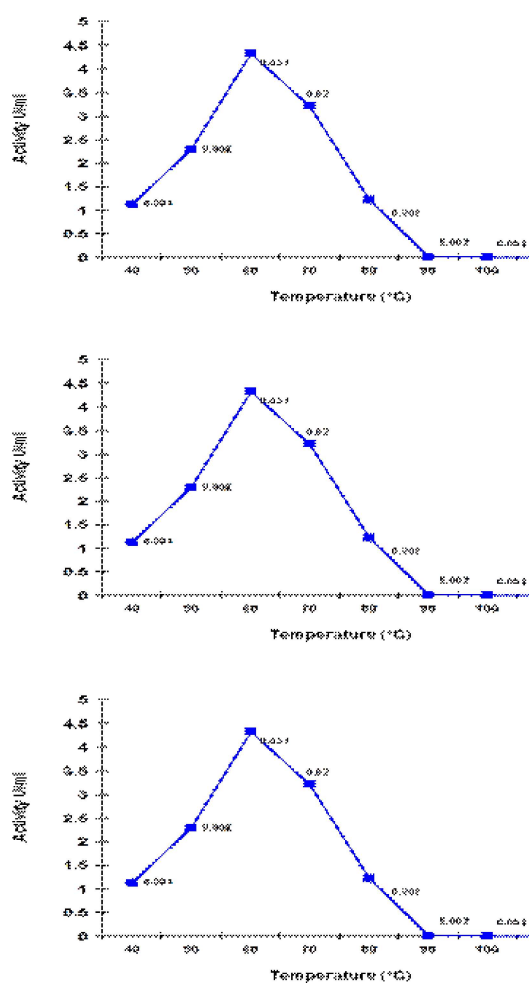


Fig.10. Effect of temperatures on stability of enzyme



for different applications has been growing rapidly^{20,21}. They are used in numerous diverse biotechnological applications ranging from biodiesel and biopolymers production to the synthesis of the chemicals for medical, agrochemical, and cosmetic applications^{2,19}. Due to this fact, novel thermostable lipases are in continuous demand for commercial applications especially in detergent, food, pulp and paper industries²². As a result, several thermophilic microbial strains able to produce thermostable lipases have been isolated²³ and the corresponding enzymes have been purified either from the wild-type culture supernatants^{5,22} or following cloning and expression in mesophilic hosts^{24,25}.

In the present study, 42 putative thermostable lipase producing thermophilic bacterial isolates from four hot water springs were isolated using tributyrin medium based on the ability of formation of zone of clearance. Similar reports of use of tributyrin medium as isolation medium for lipase producing bacteria have been reported^{26,27,28,29,30}. The 42 putative lipase producing bacterial isolates were quantitatively screened for determining the maximum enzyme activity showing bacterial isolate by using pNP-laurate as substrate for enzyme activity. The MBW2 bacterial isolate was reported to show maximum lipase activity of 4.83 U/ml after 24 hrs, was selected for morphological, biochemical and molecular characterization. Similar studies of use of pNP-laurate as substrate for enzyme assay has also been reported^{27,29,30,31}. However, other substrates like p-

nitrophenylpalmitate (p-NPP) and olive oil have also been used by many researchers for assay of lipase enzyme activity^{30,32,33,34}. *In silico* molecular analysis of 16S rDNA of the isolate indicated the similarity of MBW2 isolate to *Aneurinibacillus thermoaerophilus* strain L420-91. Thus MBW2 isolate was identified as *Aneurinibacillus thermoaerophilus* strain MBW2. Survey of the literature revealed few reports on thermostable lipase produced by *A. thermoaerophilus*^{35,36,37}. Optimization of various culture conditions revealed tributyrin medium as optimum medium, 24 hrs as optimum period of incubation, neutral pH of 7 as optimum pH and temperature of 60°C as optimum temperature for production of thermostable lipase. The lipase was partially purified by ammonium sulphate precipitation technique, gel filtration chromatography and ion exchange chromatography to 3.07 fold with 13.73 % protein recovery. However, Masomian *et al.*, 2013 purified a thermostable lipase from *Aneurinibacillus thermoaerophilus* strain HZ to 15.62 fold purification with 19.69% yield³⁷. SDS-PAGE revealed that this purified thermostable lipase possessed a molecular weight of 42.5 KDa. A lipase of 43 KDa has been reported by Hamid *et al.*, 2009 from *Bacillus* sp strain 42³⁸. Similarly a lipase of 40 kDa has been reported by Tan *et al.*, 2014 from recombinant *Escherichia coli* BL21³¹.

CONCLUSION

In the present study, a thermophilic bacteria producing thermostable lipase was isolated from Manikaran thermal spring, where water was at a temperature of 105°C and pH: 6.0. This bacteria was identified as *Aneurinibacillus thermoaerophilus* strain MBW2 after morphological, biochemical and molecular characterization by 16S rDNA technology. Extracellular thermostable lipase enzyme activity of 4.83 U/ml was found to be significant and after purification its molecular weight was determined to be 42.5 kDa. The lipase from this thermophilic bacteria revealed exceptional thermostability with high optimum activity temperatures, thus representing very promising candidate enzymes for a variety of high temperature industrial lipolytic applications. Such an endeavor would probably require their efficient cloning and overexpression

in mesophilic hosts, even though that for some of the strains lipase production was at relatively high levels compared to other wild-type thermophilic bacterial strains.

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