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Isolation and identification of thermo stable multi catalytic *Bacillus licheniformis* strain V7 from Ganeshpuri hot spring

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An efficient extracellular multi enzyme producer strain was isolated from Ganeshpuri hot spring located near Vajreshwari in Thane district Maharashtra. It was identified by the cultural, biochemical, MALDI-TOF MS analysis and 16sRNA sequencing as strain of *Bacillus licheniformis*. It was isolated at the incubation temperature of ≥ 50ºC and showed luxuriant growth and enzyme production ability in the similar temperature range. The multi enzyme producer strain was checked for amylase, mannanase, pectinase, lipase, and cellulase production ability. Its hydrolytic index was measured by taking the ratio of zone of substrate clearance to the colony size diameter. The observed hydrolytic index for cellulase was 5, for pectinase 2.75, for lipase 1.4 after incubation at 52°C for 72 h. Similarly observed hydrolytic index for mannanase was 2.8 and for amylase 3.5 after incubation at 52°C for 24 h. The study resulted in isolation of an efficient thermo stable multi enzyme producer strain of *Bacillus licheniformis* from Ganeshpuri hot water spring which encourages future studies to explore various whole cell and enzyme applications.

Keywords: Ganeshpuri, Hot spring, Multi enzyme, Physiochemical analysis, Thermostable.

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Introduction

Thermophilic microorganisms produce many biomolecules including enzymes which are of industrial importance. Due to their inherent stability, they have been implicated for various types of commercial applications since many industrial biotechnological processes utilize elevated temperature requiring operations. Thermo stable enzymes help these operations by changing the broth rheology as well as reducing the risk of contamination[1]. But due to the difficulties in isolation and maintenance of pure culture, thermophilic microorganisms have been less explored. Therefore, their diversity and application potential remain to be explored from majority of the thermal habitats. Due to the capability of growth at high temperatures and unique macromolecular properties, thermophiles can possess high metabolism, chemically & physically stable enzymes and higher product yields than similar mesophilic species[2].

Thermophilic microbes can be isolated from various habitats like soil, compost piles, hot springs, aquatic systems, etc. Isolation of the thermophiles from sources like hot water spring habitats is much preferred since they survive in hot environment and produce enzymes that are stable under high temperature conditions[3].

Many species of genera viz. *Staphylococcus, Pseudomonas, Serratia, Clostridium* and *Bacillus* including*, B. subtilis, B. cereus, B. licheniformis, B. pumilus* secrete extracellular thermo stable enzymes capable of degrading substrates like polysaccharides, lipids, proteins, etc. Many thermo stable enzymes e.g., amylase, protease, lipase, pectinase, etc. which plays important role in detergents, food, feed, starch, textile, leather, pulp and paper, and pharmaceutical industries have been purified from them[4,5].

In India, many hot springs are well known due to the religious and historical significance and it is considered auspicious to take bathe in it. It is believed to treat many ailments like skin diseases, rheumatic and stomach disorders. Many reports have shown that the hot springs water may have therapeutic effects for treating diseases like asthma, inflammatory arthritis, rheumatic disease, cardiovascular disease, atopic dermatitis, ankylosing spondylitis, and rhinosinusitis[6,7,8]. Apart from high microbial diversity and potential source of active as well as stable biomolecules, Indian hot springs serve as an important niche for novel strains of microorganisms[9].

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In this context, the authors decided to explore hot spring of Ganeshpuri which is located two km from Vajreshwari, Maharashtra (India). It is present in the radius of five kilometres around Vajreshwari temple which is present on a hillock formed by volcanic eruption. There are around 21 hot water springs in a five kilometres radius of the temple, and the temperature of these hot spring ranges from 43 to 49°C. In some hot springs, the water appears blackish due to accumulation of minerals[10].

Aim of the present study was to conduct isolation, screening and identification of industrially important efficient thermostable enzyme producing bacteria from Ganeshpuri hot spring.

Materials and Methods

Geographical location of spring

The hot spring of Ganeshpuri is present in Bhiwandi taluka, Thane district of Maharashtra having a longitude and latitude of 19°29′44.2458″ N and 73°2′6.0246″E respectively. Hot spring water is discharged in the water kund (water reservoir) near temple by pipeline. Both spring and kund (water reservoir) are visited by people to take holy hot water bath. It has one of the oldest temples near Mumbai having religious and tourist significance.

Collection of hot spring water sample

Water was collected from the site of Ganeshpuri hot spring in new plastic bottles which were rinsed with spring water three-four times before water and scraped sediment collection. Temperature and pH were recorded *in situ* using the glass thermometer and pH strips (Hi media) 1 to 14 scale with 0.5 pH increment. Capped bottles were transported to the lab and stored at 4°C until further processing[11].

Isolation of thermophilic bacterial species

The 100 µL water sample was inoculated and spread onto nutrient agar medium containing (g/L) peptone 5, beef extract 3, NaCl 5, and agar 27. The plates were incubated for 24 h at 50°C and the morphologically different colonies were isolated. Tolerance of the isolate to pH and temperature was checked in nutrient broth, adjusted to pH 5 to 9. Temperature range selected was between 40 to 60°C. Isolated colonies were cultivated on nutrient agar slants and glycerol stocks were made for maintaining the culture[12,13].

Screening for enzymes

Active culture of the bacterial isolate was spot inoculated on agar plate containing growth media for the selected enzyme production.

Cellulase

Minimal Media agar with composition, 0.2% sodium nitrate, 0.1% dipotassium hydrogen phosphate, 0.05% MgSo4.7H2O, 0.05% KCl, 0.02% peptone, 0.5% carboxy methyl cellulose (CMC) and incubation at 52°C for 72 h was used for the purpose. Cellulase activity was identified by the appearance of a clear halo around the tested strain after treatment with Gram's iodine[14].

Amylase

Amylase activity was determined on the nutrient agar medium with composition, (g/L) yeast extract 2 g, beef extract 1 g, peptone 5g, NaCl 5 g, 1% starch with incubation at 52°C for 24 h. Appearance of clear halo zone around the colonies after staining with Gram's iodine solution confirmed amylase activity[15,16].

Lipolytic enzymes

The isolate was grown on tributyrin agar base plates (0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1% (v/v) tributyrin, and 2.5% agar, pH 7.0) and incubated at 52°C for 72 h. Incubated plates were observed for the zone of clearance around colonies due to hydrolysis of tributyrin[17].

Mannanase

Nutrient agar was supplemented with 0.5% Locust Bean Gum (LBG) with incubation at 52°C for 24 h. Mannanase activity was identified by the appearance of a clear zone around the inoculated strain after treatment with Gram's iodine[18].

Pectinase

Media used was pectin agar plate containing (g/L) 10 pure Pectin, 2.0KH_2PO_4, 6.0 K_2HPO_4, and 2.0 $MgSO_47H_2O$, 2.5% agar. The plates were incubated at 52°C for 72 h. Cultures were inoculated on pectin agar and grams iodine solution was flooded onto the plates to see clear zone for pectinase production[19,20].

For enzyme screening, hydrolytic activity of the enzyme was determined by calculating enzymatic or hydrolytic index (EI) as, the ratio of diameter of substrate hydrolysis zone to the ratio of bacterial colony diameter in centimeter.

$$\text{Enzymatic index} = \frac{\text{Diameter of hydrolysis zone}}{\text{Diameter of colony}}$$

Morphological and biochemical characteristics of bacteria

Morphological, microscopic, and biochemical pattern of efficient thermo stable multi enzyme producer was recorded by observing colony

characteristics, grams staining, sugar utilization pattern [Hi Media kit] and compared with standard reference strain from Bergey's manual of systematic bacteriology for the tentative identification purpose[21]. To confirm the identity MALDI-TOF MS analysis[22,23] and 16sRNA sequencing was done.

Identification of bacteria using MALDI-TOF MS and 16sRNA gene sequencing

For MALDI-TOF MS analysis, mass spectrum of the strain under study is generated, which was compared with that of the other strains present in the reference database. The database includes biomarkers detected in MALDI spectra of intracellular proteins primarily in the range of 2 to 20 KD. Most of the biomarkers detected in MALDI-TOF spectra of intact bacterial cells have a molecular mass below 15 KD. The MALDI biotyper software 3.0 (Bruker Daltonik GmbH, Germany) was used to identify the isolates and visualize the mass spectra[24-26]. The strain showing ≥1.7 log value with the reference strain in database were confirmed as the member of that genus and strains showing ≥2.0 log values were confirmed as the member of that species.

Both MALDI-TOF MS and 16sRNA gene sequencing was done by outsourcing to the NCMR Pune by standard methods.

Phylogenetic analysis

This was done by MEGA X software. Sequences were subjected to homology search by using BLAST program of the National Centre for Biotechnology Information. (http://www.ncbi.nlm.nih.gov). First fifteen sequences were considered for further procedure of building phylogenetic tree by neighbour joining method[27].

Results

Isolation of thermophilic bacterial species

Hot spring water was slightly acidic in nature with pH 6.0 and temperature 57°C. Cultivation of water sample on nutrient agar at 50°C resulted in growth of numerous thermophilic bacterial isolates. Their colony characteristics and microscopic features were recorded. Many of them were efficient enzymes producers and one of them was selected for the present study. Bacterial isolate was capable of growth in the pH range of 5 to 9 and temperature range 40 to 60°C.

Screening for enzymes

The isolate was screened for the enzyme pectinase, amylase, lipase, cellulase, dextranase (data not shown) and mannanase. The greater is the substrate clearance zone, the higher is the hydrolytic index value. The isolate with hydrolysis zone ≥1.0 cm is considered significant. The observed hydrolytic index for cellulase was 5, for pectinase 2.75, for lipase 1.4 after incubation for 72 h at 52ºC. The observed hydrolytic index for mannanase was 2.8 and for amylase 3.5 after incubation at 52ºC for 24 h. It showed an excellent hydrolysis zone for cellulase and amylase followed by mannanase, pectinase and lipase. Result of the Plate assay is shown in (Fig. 1).

Identification of bacteria

For identification purpose, its morphological features were recorded as described in (Table 1). Isolate was Gram-positive rods with high motility. Results of the enzyme tests were positive for all enzymes as described in (Table 2). Biochemical tests of the sugar utilization were positive for fructose, salicin and esculin hydrolysis as described in (Table 3).

Good quality MALDI-TOF MS spectra was generated for the bacteria and its comparison with the Bruker taxonomy database using Biotyper 3.1 software resulted in the identification of the isolate as *Bacillus licheniformis* with best match score of 2.08 with reference strain *Bacillus licheniformis* 992000432 LBK.

16s RNA sequencing and phylogenetic analysis confirmed the isolate as strain of *Bacillus licheniformis.* The evolutionary relationship of taxa is shown in (Fig. 2). The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.03937451 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. This analysis involved 16 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pair wise deletion option). There were a total of 1550 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. It was labelled as *Bacillus licheniformis* V7 strain and deposited at the NCMR, Pune with accession number MCC4395. 16S rRNA gene

Fig. 1 — Screening for thermostable enzymes.

Table 1 — Morphological characters

S. No.	Characters	Isolate
1	Size	2 mm
2	Shape	Circular
3	Colour	White
4	Opacity	Opaque
5	Elevation	Raised
6	Surface	Smooth
7	Consistency	Non-sticky
8	Margin/Edge	Irregular
9	Gram's nature	+ve rod
10	Motility	Highly motile

Table 2 — Biochemical characters

S. No.	Characters	Isolate
1	Catalase test	+
2	Amylase	+
3	Mannanase	+
4	Pectinase	+
5	Lipase	+
6	Dextranase	+
7	Cellulase	+

+ Positive; - Negative

Table 3 — Carbohydrate utilization of isolate

S. No.	Sugar profile	Result	S. No.	Sugar profile	Result
1	Lactose	-	19	Sorbitol	-
2	Xylose	-	20	Mannitol	-
3	Maltose	-	21	Adonitol	-
4	Fructose	+	22	Arabitol	-
5	Dextrose	-	23	Erythritol	-
6	Galactose	-	24	α-methyl Dglucoside	-
7	Raffinose	-	25	Rhamnose	-
8	Trehalose	-	26	Cellobiose	-
9	Melibiose	-	27	Melezitose	-
10	Sucrose	-	28	α-methyl D mannoside	-
11	L-Arabinose	-	29	Xylitol	-
12	Mannose	-	30	ONPG	+
13	Inulin	-	31	Esculin hydrolysis	+
14	Sodium gluconate	-	32	D-Arabinose	-
15	Glycerol	+	33	Citrate utilization	+
16	Salicin	+	34	Malonate utilization	-
17	Dulcitol	-	35	Sorbose	-
18	Inositol	-	36	Negative control	-

+ Positive; - Negative

sequence was submitted to the NCBI genebank with accession number MW509845.

Discussion

Hot springs are considered important habitat for thermostable enzyme producing microorganisms and numerous reports of microbial exploration from different hot springs worldwide can be found in the literature. In the present study from Ganeshpuri hot spring, two strains of *Bacillus licheniformis* were obtained with thermostable multi enzyme production potential[28]. Out of these, the one with the higher efficiency of enzyme potential was further

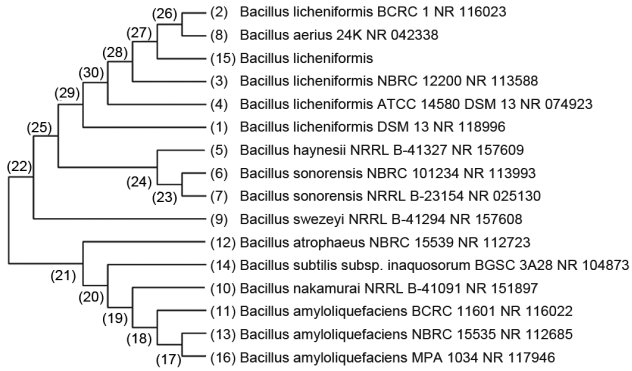

Fig. 2 — Evolutionary relationships of taxa.

investigated in the present study and identified by 16 rRNA gene sequencing and phylogenetic analysis.

Enzyme production from *Bacillus licheniformis* strains has been reported extensively earlier and some of the reports include, thermostable and acidophilic amylase[29,30], peptidase[31], mannanse[32,] thermostable pectinase[33] production from *Bacillus licheniformis* strains. Majority of the studies described single enzyme potential and very few reports have shown more than one enzyme potential from *Bacillus licheniformis* strains. Multienzyme study reports include amylase, protease, cellulase, gelatinase, producing strains from Jordanian Hot Springs[34], alkaline proteases and thermostable α-amylase co-production from *Bacillus licheniformis*[35], α-amylase, protease and lipase production from *Bacillus* species of *B. licheniformis*, *B. sonorensis, B. aerius* from Saudi hot springs[36,37]. *Bacillus licheniformis* shows genotypic diversity[38] from various sources and its versatility for industrial applications can be realized from various study reports. Efficient and thermostable enzyme production can also be utilized for environment management by waste treatment and composting. Use of Mannanase producing *Bacillus* species[39] in waste composting and thermostable microbial inoculums use for composting and waste management[40,41] is tested and reported earlier. *Bacillus licheniformis* V7 can exhibit such potential due to its thermostable multi catalytic property and needs further investigation.

Conclusion

Ganeshpuri hot spring harbors thermostable enzyme producers and the present study has resulted in the isolation of an efficient thermo, pH stable bacterial species identified as *Bacillus licheniformis* V7 strain. It's tolerance to the pH and temperature can result in enzymes capable of withstanding similar stress conditions. It can be utilized not only to produce screened enzymes separately to be used in the industrial processes but also for applications where multienzyme or whole cell action is needed like solid waste composting. Hence, the thermophilic isolate and its thermo stable enzymes viz. amylase, pectinase, cellulase, mannanase can find potential applications in biotechnological industries. Preliminary studies on pectinase and mannanase crude enzyme activity are showing promising results. Further studies on the purified enzymes are needed to explore its true potential.

Conflict of interest

The authors declare that they have no conflicts of interest.

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