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NOVEL THERMO-ALKALIPHILIC STRAIN OF BREVIBACILLUS BORSTELENSIS: POTENTIAL EXTRACELLULAR LIPASE PRODUCER ISOLATED FROM HOT SPRINGS OF GANESHPURI, MAHARASHTRA, INDIA

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ABSTRACT

Everlasting search for newer and more robust hydrolases of bacterial origin has leaded this study to screen various hot spring samples from Thane district, Maharashtra, India. Lipases (EC 3.1.1.3), owing to broad range of industrial applications are potential candidate biomolecules for research explorations. A thermo-alkaliphilic gram positive, sporulating bacterial strain GP-1 has been isolated from Ganeshpuri sulfur springs, with extracellular lipase production. GP-1 was characterized using various biochemical reactions and 16S rDNA characterization methods as *Brevibacillus borstelensis*. The 16S rRNA sequence is submitted at GenBank database, National Center for Biotechnological Information, with accession no. MN282927. *Brevibacillus borstelensis* GP-1 was further optimized for lipase production using one-factor-at-a-time method for various media and physical parameters, for submerged conditions. Maximum lipase production was obtained with coconut oil, yeast extract, 8 gm% inoculum with initial pH 9.0, incubation temperature of 50°C and 48 hours of incubation period. Results clearly suggest that the novel strain of *Brevibacillus borstelensis* GP-1 is a prospective candidate for lipase production. Further scope may involve statistical optimization and enzyme characterization.

Keywords: Lipases, Thermo-alkaliphilic, *Brevibacillus borstelensis*, 16S rDNA, coconut oil

1. INTRODUCTION

Hydrolases have been of prospective concern for researchers due to their enormous applications in various industries. Among excess of sources for hydrolases, massive research on bacterial enzymes has been continued, owing to small size of bacterial cells, short generation times and ease of manipulation of bacterial genes. Lipases (EC 3.1.1.3) being one of the most appealing hydrolytic enzymes (catalyzing conversion of triglycerides to di- or mono-glycerides, glycerol and fatty acids), since last few decades, due to its applicability in various fields [1, 2]. Besides, extracellular lipases from bacterial strains have gained detailed consideration due to their stability at extremes of temperature and pH. All such factors lead to compose bacterial lipases a potential candidate, as these can work well with the vigorous industrial processes. Among the plethora of applications of bacterial lipases, few to list are detergent formulations, food processing, cosmetics and leather industry, esterification reactions to biodiesel production [3, 4].

Hence, there is an unsettled demand of novel extremophilic bacterial strains producing extracellular lipases, having tolerance and activity under extreme reaction conditions. Looking at huge demand of extremophilic lipases by industries, extremophilic bacteria, particularly thermophilic and alkaliphilic bacterial strains have gained attention of researchers since last two decades [2, 3, 5, 6]. Thermostable and alkaliphilic lipases from *Bacillus* species have been reported earlier [7-9]. Extracellular lipases from *Serratia marscencens* [10] and *Staphylococcus aureus* [11] are also reported. Thermophilic strains of *Brevibacillus borstelensis* are also reported to produce variety of hydrolytic enzymes, including amylase, cellulase and lipase [12].

Hot springs have been one of the most captivating environmental sources of novel extremophilic strains of bacteria with varied potential, production of hydrolytic enzymes being the major attraction. Studies have been done for isolation of thermophiles from hot springs of Sarudi Arabia, reporting production of extracellular lipase, along with other hydrolytic enzymes amylase and

cellulase [12]. Metagenomic analysis of hot spring water samples near Mumbai, India region for exploring bacterial diversity is previously reported [13]. The potential of thermophilic strains of various bacteria including *Brevibacillus* species, isolated from Armenian hot springs, as candidates for commercial production of thermostable lipases is also reported [14]. Works also revealed about thermophilic Bacillus strains for developing plant growth promoting consortium from hot springs of Leh and Ladakh region of India, that were growing upto 60°C [15]. Identification of at least 22 different strains of thermophilic bacteria, producing extracellular hydr lases, including lipase, had been successfully reported from geothermal springs in Armenia and Nagorno-Karabakh [16].

The current study focuses on thrust of screening various hot spring water samples and isolating bacteria producing robust lipases, capable of thermal and alkaline stability. It focuses on screening, isolation and characterization, including 16S rDNA characterization, of lipolytic bacterial isolates from Hot spring water samples near Virar, Dist. Thane, Maharashtra, India. The study also involved optimizing various physico-chemical parameters using one-factor-at-a-time (OFAT) method. The parameters involved carbon source, nitrogen source, mineral salts, pH, temperature, and inoculum size and incubation period for lipase production by the selected thermophilic bacterial isolate.

2. MATERIALS AND METHODS

2.1. Screening and isolation of thermo-alkaliphilic lipolytic bacteria

Hot water samples were collected from different hot water springs near Virar, Thane, Maharashtra, India, in sterile plastic bottles with transport medium. The samples collected were screened for the presence of cultivable lipolytic bacterial strains by isolation on Rhodamine B agar with composition (% w/v): Rhodamine B dye, 0.001; nutrient broth, 0.8; NaCl, 0.4; agar, 2 and olive oil, 3 (v/v) in distilled water, with pH 6.5. The plates were incubated at 55 °C for 18 hours. Those isolates giving orange fluorescent halos, upon exposure to UV light (350 nm), were then selected for further studies [17].

2.2. Quantitative screening for selection of most potential isolate

Quantitative screening of screened lipolytic bacterial isolate was further carried out by determining the enzyme units per ml of lipase produced by individual isolates.

2.3. Spectrophotometric method

Spectrophotometric method for determining EU of lipase by the isolates was also carried out as per Winkler and Stuckman (1979), with slight modifications as applied by Bussamara [18, 19]. The method involves incubating substrate emulsion, 9.0 ml with enzyme solution (cell-free supernatant) 1.0 ml. The substrate solution was prepared by adding 30 mg para-nitrophenyl palmitate dissolved in iso-propanol (10 ml) and a mixture of 50 mM Tris-HCl buffer (pH 8.0) with Triton X-100 and Gum Arabic, mixed with continuous stirring. The mixture was incubated at 37°C, for 15 minutes and then determining the amount of p-nitrophenol formed by measuring absorbance at 410 nm, using standard curve of p-nitrophenol in Tris.HCl buffer. One micromole of p-nitrophenol released per minute, under standard assay conditions, was expressed as one unit of lipase activity.

2.4. Characterization of most potential isolate

The most efficient isolate GP-1 was further characterized using morphological, cultural and biochemical characteristics. The GP-1 was even identified by using molecular characterization methods, involving 16S rDNA characterization methods.

2.4.1. Morphological, Cultural and Biochemical characterization

GP-1 was studied for its morphological traits, such as size, shape, arrangement of cells and Grams nature. Endospore production, motility and capsule production was also studied for the most potential isolate [20]. GP-1 was also subjected to various biochemical tests as per Bergey's manual and identification of the isolate up-to genus and species level was carried out. Various biochemical tests performed includes growth at temperature of 4, 25, 40, 55, and 65°C , tolerance of NaCl at concentrations of 2-5 %, hydrolysis of casein, gelatin, starch and urea, nitrate reduction, acid production from glucose, fructose, glycerol, maltose, maanitol, sucrose & xylose, catalase & oxidase production and growth at different pH from 4-12.

2.4.2. Molecular characterization using 16S rRNA sequence

Molecular characterization of the most potential isolate, GP-1 was carried out in two discrete steps; DNA

preparation and PCR amplification, followed by 16S rRNA sequencing and Data analysis.

2.5. DNA Preparation and PCR Amplificaiton

Genomic DNA was extracted from the isolates using Genomic DNA isolation kit. Each genomic DNA to be used as a template was amplified using Polymerase Chain Reaction (PCR) with the aid of 16S rDNA Universal Primers:

8F: 5'-AGAGTTTGATCCTGGTCAG-3'

1492R: 5'-GGTTACCTTGTTACGACTT-3'

The PCR product was further subjected to agarose gel electrophoresis (1% agarose) to check the quality. Sequencing analysis was performed on single discrete 1500 bp PCR amplicon. The 16S rDNA sequences were aligned and compared with other 16S rRNA genes in GenBank by using NCBI Basic Local Alignment Search Tools (BLASTn) program, as per NCBI database. The identified 16S rRNA was searched against NCBI-NT using blastn and on the basis of results of top 15 hits, Phylogenetic tree was constructed. The ancestral states were inferred using Maximum Likelihood method and Tamura Nei model [21].

2.6. Optimization of physico-chemical parameters for lipase production using OFAT method

2.6.1. Effect of Carbon source on lipase production

Lipase production by the isolate was studied by providing different carbon sources, which included carbohydrates in media, glucose, lactose, sucrose, xylose, maltose, mannitol and starch at concentrations of 2 gm%. Various different oils were also tested for their effect on growth and lipase production, which included olive oil, coconut oil, sunflower oil and eucalyptus oil, at 2% (v/v) concentrations (all market available oil compositions were used). A control flask was kept without inducer substances.

2.6.2. Effect of Nitrogen source on lipase production

Various nitrogen sources were added to the media, at concentration of 3.5 gm/litre, to evaluate their effectiveness to growth and lipase production. The inorganic nitrogen sources included potassium nitrate, ammonium chloride, ammonium nitrate and sodium nitrate. The organic nitrogen sources included peptone, tryptone, casein and beef extract. A control flask was kept without nitrogen source included.

2.6.3. Effect of pH and temperature on lipase production

Growth and lipase production was also evaluated by growing GP-1 at different initial pH, ranging from 4 to 12. Effect of temperature was analyzed by incubating the production media at temperatures of 10°C, 25°C, 37°C, 45°C, 50°C, 55°C and 60°C .

2.6.4. Effect of inoculum size on lipase production

Growth and lipase production using GP-1 isolate was evaluated by inoculating different inoculum sizes, ranging from 2 % to 20 %. Other conditions of media and incubation were kept optimum.

2.6.5. Effect of incubation period on lipase production

Lipase production and growth of GP-1 was also evaluated by adding appropriate inoculum into production media with composition as discussed above. The production of lipase was evaluated at regular time intervals of 24, 48, 72, 96, 120 and 144 hours of incubation. Other conditions of media and incubation were kept optimum.

3. RESULT & DISCUSSION

3.1. Screening and isolation of thermo-alkaliphilic lipolytic bacteria

Extreme ecosystems such as hot springs are of great interest as a source of novel extremophilic species, enzymes, metabolic functions for survival and biotechnological products [3]. With the same context, different hot spring samples from Thane district, Maharashtra, India were collected and analyzed for presence of thermo-alkaliphilic lipase producers. Out of total 5 different hot spring water samples that were tested, total of 19 thermophilic lipolytic bacterial isolates were obtained, showing orange fluorescence on Rhodamine B agar, at 350 nm, selected for further studies. Secondary screening revealed that GP1 was showing highest lipolytic activity of 59.75 U/ml, isolated from Ganeshpuri hot water springs (Data not presented here). Ganeshpuri hot water spring had been an exploratory source of bacteria Bacillus sp. producing thermostable lipase [22]. Brevibacillus sp. have also been isolated from Algerian hot springs [23]. Also, geothermal springs in Armenia have been reported as potential sources of hydrolase producing thermophilic bacilli [24]. GP-1 was further characterized and then optimized for lipase production.

3.2. Characterization of the most potential isolate

Morphological, cultural and biochemical characteristics of GP-1 isolate were reported and given in table 1. The isolate was tentatively identified as *Brevibacillus* species. Further, the isolate identification was confirmed to be *Brevibacillus borstelensis* GP-1strain, based on 16S rDNA analysis. The sequence data is submitted at NCBI gene database, gene accession number MT292327. Fig 1 shows agarose gel electrophoresis results of GP-1 with 0.8% agarose. Single thick band in GP-1 lane indicates good quality genomic DNA isolated. The Phylogenetic tree constructed showed the closest strain to be *Brevibacillus borstelensis* strain IPH-801 (Fig 2). This strain was reported to be a thermophilic bacterium isolated from soil, with good activities of hydantoinase and carbamoyla. Previous studies reported about thermophilic *Brevibacillus borstelensis* strain producing extracellular hydrolases such as lipase, amylase and cellulase, with excellent activities at 55 °C, which was isolated from hot springs in Saudi Arabia [12]. Norashirene et al. also reported various strains of *Brevibacillus borstelensis* from Selayang hot spring, Malaysia [25]. Isolation of thermophilic bacterial strains of *Brevibacillus borstelensis* from Attri sulfur hot water springs in Odisha is also reported earlier [26]. In another study conducted regarding diversity analysis of Algerian hot springs, for endospore forming bacteria, using cultural and non-cultural methods, *Brevibacillus* sp was one of the potential isolates.

The studies also reported that all the isolates were producing at least one extracellular hydrolase [23]. A study also demonstrated strain of *Brevibacillus borstelensis* producing lipase, along with protease and amylase [16]. This organism is also attributed by various research groups towards biodegradation of polyethylene, one of the promising pollution problem [27, 28] and has even been reported to grow in synthetic medium with polyethylene films as sole carbon source [29]. Apart from lipase, *Brevibacillus borstelensis* strains are reported to produce enantioselective thermostable dipeptidase [30]; cellulase [31], and alpha-amylase [32], to list a few.

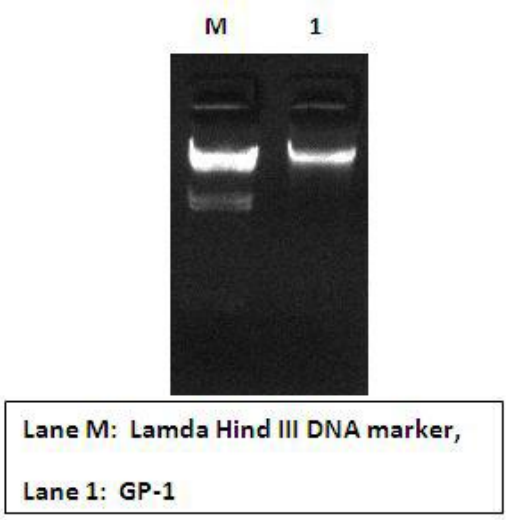

Fig. 1: Agarose gel electrophoresis (0.8% agarose) of genomic DNA isolated of GP-1 isolate

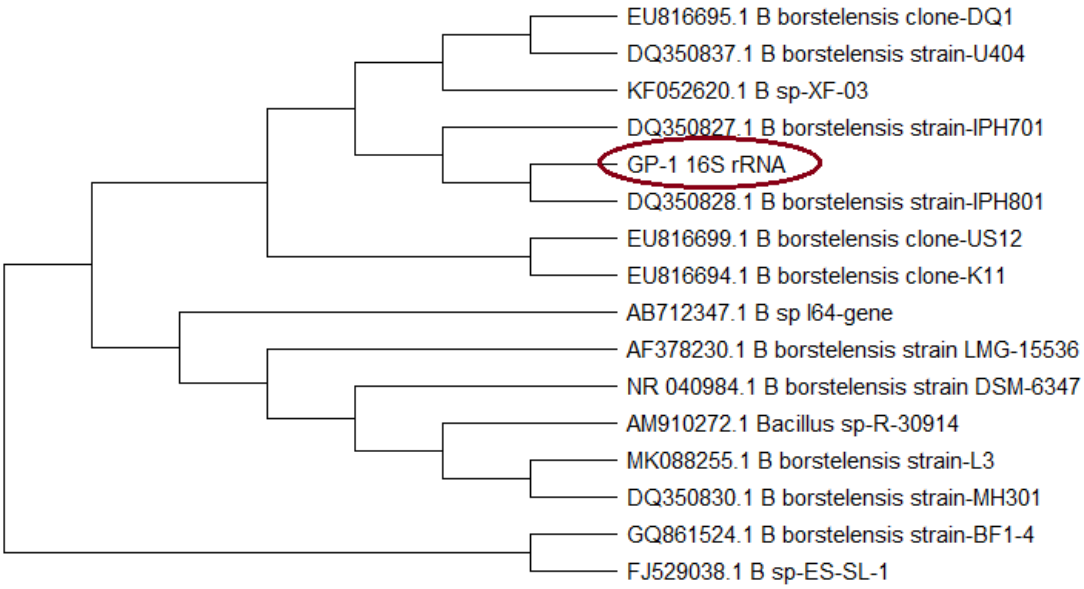

Fig. 2: Phylogenetic tree for GP-1 constructed using Maximum likelihood method

3.3. Optimization of physico-chemical parameters for lipase production

Optimization of submerged culture conditions such as carbon and nitrogen sources, other nutrients, pH, temperature, along with microbial physiology has profound impact on the qualities and quantity of lipase production.

3.3.1. Effect of Carbon source on lipase production

Cellular growth and metabolism are primarily regulated by the type of carbons source incorporated in the growth medium, which controls the production of different metabolites. The easily assimilable sugars contribute to fast growth and thereby increase enzyme production. Evaluation of various carbon sources including carbohydrates revealed that the easily assimilable sugars contribute fast growth but have adverse effect on lipase production. Fig. 3 shows that oils serve as inducers of lipase production and vegetable oils, such as sunflower oil, groundnut oil, olive oil and coconut oil are showing enhanced lipase production as compared to carbohydrate sources. *Brevibacillus borstelensis* GP-1 strain showed induction of more lipase with maximal production using coconut oil, 74.52 U/ml, which is 1.53 times higher compared to control without oil inducers (Fig. 3).

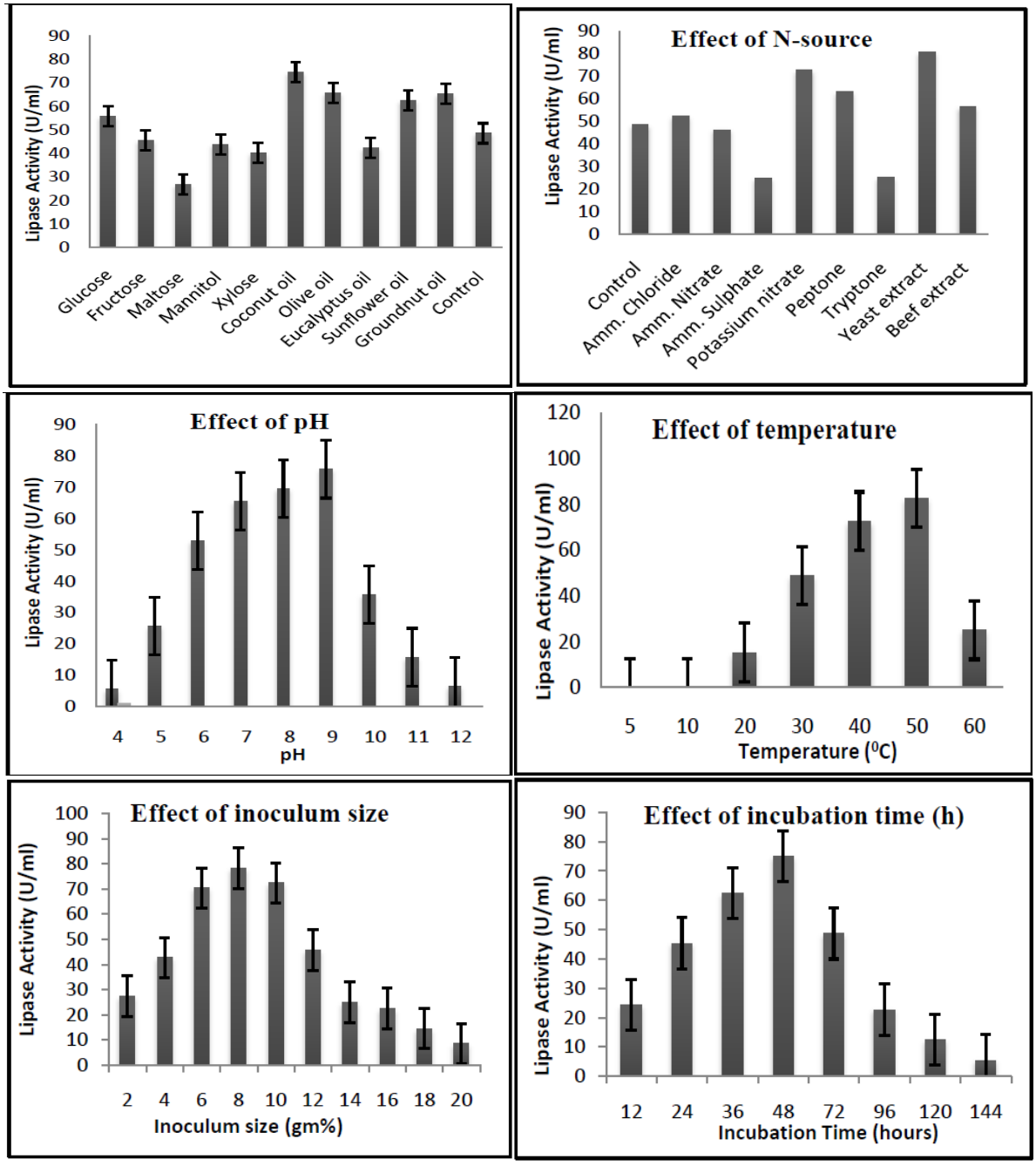

Fig. 3: Optimization of various physico-chemical parameters on lipase production by GP-1

Table 1: Biochemical characterization of GP-1

Isolates	GP-1
Morphological Characteristics	
Size & shape	Long rods
Arrangement	Single/pairs
Gram reactivity	Positive
Spores	Ellipsoidal
Cultural Characteristics	
Size	Large
Shape	Circular
Margin	Entire
Elevation	Flat
Opacity	Opaque
Pigmentation	Brownish
Consistency	Soft
Biochemical Characteristics	
Sugar fermentation	
Glucose	-
Glycerol	-
Fructose	-
Maltose	-
Mannitol	-
Sucrose	-
Xylose	-
Nitrate reduction	+
Indole test	-
Citrate utilization	+
Growth at	
4 °C	-
25 °C	+
40 °C	++
55°C	++
Catalase	+
Oxidase	-
Hydrolysis of: Casein	++
Gelatin	++
Starch	-
Urea	-
Tolerance to NaCl: 2%	-
3%	-
4%	-
5%	-

One of the most prominent lipase applications is degradation of oil cakes, with the most dominant one being the coconut oil cake. This suggests that lipases can efficiently degrade the lipid present in coconut oil. Also, proficient growth of lipase producer on coconut oil cakes is indicative that it must be inducing lipase in bacterial strains. Many groups reported lipase production using coconut oil cake with bacterial strains including *Bacillus subtilis* [33], *Streptomyces indiaensis* [34], *Staphylococcus pasteuri* [35], *Bacillus coagulans* VKL1 strain [36] and a yeast strain *Candida rugosa* for hydrolysis of virgin coconut oil [37]. However, olive oil has also been reported as the most efficient carbon source for lipase production [9].

3.3.2. Effect of nitrogen source on lipase production

Nitrogen is the second most significant element next to carbon in deciding growth and metabolism of any living cell. In the same context, various nitrogen sources were evaluated for their effect on lipase production by *Brevibacillus borstelensis* GP-1 strain. Maximum lipase activity was obtained with potassium nitrate (72.65 U/mL) among inorganic nitrogen source and yeast extract (80.52 U/mL) among organic nitrogen source tested (Fig 3). Although, very limited information about *Brevibacillus* lipase productivity has been found, but the findings of yeast extract as the best nitrogen source are in agreement with previous studies on bacterial strains such as *Burkholderia multivorans* [38], *Bacillus licheniformis* [39] and *Bacillus coagulans* VKL1 strain [36]. Although yeast extract is also reported to be the best source of nitrogen for fungal species such as Candida viswanathii [40].

3.3.3. Effect of pH and temperature on lipase production

pH plays pivotal role in the production of any metabolites and growth of the bacteria. It was found that *Brevibacillus borstelensis* GP-1 strain showed maximum Lipase production *i.e.* maximum of 75.65 U/ml at pH 9 (Fig 3). This may be because this bacterium can grow up-to pH 10 and hence can produce lipase enzyme at alkaline pH to maximal concentration. These findings are in agreement with previous reports of maximum lipase production with initial alkaline pH by [41] and *Bacillus* [7] and optimum pH of 8.0 for [42]. Temperature is another crucial factor for growth and metabolism of any living cell. Extremophilic bacteria have been served as potential sources of many enzymes since many years. *Brevibacillus borstelensis* GP-1 isolate showed maximum lipase production at 50°C, making it one of the potential candidate as an industrial strain (Fig. 6). As stated previously, *Brevibacillus borstelensis* is a thermophilic organism growing upto 50°C and hence the potential of this strain to produce lipase at 50°C is justifiable. Earlier, 55°C is reported as optimum temperature for lipase

production [43] whereas high yields of lipase by *Bacillus sterothermophilus* are reported up to 55°C and 60°C [44].

3.3.4. Effect of Inoculum size on lipase production

Inoculum size has significant contribution towards growth of bacteria and hence affects various metabolites, including enzymes. Experimental studies indicated that amongst the cell mass concentrations evaluated, maximal lipase production was observed at 8 gm% (Fig. 3). No noteworthy increase in growth and lipase production by GP-1, by further increase in inoculum size. Similar studies were carried out by scientists earlier, which proved about 4-8 % inoculum giving maximum lipase production [42].

3.3.5. Effect of Incubation time on lipase production

Time of incubation plays key role in maximizing production of a particular metabolite, such as hydrolytic enzymes, for example lipase. Enzymes are normally primary metabolites and hence normally produced during exponential growth. Maximum lipase production was observed after 48 hour of incubation (Fig 3). Lipase activity was shown to decrease with further incubation gradually, upto 120 hours tested. Gradual decrease in enzyme production can be traced to toxic metabolites accumulation [41]. Earlier studies revealed maximum lipase production at the end of 12 hours [45], 48 hours [46] and also after 72 hour of incubation, increasing upto 7 days [47].

4. CONCLUSION

The robust environment of hot water springs have been the ever-lasting source of novel strains with potential industrial interests. Thermophilic strain of *Brevibacillus borstelensis* GP-1 isolated from Ganeshpuri hot water spring produced extracellular lipase. The Phylogenetic studies revealed that it is closely related to strain *Brevibacillus borstelensis* IPH-801, which has potential to produce interesting enzymes like hydantoinase and carbamoylase. Further, it was found that *Brevibacillus borstelensis* GP-1 could produce maximum lipase with coconut oil, yeast extract, pH 9 and incubation temperature of 50°C, with 8% inoculum size and 48 hours of incubation period. Thus, it is very much obvious that *Brevibacillus borstelensis* GP-1 is a prospective candidate and its other potentials need to be explored in further research. Further research shall be projected towards statistical optimization of media and fermentation conditions, followed by purification and characterization of the enzyme.

Conflicts of interest

Authors declare that there are no conflicts of interest.

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