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# *Brevibacillus sp*: A Novel Thermophilic Source for the Production of Bile Salt Hydrolase

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**Abstract** A thermophilic microorganism growing within the temperature range of 40–65 °C (optimum at 55 °C) was isolated from hot water springs near Konkan, Maharashtra, India. Based on 16S rDNA sequence analysis, it was concluded that the isolate belongs to the genus *Brevibacillus*. The present paper reports the isolation, identification, and standardization of fermentation conditions for the production of enzyme, bile salt hydrolase (EC 3.5.1.24) which is produced intracellularly at high temperatures. This is the first report regarding the production of bile salt hydrolase from a thermophilic source. Optimization of fermentation conditions resulted in a 2.9-fold enhancement in enzyme production.

**Keywords** *Brevibacillus sp* · Thermophile · Bile salt hydrolase · Glycodeoxycholic acid · Hot springs · Fermentation

## Introduction

Bile salt hydrolysis is a biologically significant reaction among the bacterial alterations of bile acids. The enzymes responsible for bile salt hydrolysis are bile salt hydrolases (BSHs; EC 3.5.1.24). Based on the sequence similarity between BSH and penicillin V acylase, it was suggested that the enzyme belongs to the N-terminal nucleophile hydrolase superfamily [1]. BSH catalyzes the hydrolysis of glycine- and taurine-conjugated bile salts to amino acid residues and free bile salts (bile acids).

High blood cholesterol is a major risk factor for coronary heart disease. Although different drugs are available for the treatment, they are all known to have side effects. The possibility of using bile salt deconjugation to lower serum cholesterol levels in hypercholesteremic patients and prevent hypercholesteremia in normal people is of great interest at present [2]. The microorganisms exhibiting BSH activity could be beneficial because they have the potential to reduce serum cholesterol [3, 4].

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In humans and other mammals, cholesterol balance is achieved by modulating both synthesis and excretion. Cholesterol excretion is mediated by bile acids, the water-soluble amphipathic molecules formed from cholesterol in the liver [5]. Bile salts play an important role in lipid digestion and absorption in the small intestine. Bile salts circulate back to the liver through a process known as enterohepatic circulation [6]. BSH generates less water-soluble bile acids (to be excreted via feces) with its deconjugation property. This drain of bile salts results in a reduction of serum cholesterol because the replacement of bile salts would require the utilization of cholesterol in the body.

BSH activity has been detected in *Lactobacillus sp* [7], *Bifidobacterium* [8], *Clostridium* [9], *Enterococcus* [10], and *Bacteroides* [11]. BSH activity is mainly observed in bacteria isolated from the gastrointestinal tract. In addition to autochthonus intestinal microbiota, BSH activity was also reported from an enteropathogenic strain of *Listeria monocytogenes* [12] and bile adapted strain of *Xanthomonas maltophila* [13]. Although the production of BSH from mesophiles is well known, little is known about the production of BSH from thermophiles. To investigate the production of BSH from thermophilic source, we have isolated a novel thermophilic microorganism from hot water springs near Konkan, Maharashtra, India. The present paper reports the isolation of a thermophilic bacterium with BSH activity and optimization of growth conditions for the production of BSH. In the present study, we report for the first time the production of BSH enzyme from a thermophilic source.

## Materials and Methods

### Source and Isolation of Thermophile

A combined sediment-water samples were collected from hot water springs near Konkan, Maharashtra, India. Location and altitude of the sample collection site is 18°05′ S, 73°020′ E and 40 m above the sea level. The temperature at the site of collection was 60 °C. These samples were inoculated (onsite) into tubes containing 10-ml nutrient broth and incubated at 65 °C for 96 h with shaking at 200 rpm. Tubes showing growth were subcultured for the desired isolation. Isolated colonies on lysogeny broth agar plates containing 0.025% glycodeoxycholic acid were further characterized.

### Bile Salt Hydrolase Assay

The isolate was checked for its ability to produce bile salt hydrolase by modified ninhydrin method according to Suresh Kumar et al. 2006 [1]. Briefly, the culture was grown for 12 h and centrifuged at 12,000×*g* at 4 °C for 2 min. The cell pellet was washed with potassium phosphate buffer pH 6.5 and centrifuged and the wet weight of the pellet was determined. The cell pellet was suspended in 100 μl reaction mixture (10 mM potassium phosphate buffer pH 6.5, 10 mM DTT, 10 mM sodium glycodeoxycholate). The mixture was incubated at 40 °C for 30 min and then terminated by adding 100 μl of trichloroacetic acid (15% *w*/*v*). The mixture was centrifuged, and 50 μl of the supernatant was mixed with 50 μl of 2% ninhydrin reagent solution. The preparation was thoroughly mixed and boiled for 14 min. The absorbance of the cooled sample was recorded at 570 nm. BSH activity per gram of whole cells was determined by using a standard curve prepared with glycine. One

unit of BSH activity was defined as the amount of enzyme that liberated 1 μmol of amino acid from the substrate per minute per gram wet weight of cells.

## 16S rDNA Sequencing

Genomic DNA was isolated and quantified according to Marmur [14]. The 16S r DNA was amplified from 100 ng of genomic DNA using primers 121 F (5′GGC GGA CGG GTG AGT AAT 3′) and 1488 R (5′CGG TTA CCT TGT TAC GAC TTC ACC 3′), respectively [15]. Thermal cycling was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1.5 min, and a final extension step of 72 °C for 10 min. The amplified polymerase chain reaction (PCR) product was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany). The amplicon was cloned in pGEMT easy vector, and the nucleotide sequences were determined using Big Dye terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, and Applied Biosystems 3730 DNA Analyzer. The partial 16S rDNA sequence was searched for highly similar nucleotide sequences using National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) program. Retrieved sequences were aligned by CLUSTAL W program and manually edited. The phylogenetic tree was constructed by the neighbor joining method, using Jukes–Cantor algorithm in Molecular Evolutionary Genetic Analysis Programs version 4.0 [16]. Bootstrap values were calculated for 1,000 trees. The 16S rDNA sequence determined in this study has been deposited in the NCBI data bank with the accession number EU 251074.

## Optimization of Fermentation Parameters

Optimization of different nutrient and physical parameters for BSH production was studied by maintaining all factors at constant level except the one being studied. Erlenmeyer flasks (250-ml) containing 50 ml of sterilized media were inoculated with 10% inoculum and shaken at 160 rpm, 55 °C for 12 h. Effect of pH on BSH production was determined by cultivating the isolate in different initial pH values of the selected medium. The influence of temperature was studied by cultivating the isolate at various incubation temperatures ranging from 40 to 65 °C. The effect of different carbon sources on growth and bile salt hydrolase activity produced in shake flasks was assayed by substituting minimal medium with various carbon sources (0.5%). Simple and complex carbon sources including glucose, xylose, lactose, mannose, fructose, mannitol, galactose, glucose, and sorbitol were used. The influence of various nitrogen sources on cell growth and enzyme yield was also tested. Nitrogen sources (0.6%) such as yeast extract, peptone, tryptone, and beef extract supplemented separately in minimal medium were used. Minimal medium without carbon and nitrogen source, respectively, was served as control.

## Substrate Specificity

BSH production profile and cell growth of the culture was monitored under shake flask conditions up to 72 h. Substrate specificity was determined by measuring the BSH activity under standard assay conditions with six major human bile salts, i.e., Glycocholic acid, glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, and taurochenodeoxycholic acid, respectively.

## Results

### Source and Isolation of Thermophile

Five sediment-water samples were collected from hot water springs of Konkan areas in India. These samples were used as inocula for screening and enrichments of aerobic, thermophilic microorganisms. Single colony was picked up after incubation on the nutrient agar plate at 65 °C for 48 h. The isolate was observed to be BSH positive by the Ninhydrin method described above.

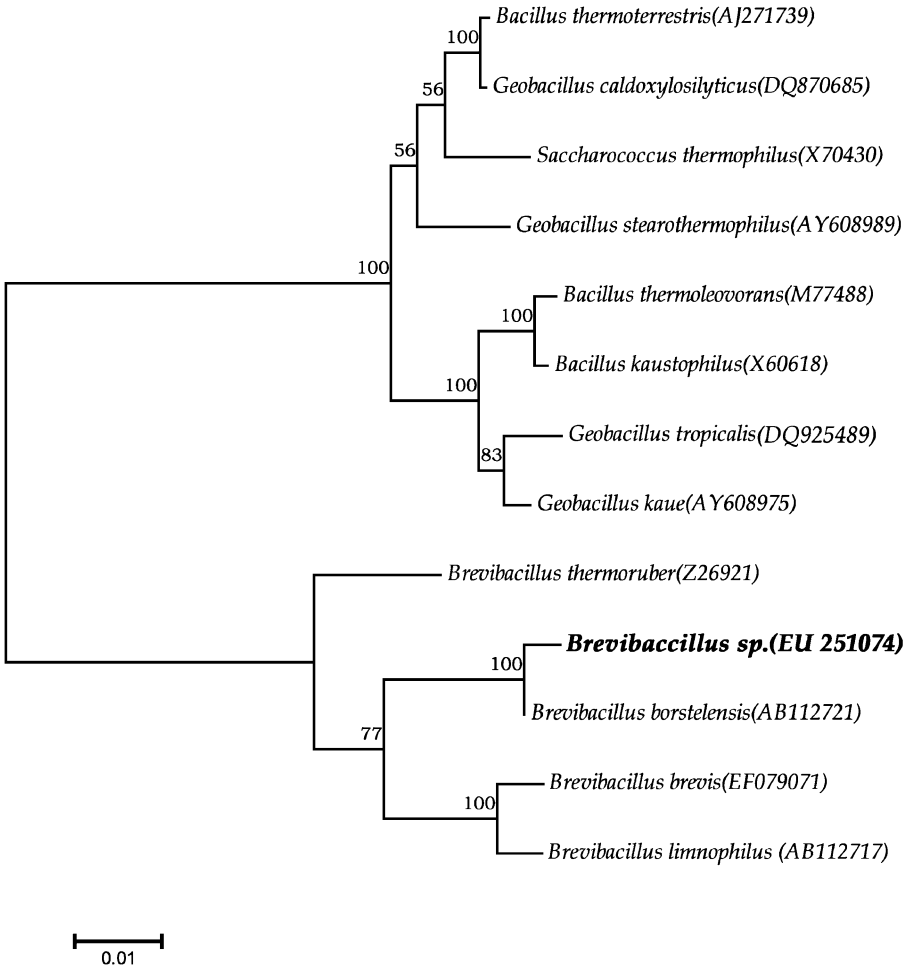


**Fig. 1** Phylogenetic tree based on 16S rDNA sequences showing the position of isolate among its closely related organisms. Database accession numbers are shown in *parentheses* after species, strain, or name. Values displayed at the nodes ($n$=1,000) indicate bootstrap values. The tree was constructed by the neighbor-joining method. The *scale bar* represents 0.01 nucleotide substitution per position

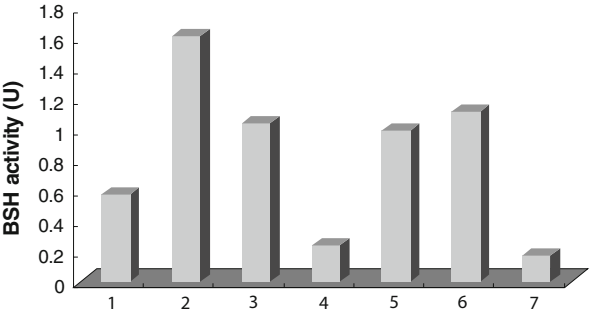


**Fig. 2** Media optimization of bile salt hydrolase. The following different media were used *1* Luria bertini *2* Sodium glutamate *3* Nutrient broth *4* Minimal medium *5* EMS *6* MGYP *7* Skimmed milk

## 16S rDNA Sequencing

About 1,400 bp sequence of the 16S rDNA was compared to sequences of related bacteria. BLAST analysis showed high nucleotide sequence similarity (99%) with *Brevibacillus borstelensis* DSM 6347T (AB112721). The subsequently constructed phylogenetic tree revealed the isolate clusters with representatives of the genus *Brevibacillus* (Fig. 1).

## Optimization of Fermentation Parameters

This study was done to increase the yield of BSH production from *Brevibacillus sp.* Optimum BSH production was observed in sodium glutamate medium (yeast extract—0.5%, peptone—1%, sodium chloride—0.2%, sodium glutamate—0.5%; Fig. 2). So, this medium was used for the rest of the experiments. Time course for growth of *Brevibacillus* and BSH production was observed in shake flask conditions (Fig. 3). In effect, the maximal growth was attained after 24 h and slowed down later. The enzyme activity appeared to be maximum after 12 h fermentation and decreases sharply between 24 to 72 h. The activity profile of BSH from *Brevibacillus sp* was determined in sodium glutamate medium adjusted to different pH values ranging from 5.0 to 9.0. BSH activity was detected to be optimum in pH 6.0; there was no considerable change in the enzyme production after this pH (Fig. 4). Temperature profile for BSH activity from the isolate showed that BSH activity was maximum at 55 °C. When incubated at different temperatures ranging from 30 to 75 °C, the culture failed to grow below

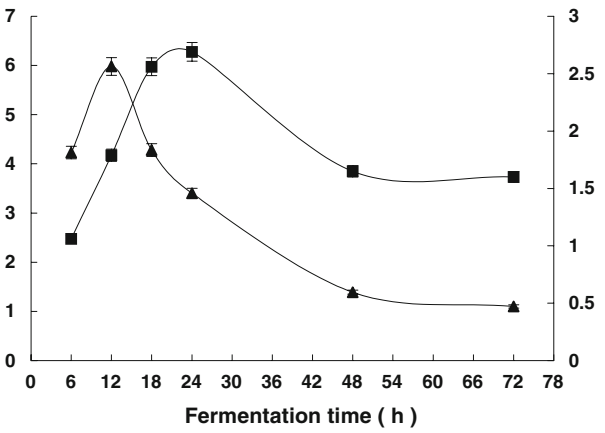


**Fig. 3** Effect of Fermentation time on BSH production from *Brevibacillus sp.* at 55 °C, pH 6.0, 160 rpm in sodium glutamate medium. Samples were removed at different intervals and assayed for growth (*filled square*) and BSH activity (*filled triangle*) simultaneously

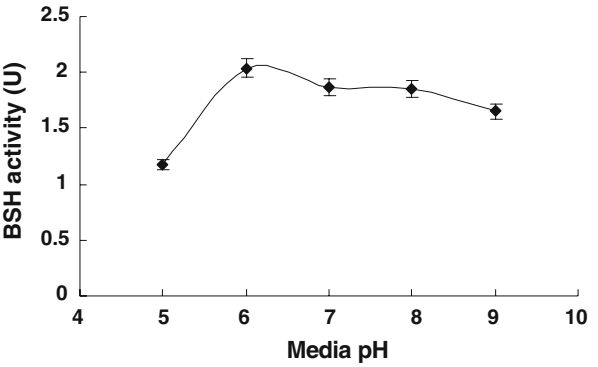


**Fig. 4** Effect of initial pH of the culture medium on BSH activity of *Brevibacillus sp*

40 and above 65 °C. The enzyme activity was almost stable from 50 to 55 °C temperature range (Fig. 5). Influence of various carbon sources were tested for BSH production. Among all sugars, lactose was found to be the most suitable substrate for BSH activity (Fig. 6). Supplementation of lactose increased BSH activity by 1.7-fold as compared to control. Mannose and mannitol does not have considerable effect on activity. The other carbon sources had inhibitory effect on BSH activity. On the other hand, presence of nitrogen source has a significant effect on BSH activity. The best nitrogen source for BSH production by *Brevibacillus sp.* was yeast extract, followed by tryptone and peptone. The BSH activity was increased 2.9-fold when yeast extract was used as nitrogen source (Fig. 7).

Substrate Specificity

Bile salt hydrolase from *Brevibacillus sp* showed a broad substrate range for six major human bile salts. This enzyme showed highest activity with GDCA and exhibited preference for glycine-conjugated bile salts over taurine-conjugated forms (Fig. 8).

## Discussion

The phylogenetic analysis, based on the 16S rDNA sequence, showed that the isolated thermophile is phylogenetically related to the genus *Brevibacillus*. The threshold for

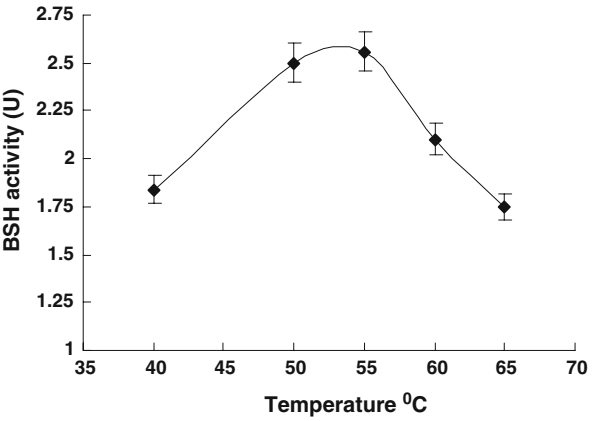


**Fig. 5** Effect of incubation temperature on BSH activity of *Brevibacillus sp*

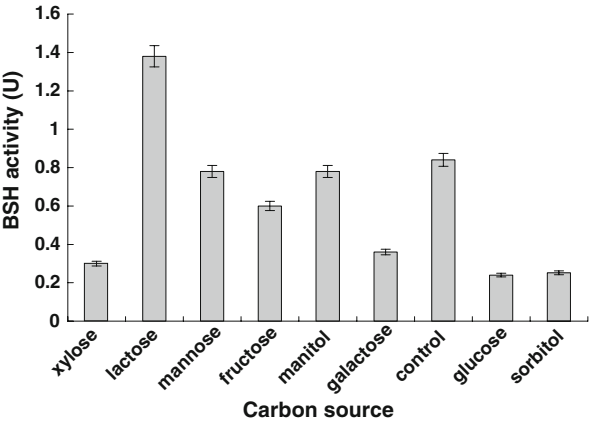


**Fig. 6** Effect of carbon source on BSH production during cultivation of *Brevibacillus sp*

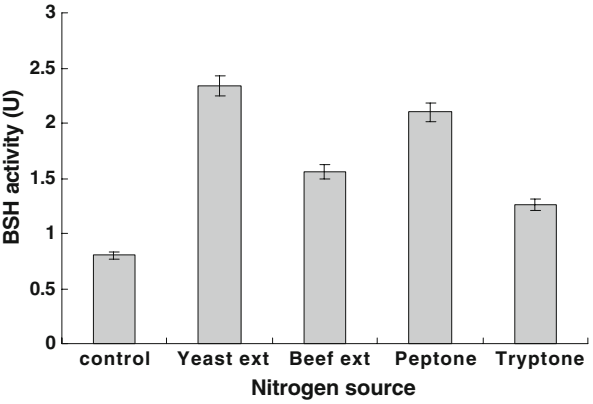


**Fig. 7** Effect of nitrogen source on BSH production during cultivation of *Brevibacillus sp*

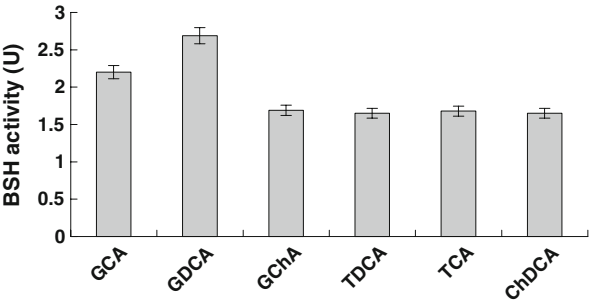


**Fig. 8** Substrate specificity of BSH from *Brevibacillus sp*

similarity to be considered as the same species is 97%, according to Stackebrandt and Goebel [17]. 16S rDNA analysis of the isolate showed the sequence to be 99% similar to *B. borstelensis* type strain DSM 6347 T (AB 112721). The isolate was Gram-positive, aerobic, rod-shaped, catalase-, and oxidase-positive. Nitrate reduction tests were negative. Growth of the isolate was observed within a temperature range of 40–65 °C and pH range of 5.0–9.0. Acid production is seen in presence of various sugars, but formation of gas is not observed. Members of the genus *Brevibacillus* share similar biochemical characteristics [18]. These data suggest that this isolate does belong to this genus—more specifically to *B. borstelensis*. The isolated strain of *Brevibacillus sp* constitutes the first report of a thermophilic producer of BSH. Conditions for the optimum production of bile salt hydrolase from this thermophile was screened. The production of high titres of any enzyme by optimizing the growth parameters is of prime importance in enzymology. The optimization of various nutritional and physical parameters to which an organism exposed is known to significantly increase the product yield. Since BSH is a pharmaceutically important enzyme, optimization of its production to increase yield is of great importance. A 2.9-fold increase in BSH production was achieved through optimization of cultural parameters. Optimum production of the enzyme was achieved in sodium glutamate medium with pH adjusted to 6.0, incubated at 55 °C in shake flasks at 160 rpm for 12 h. The initial pH of the medium has been reported to strongly influence many enzymatic systems. The pH affects the ionization and therefore the binding and interaction of a myriad of molecular processes; this includes very basic things such as nutrient availability. Similarly, pH also affects the solubility of many substances that bacteria need [19]. Slightly, acidic pH favors the BSH production from *Brevibacillus sp*, which is similar to the intestine of healthy humans [20]. The isolate showed preference to glycine-conjugated bile salts than taurine-conjugated ones. This property of substrate specificity is similar to the reported BSH from mesophilic bacteria [21].

Some properties of *Brevibacillus* BSH (appreciable thermal stability and favorable substrate affinity) seem to be useful for carrying out biotransformation of bile salts for the production of ursodeoxycholic acid, which has potential applications in solubilizing gallbladder cholesterol stones. Bile salt deconjugation liberates amino acids such as glycine and taurine, which can be used as carbon source by bacterial strains [22]. The property of deconjugation of bile salts exhibited by gastrointestinal bacteria may be a detoxification mechanism, and BSH produced by this bacteria may play a role in bile tolerance and consequently in survival in the gastrointestinal tract. To date, BSH activity has not been detected in bacteria isolated from environment in which bile salts are absent [23]. Physiological role of BSH production in the isolated thermophile needs to be explored. Purification and characterization of this enzyme are underway.

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