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Isolation and identification of lipase producing thermophilic *Geobacillus* sp. SBS-4S: Cloning and characterization of the lipase

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A thermophilic microorganism, SBS-4S, was isolated from a hot spring located in Gilgit, Northern Areas of Pakistan. It was found to be an aerobic, gram-positive, rod-shaped, thermophilic bacterium that grew on various sugars, carboxylic acids and hydrocarbons at temperatures between 45°C and 75°C. Complete 16S rRNA gene sequence of the microorganism exhibited homology to various species of genus *Geobacillus*. A highest homology of 99.8% was found with *Geobacillus kaustophilus*. A partial (0.7 kbp) chaperonin gene sequence also showed a highest homology of 99.4% to that of *G. kaustophilus* whereas biochemical characteristics of the microorganism were similar to *Geobacillus uzenensis*. Based on biochemical characterization, 16S rRNA and chaperonin gene sequences, we identified SBS-4S as a strain of genus *Geobacillus*. Strain SBS-4S produced several extracellular enzymes including amylase, protease and lipase. The lipase encoding gene was cloned, expressed in *Escherichia coli* and the gene product was characterized. The recombinant lipase was optimally active at 60°C with stability at wide pH range (6–12). The enzyme activity was enhanced remarkably in the presence of Ca^{+2}. The K_m and the V_{max} for the hydrolysis of *p*-nitrophenyl acetate were 3.8 mM and 2273 μmol min^{-1} mg^{-1}, respectively. The ability of the recombinant enzyme to be stable at a wide pH range makes it a potential candidate for use in industry.

[**Key words:** 16S rRNA; Chaperonin; *Geobacillus* SBS-4S; Thermophiles; Thermostable lipase]

Thermophiles prefer to live at high temperatures where most of the other organisms fail to survive. There has been increasing interest in thermophilic bacteria because of their thermostable enzymes (1). Adaptation of these bacteria to hot environments (2), production of heat-stable enzymes (3), structural and functional relationships of these enzymes (4) and their biotechnological and industrial applications (5,6) are among the major areas of research on these microorganisms. Thermophiles have been isolated from hot springs and other thermal environments. Thermophilic Bacillus-related species have been isolated from various terrestrial soils and deep-sea sediments (7–11). The aerobic thermophilic bacteria *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius*, and *Bacillus thermodenitrificans* are reclassified into the new genus *Geobacillus* (9).

Species belonging to genus *Geobacillus* are reported to produce extracellular enzymes including amylase (12), xylanase (13) and lipase (14). Lipase hydrolyzes triglycerides to diglycerides, monoglycerides, glycerol and fatty acids. They have been characterized for industrial uses including digestive aids, food additives for flavor, reagents for the synthesis of useful compounds and treatment of domestic sewage. Many of these processes require thermostable lipases. Thermophilic bacteria could be good candidates for these lipases. However, low expression level of these proteins is a hurdle in their structure function relationship studies and to evaluate their biotechnological potential. Therefore, cloning such thermophilic enzymes into more suitable mesophilic hosts has been achieved in order to produce high levels of thermostable proteins (15).

In this study, we report on identification and characterization of a thermophilic bacterium isolated from a hot spring located in the Northern Areas of Pakistan. Furthermore, cloning and characterization of the thermostable lipase have also been described.

MATERIALS AND METHODS

Isolation of SBS-4S Water samples were collected in sterile conical tubes (Sterilin, U.K.) from a hot spring located in Gilgit, Pakistan. The collected samples were transported to the laboratory and kept at 4°C prior to the isolation procedure. For isolation of microorganisms samples were directly spread on solidified Luria Bertani (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 2.5% agar) plates. Plates were incubated at 55°C for 48 h.

Biochemical characterization Biochemical characterization was performed by using QTS-24 miniaturized identification system (DESTO Laboratories, Karachi, Pakistan). All methods and reagents preparations were carried out according to the instructions of the manufacturer. In addition to the biochemical tests given in the QTS-24 kit, production of oxidase, catalase, urease, xylanase, protease and amylase was examined.

Growth media For routine growth of bacterial cells LB medium was used. The growth of the microorganism was also examined on Modified LB, Tryptone Salt Medium, Nutrient Broth and Minimal Glucose Medium (16).

To examine the effect of salt concentration on growth, the microorganism was grown in TY medium (tryptone 1% and yeast extract 0.5%) with the addition of 0, 0.5, 1, 2, 3, 4 and 5% (final concentration) of NaCl in triplicate.

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TABLE 1. Primers used in this study and their nucleotide sequences.

Sr. no.	Primer name	Nucleotide sequence (5′–3′)
1	FGPS4-281 bis	AGAGTTTGATCCTGGCTCAG
2	FGPS1509-153	AAGGAGGTGATCCAGCCGCA
3	H729	CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCIGGIGAYGGIACIACIAC
4	H730	AGCGGATAACAATTTCACACAGGAYKIYKITCICCRAAICCIGGIGCYTT
5	SBS-LIP-N	CATATGGCGGCTTCGCGAGCCAACGATGCGCC
6	SBS-LIP-C	TTAAGGTTGCAAGCTCGCCAACTGCTCG

Underlined sequences are the nucleotides of M13/pUC sequencing primers.

Optimum pH for growth Buffers of various pH ranging from 3 to 9 were prepared and utilized for the preparation of LB medium. The following are the buffers and their pH range: 50 mM sodium acetate (pH 3–5), 50 mM sodium phosphate (pH 6–7) and 50 mM Tris–HCl (pH 8–9).

Antibiotic sensitivity In order to examine the sensitivity/resistance against antibiotics, the isolate was grown in LB medium containing ampicillin, amoxicillin, carbenicillin, choloramphenicol, gentamycine, kanamycin, rifampicin, streptomycine, spectinomycin and tetracycline. All antibiotics were tested at a final concentration of 50 μg/mL except for tetracycline which was used at a final concentration of 12.5 μg/mL in three independent experiments. Cultures were inoculated in duplicate for all the experiments and incubation was made at 55°C. The reading in all the cases was taken after 24 and 48 h.

Cloning of 16S rRNA gene The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers FGPS4-281 bis and FGPS1509-153 (Table 1) as forward and reverse primers, respectively (17) and genomic DNA of strain SBS-4S as template. The PCR amplified DNA fragment was ligated in pTZ57R/T and *Escherichia coli* DH5α cells were transformed. Recombinant clones were detected by blue/white screening. Cloning of the gene was confirmed by single digestion of the recombinant plasmid with *Eco*R1 and double digestion with *Eco*R1 and *Hin*dIII. Recombinant plasmid DNA was extracted and DNA sequencing from both the strands was performed using ABI-310 single capillary automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing was done in triplicate in order to get the correct sequence. Database homology searches were performed by using Basic Local Alignment Search Tool (BLAST) programme (18). Multiple sequence alignment and phylogenetic analyses were performed by using Clustal W programme (19) provided by DNA Data Bank of Japan.

Chaperonin gene amplification and homology comparison A part of chaperonin gene was amplified by PCR using H729 and H730 primers (Table 1) as priming strands and the genomic DNA of the isolate as a template. The primers H729 and H730 contained M13 forward and reverse sequencing primers respectively, therefore the PCR product was directly sequenced and the resulting DNA sequence was used for alignment and homology search.

Cloning of the lipase gene For cloning of the lipase gene forward (SBS-LIP-N) and reverse (SBS-LIP-C) primers were designed based on the DNA sequence of closest homologue of strain SBS-4S (*G. kaustophilus* strain HTA 426). The lipase gene was amplified by PCR using the primers SBS-LIP-N and SBS-LIP-C (Table 1). The purified PCR product was ligated in pTZ57R/T and *E. coli* DH5α cells were transformed. The resulting plasmid was named as pTZ-lip. Plasmid DNA was isolated from transformants and the restriction digestion (single and double) was done in order to confirm the exact size of the insert. DNA sequencing, database homology, multiple sequence alignment and phylogenetic analyses were performed as described above.

Expression of the lipase gene For expression, the gene fragment was cleaved from pTZ-lip utilizing the *Nde*I and *Hin*dIII restriction enzymes and ligated in the pET-21a expression vector previously digested with the same restriction enzymes. The resulting plasmid was named as pET-lip. This recombinant plasmid was used for the expression of the lipase gene in *E. coli* BL21(DE3). Cells carrying recombinant vector were grown for 16 h at 37°C in LB medium containing ampicillin (100 μg/mL). The preculture was inoculated (1%) into fresh LB medium containing ampicillin and cultivation was continued until the optical density at 660 nm reached to 0.4. The gene expression was induced with 0.1 mM (final concentration) isopropyl-β-D-

TABLE 2. Characteristics differentiating strain SBS-4S from the closely related species of the genus *Geobacillus*.

Characteristics	Microorganisms									
	SBS-4S	1	2	3	4	5	6	7	8	9
Motility	+	+	+	+	+	-	ND	ND	+	+
Production of acid from										
adonitol	−	−	v	−	−	v	+	ND	−	−
L-arabinose	+	−	−	−	−	−	−	+	+	−
inositol	+	−	−	−	−	−	+	ND	−	−
rhamnose	+	−	−	−	+	−	−	V	−	−
sorbitol	−	−	−	+	+	−	−	ND	−	−
glucose	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
mannose	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
maltose	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
sucrose	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
melibiose	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
raffinose	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hydrolysis of										
starch	+	+	+	+	+	+	+	V	+	+
gelatin	+	−	+	−	−	−	+	ND	+	−
urea	−	−	−	−	+	ND	−	−	−	−
citrate	−	−	v	+	−	+	+	V	−	−
malonate	+									
o-nitro-phenyl-β-D-galactopyranoside	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
lysine decarboxylase	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
arginine decarboxylase	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
ornithine decarboxylase	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
tryptophan deaminase	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
H_2S production	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
indole production	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
Voges–Proskauer test	−	−	−	−	−	−	−	−	−	−
gas from nitrate	+	−	v	+	−	−	−	+	−	+
oxidase	+	ND	V	+	ND	+	+	ND	+	ND
catalase	+	ND	v	+	ND	+	+	ND	+	ND
NaCl tolerance (%, w/v)	0–4	0–1	0–≤5	0–4	0–1.5	ND	0–≤5	0–3	0–4	0–≤5
pH range	6–9	5.5–8.5	6–8	ND	6.5–8.5	6.2–7.5	6–8	6–8	6.2–7.8	6–7.6

The numbers given at the top of each column are: 1, *G. gargensis* strain GaT; 2, *G. stearothermophilus*; 3, *G. thermocatenulatus*; 4, *G. thermoglucosidasius*; 5, *G. thermoleovorans*; 6, *G. kaustophilus*; 7, *G. thermodenitrificans*; 8, *G. uzenensis*; and 9, *G. subterraneus*. Symbols are: +, growth/activity observed; −, no growth/activity observed; ND, not determined; V, variable within group. Data are from the present study (*Geobacillus* SBS-4S) or from references 9 (*G. stearothermophilus*); 9 (*G. thermocatenulatus*); 28 (*G. thermoglucosidasius*); 9,27 (*G. thermoleovorans*); 9 (*G. kaustophilus*); 8 (*G. thermodenitrificans*); and 9 (*G. uzenensis*; *G. subterraneus*).