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*Full Length Research Paper*

# Isolation and screening of protease producing thermophilic *Bacillus* strains from different soil types of Pakistan

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**Thermophilic bacteria produce commercially useful proteases, active at high temperature. Due to growing demand of proteases application in different industries, the present study was designed to isolate protease producing bacillus strains. Nine strains were isolated from three different ecological sources and qualitatively screened on skim milk agar. The *Bacillus* strain PB1 showed 5.4 cm zone of hydrolysis. The screened protease producing strain was further identified on the basis of morphological, biochemical (API 20 and API 50 CHB) and 16 s rRNA molecular identification as *Bacillus licheniformis* PB1 (Accession EU650317).**

**Key words:** Thermophilic bacteria, proteases, 16S rRNA.

## INTRODUCTION

Thermophiles produce thermostable enzymes (Adams and Kelly, 1998) not only active at high temperature but are also resistant to and active in presence of organic solvents and detergents (Jaenicke et al., 1996) with advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in solubility of nongaseous reactants and products and reduced incidence of mesophilic organism contamination. Thermophilic bacteria from hot springs produced unique thermostable enzymes and proteases secreted from thermophilic bacteria are thus of particular interest and have become increasingly useful in a range of commercial applications. (Sonnleitner, 1983; Rahman et al., 1994; Rao et al., 1998; Zeikus et al., 1998; Singh et al., 2001). The genus *Bacillus* has been studied in considerable depth, and the ability of *Bacillus* strains to produce and secrete large quantities (20 – 25 g/l) of extracellular enzymes has placed them among the most important industrial enzyme producers (Schallamey et al., 2004). In industry, *Bacillus licheniformis* is used for the production of proteases or α-amylase. These hydrolases play an important role during vegetative growth and other phenomena occurring during the bacterium's cell cycle. The requirement of proteases for sporulation has been demonstrated by the use of protease inhibitors (Rao et al., 1998). There is great diversity in physiology among members of the genus, whose collective features include degradation of many substrates derived from plant and animal sources, including cellulose, starch, pectin, proteins, agar, hydrocarbons, antibiotic production, nitrification, denitrification, nitrogen fixation, facultative lithotrophy, autotrophy, acidophily, alkaliphily, psychrophily, thermophily and parasitism (Senesi et al., 2001). Spore formation, universally found in the genus, is believed to be a strategy for survival in the soil environment, wherein the bacteria predominate (Smith et al., 1952). Members of the genus *Bacillus* have played a significant dual role in many human activities. Species such as *Bacillus subtilis*,

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*Bacillus amyloliquefaciens* and *B. licheniformis* are used industrially for the production of enzymes, antibiotics, solvents and other molecules (Gerhartz, 1990), and *Bacillus thuringiensis* and *Bacillus sphaericus*, because of their insecticidal activity, are used in crop protection (Bourque et al., 1995) while *Bacillus mycoides* has the ability to promote plant growth (Petersen et al., 1995). Where as *B. subtilis*, *Bacillus clausii* or *Bacillus alcalophilus* have been used as an oral bacteriotherapeutic agent for the treatment of gastro-intestinal disorders (Hoa et al., 2000; Senesi et al., 2001; Casula and Cutting, 2002).

The objective of the current research study was to isolate, screen and identify the *Bacillus* strains having ability to produce low cost thermostable proteases with commercial applications.

## MATERIALS AND METHODS

### Isolation of thermophilic organisms (*Bacillus* Species)

For isolation of protease producing thermophilic organisms, soil samples from three ecological sources were collected. Briefly 1 g of the each soil sample was suspended in 100 ml sterile distilled water and agitated for 1 h at 60°C in shaking incubator (modified from Naidu and Devi, 2005), 0.1 ml was spread on nutrient agar and incubated further at 60°C for 24 h. Three colonies from each source were screened on skim milk agar containing NaCl 0.5%, peptone 0.3%, skimmed milk 0.3% and agar as 2.0%. Nine strains of aerobic thermophilic *Bacillus* were selected for study. Three strains, that is PB-I, PB-II and PB-III were isolated from soil of desert area of Sindh, Pakistan. Three other strains PB-IV, PB-V and PB-VI, were isolated from hot springs of Himalayan Mountain Range (Koh Himalaya) District Diamer Northern Areas of Pakistan. Strains PB-VII, PB-VIII and PB-IX were isolated from hot springs of Tata Pani, District Kotli, Azad Kashmir, Pakistan (Table 1). The cultures were routinely maintained at 60°C on Thermus Tryptone agar containing ingredients as tryptone 1.0%, NaCl 0.5% and agar as 2.0% at pH 7.5.

### Identification of bacterial isolates

These strains were cultured, maintained and identified in Microbiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan. The strains were identified on the basis of standard morphological methods and Gram's reaction. While the best enzyme producing strain was further followed for identification on the basis of API-20E, API-50CHB and 16S rRNA.

### Isolation of genomic DNA

50 µl lysis solutions was placed into eppendorf tubes, a part of colony with a sterile tooth pick was suspended into lysis solution. The suspension was heated at 95°C for 15 min at 500 – 700 rpm. The suspension cooled to room temperature and topped with 450 µl sterile dist.$H_2O$. The tubes were vortexed and centrifuged for 4 min at 6000 rpm, 100 µl of supernatant was placed in new tube as template DNA stock.

### Polymerase chain reaction amplification

Bacterial isolates were identified by polymerase chain reaction (PCR). Amplification was performed for 30 cycles with denaturation at 94°C for 3 min, annealing at 56°C for 1 min, extension at 72°C for 2 min by using the bacterial Universal primers 27_forward 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492_reverse 5'-TACGGTTACCTTGTTACGACTT-3' as previously described by (Christner, 2002). DNA was observed following electrophoresis in 1% agarose in TPE buffer (90 mM Tris-phosphate, 2 mM EDTA) and staining with ethidium bromide (1 µg $ml^{-1}$ in TPE buffer) (Webb et al., 2000).

### Purification

Amplified products were purified using the QIA quick PCR purification kit (Qiagen Ltd., Crawley, United Kingdom). Quantification of the DNA was done by nanodrop spectroscopy.

### 16S rRNA gene sequencing and phylogenetic analysis

PCR products were sequenced in house, forward and reverse sequences were aligned using ABI Auto-assembler software (Applied Biosystems Inc.), Both strands of the amplified products were sequenced using the ABI BigDye Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Warrington, United Kingdom). The resulting sequence was edited to a total length of 1020 bp. The deduced sequence was subjected to BLAST search tool, for the closest match in the NCBI database. Phylogenetic analysis was performed by subjecting the deduced sequence to the 16 S rRNA database to obtain closely isolated sequences and the phylogenetic tree was constructed based on evolutionary distances (Webb et al., 2000; Atiq et al., 2010).

## RESULTS

### Isolation and identification of bacterial strains

Nine strains of aerobic thermophilic *Bacillus* species isolated from different sources as PB I-III from desert of Sindh, PB IV-VI from hot spring Diamer (Northern areas) and PB VII-IX from Tata pani (Kotli, AJK) were used in this study. Hot springs and dry hot areas are the major sources of the thermophilic microorganisms.

### Qualitative test / proteolytic ability of bacterial isolates and growth pattern of *Bacillus* strain PB1 on different media

The extracellular thermostable protease producing ability was screened. All the nine strains were inoculated on skim milk agar for 24 to 72 h at 60°C, and after growth the zone of hydrolysis was measured. The results (Figure 1A and Table 1) clearly showed that maximum zone of hydrolysis (5.4 cm) was shown by only one *Bacillus* strain PBI isolated from desert of Sindh, whereas no zone was shown by other isolated strains PB II – PB IX. *Bacillus*

**Table 1.** *Bacillus* strains PB1-IX showing zone of hydrolysis, isolated from 3 different ecological sources of Pakistan.

| S/N | Thermophilic *Bacillus* strains | Zone of hydrolysis (cm) | Source of isolation |
|---|---|---|---|
| 1 | *Bacillus* strain PB-I | 5.4 | Soil (*Sindh Desert*) |
| 2 | *Bacillus* strain PB-II | Nil | Soil (*Sindh Desert*) |
| 3 | *Bacillus* strain PB-III | Nil | Soil (*Sindh Desert*) |
| 4 | *Bacillus* strain PB-IV | Nil | Hot springs (*Diamer*) |
| 5 | *Bacillus* strain PB-V | Nil | Hot springs (*Diamer*) |
| 6 | *Bacillus* strain PB-VI | Nil | Hot springs (*Diamer*) |
| 7 | *Bacillus* strain PB-VII | Nil | Tata Pani (*Kotli*) |
| 8 | *Bacillus* strain PB-VIII | Nil | Tata Pani (*Kotli*) |
| *9* | *Bacillus* strain PB-*IX* | Nil | Tata Pani (*Kotli*) |

strain PB I was used for further study. The different patterns of growth were also checked on various media (Figures 1B, C and D).

After screening on skim milk agar, one *Bacillus* strain PB1 was selected as good enzyme producer. This strain was identified on the basis of standard basic morphological, physiological and by using API 20E (for addition of 12 tests), API 50 CHB and 16S rRNA technique.

### Morphological and physiological features

The bacterium was rod shaped hemolytic with bulging central spores, exhibited gram positive staining as revealed by light microscopy and was motile as observed by hanging drop method. The isolate could ferment various sugars as revealed by API 20E and 50 CHB test results (Table 2). The spores were abundant in late stationary phase and showed direct relationship with protease production. The isolate grows in temperature ranges between 30 - 75°C, pH 4.0 - 12.0 and up to NaCl concentration 15%.

### 16S rRNA gene sequencing and phylogenetic analysis

About 98% homology of sequences shows with *B. licheniformis.* NCBI BLAST results (tree list) are attached (Figure 2).

## DISCUSSION

In the present study nine strains were isolated, PB I-IX, and screened on skim milk agar and one strain PB1 was found as protease producer. As the organism was isolated from desert soil of Sindh and genus *Bacillus* are generally found in soil, the proteases of these microorganisms play an important role in nitrogen cycle, which contributes to the fertility of soil. Soil protease is thought to be mainly supplied by soil microorganisms (Godfrey and Reichelt, 1983; Watanabe and Hayano, 1993). *Bacilli* have great capacity to produce variety of extracellular proteins and secret large quantities (20 - 25 g/L), and this property has placed them amongst the most important industrial protein producers (Chang et al., 1982; Schallamey et al., 2004).

In the present study, the isolated nine strains were screened qualitatively on skim milk agar and the alkaline proteolytic activity was correlated with the zone of protein hydrolysis around bacterial colonies. The qualitative test revealed the result that (Figure 1A, Table 1) one strain out of nine produced zone of hydrolysis (5.4 cm) and this strain as hyper-protease producer was used for further studies. It has been reported that *B. licheniformis* produces very narrow zone of hydrolysis on casein-agar despite being very good protease producers in submerged cultures (Mao et al., 1992), but *B. licheniformis* PB1 produced very prominent and distinct zone of hydrolysis.

The isolated strain was identified on morphological, biochemical and molecular basis. The bacterium was rod-shaped forming spores central and abundant in early stationary phase. Cell exhibited gram positive morphology when Gram's stained and observed under light microscope. The organism was hemolytic in nature and motile, grows in Nutrient Agar in temperature ranges between 30 - 75°C, pH ranges 4 - 12 and up to 15% NaCl concentration. The isolate could ferment sugars (results decarboxylase as positive, but $H_2S$ and indole negative. It cannot ferment inositol, rhamnose and melebiose. Details of 50 CHB API tests results are given in Table 2.

The isolate was also identified on 16S rRNA genetic analysis. BLAST analysis of the 70 S bases of 16 S rRNA of API 20E), mainly glucose, mannose, sorbitol, saccharose, with vogus prausker, urease, citrate, orthonitrophenyl β-D galactopyranoside, amylase, L-lysine decarboxylate, arginine dihydrolase, orthinine sequence of *Bacillus* strain PB-1 (Figure 2) revealed

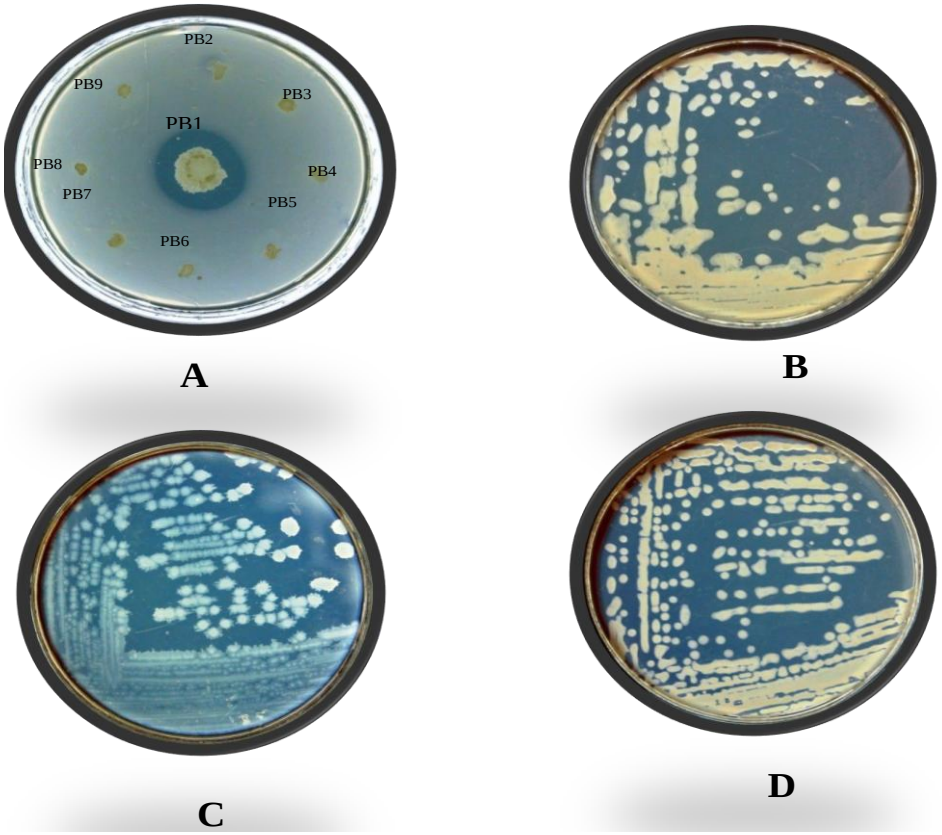


**Figure 1A.** Comparison of zone of hydrolysis on skim milk agar by all 9 isolated *Bacillus* strains PB1-9, (B) Growth pattern of *Bacillus* strain PB1 on Nutrient agar. (C) Growth pattern of *Bacillus* strain PB1 on skim milk agar, (D) Growth pattern of *Bacillus* strain PB1 on Thermus tryptone agar.

**Table 2.** Biochemical tests for Isolate *B. licheniformis* PB1 using API 50 CHB.

| S/N | Test | Results | | S/N | Test | Results | |
|---|---|---|---|---|---|---|---|
| | | 24 h | 48 h | | | 24 h | 48 h |
| 1 | Control | -ve | -ve | 26 | ESC (Esculine Citrate) | + ve | + ve |
| 2 | Gly (Glycerol) | +ve | + ve | 27 | SAL (Salicine) | + ve | + ve |
| 3 | ERY (Erytheritol) | - ve | - ve | 28 | CEL (D-Cellobiose) | + ve | + ve |
| 4 | DARA (D-Arabinose) | - ve | - ve | 29 | MAL (D-Maltose) | + ve | + ve |
| 5 | LARA (L-Arabinose) | + ve | + ve | 30 | LAC (D-Lactose) | - ve | + ve |
| 6 | RIB (D-Ribose) | + ve | + ve | 31 | MAL (D-Mellibiose) | - ve | + ve |
| 7 | DXYL (D-Xylose) | + ve | + ve | 32 | SAC (D-Saccharose) | + ve | + ve |
| 8 | LXYL (L-Xylose) | - ve | - ve | 33 | TRE (D-Trehalose) | - ve | - ve |
| 9 | ADO (D-Adonitol) | - ve | - ve | 34 | INU (Inuline) | - ve | - ve |
| 10 | MDX (Methyl β-D-Xylopyranoside) | - ve | - ve | 35 | MLZ (D-Melezitose) | - ve | + ve |
| 11 | GAL (D-Galactose) | + ve | + ve | 36 | RAF (Raffinose) | - ve | + ve |
| 12 | GLU (D-Glucose) | + ve | + ve | 37 | AMD (Amidone) | + ve | + ve |
| 13 | FRU (D-Fructose) | + ve | + ve | 38 | GLYG (Glycogene) | + ve | + ve |
| 14 | MNE (D-Mannose) | + ve | + ve | 39 | XLT (Xylitol) | - ve | + ve |
| 15 | SBE (L-Sorbose E) | - ve | - ve | 40 | GEN (Gentiobiose) | + ve | + ve |
| 16 | RHA (L-Rhamnose) | - ve | + ve | 41 | TUR (D-Turanose) | + ve | + ve |
| 17 | DUL (Dulcitol) | - ve | - ve | 42 | LYX (D-Lyxose) | - ve | - ve |

**Table 2.** Contd.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 18 | INO (Inositol) | + ve | + ve | 43 | TAG (D-Tagatose) | + ve | + ve |
| 19 | MAN (D-Mannitol) | + ve | + ve | 44 | DFUC (D-Fucose) | - ve | - ve |
| 20 | SOR (D-Sorbitol) | + ve | + ve | 45 | LFUC (L-Fucose) | - ve | - ve |
| 21 | MDM (Methyl α-D Mannopyranoside) | - ve | - ve | 46 | DARL (D-Arabitol) | - ve | - ve |
| 22 | MDG (Methyl α-D Glucopyranoside) | + ve | + ve | 47 | LARL (L-Arabitol) | - ve | - ve |
| 23 | NAG(N-acetyl Glucoseamine) | - ve | - ve | 48 | GNT(Potassium luconate) | - ve | - ve |
| 24 | AMY (Amygdaline) | + ve | + ve | 49 | 2KG (Potassium 2-cetogluconate) | - ve | - ve |
| 25 | ARB (Arbutine) | + ve | + ve | 50 | 5KG (Potassium 5-cetogluconate) | - ve | - ve |

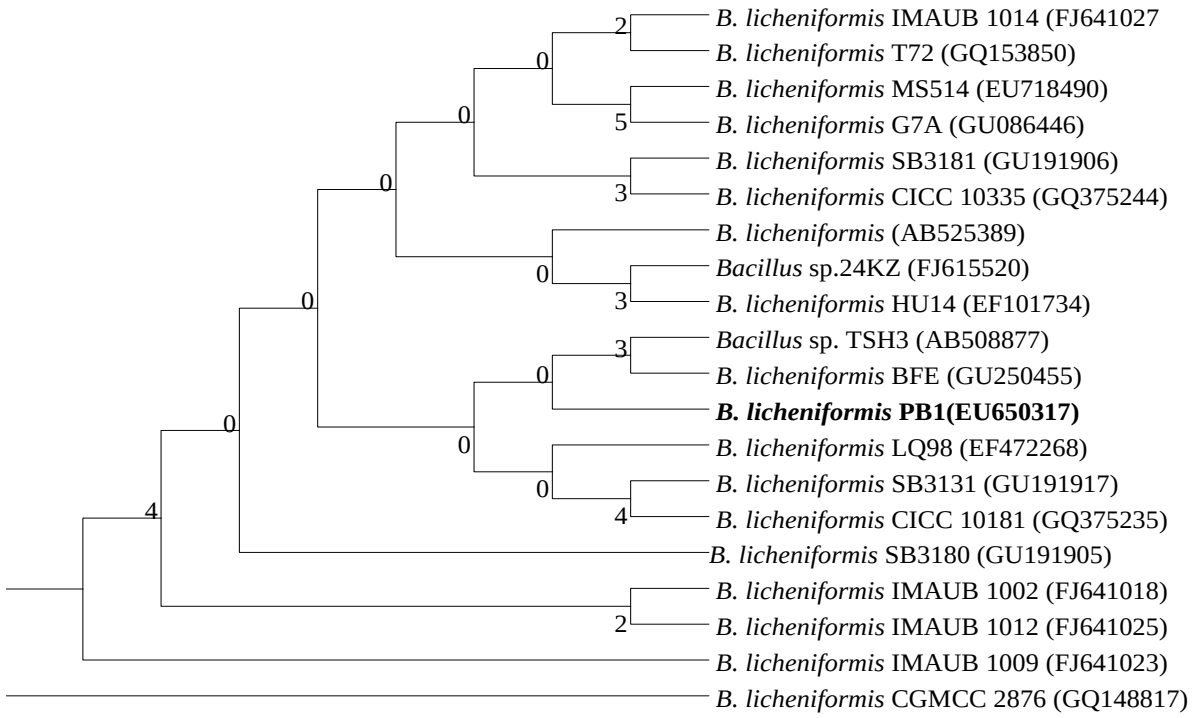


**Figure 2.** Phylogenetic relationship of *B. licheniformis* PB1.

that it had a closest match (98% homology) with *B. licheniformis*. The sequence is deposited in NCBI Gene Bank with accession no EU650317. The API 50CHB test results were also confirmed by software version 4.0 from Boehringer pvt. Ltd. Which showed 98.9% I.D. relation with *B. licheniformis*. The overall API based biochemical and physiological traits also suggest that this isolate can be among the members of *Bacillus* species of phylogenetic Group 1 which comprised 3 of 22 *Bacillus* species (Buchanan et al., 1974).

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