Annals of Microbiology, **57 (3)** 369-375 (2007)

# Isolation and identification of alkaline protease producer halotolerant *Bacillus licheniformis* strain BA17

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*Received 5 February 2007 / Accepted 5 June 2007*

**Abstract -** An alkaline protease producer *Bacillus licheniformis* strain was isolated from Van Lake in Turkey. The strain is Gram positive, aerobic, motile, sporulating rod-shaped bacterium. Spores were ellipsoidal and positioned central in nonswollen sporangium. The cells were able to grow well at a pH range of 5.7-10. The optimal growth temperature was found to be 37 °C. Growth at a wide range of NaCl concentration (from 0 to 20%) showed that BA17 is halotolerant. Main fatty acid composition of BA17 was anteiso-C15:0 and iso-C15:0. The strain was presumptively identified as *B. licheniformis* according to 16S rDNA gene sequence analysis. The most appropriate medium for the growth and protease production is composed of 0.5% yeast extract, 0.5% $NaNO_3$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% $K_2HPO_4$ and 0.5% maltose. The optimum temperature and pH of the alkaline protease of strain BA17 were found to be 60 °C and pH 11, respectively. The activity was completely lost in the presence of PMSF, suggesting that the preparation contains serine-alkaline protease(s).

**Key words:** *Bacillus licheniformis*, halophiles, alkaline protease, protease production.

## INTRODUCTION

Extremozymes have a great economic potential in many industrial processes, including agricultural, chemical and pharmaceutical applications (van den Burg, 2003). Since the present enzymes are not sufficient to meet most of the industrial demand, isolation and characterisation of extremophiles have attracted attention from researchers (Patel *et. al.*, 2005).

Halophiles are salt-loving organisms that inhabit hypersaline environments. Halotolerant and halophilic microorganisms grow in 1 to 20% NaCl (w/v) and some can grow in NaCl (> 30% w/v) saturated waters. Halophiles can survive in hypersaline habitats by their ability to maintain osmotic balance. They accumulate salts such as sodium or potassium chloride (NaCl or KCl), up to concentrations that are isotonic with the environment. As a result, proteins from halophiles have to cope with very high salt concentrations (e.g. KCl concentrations of > 4 M and NaCl concentrations of > 5 M) (Danson and Hough, 1997; Demirjian *et al*., 2001). The production of halophilic enzymes, such as xylanases, amylases, proteases and lipases, has been reported for some halophiles. Low solubility of halophilic enzymes has been taken advantage of by applying them in aqueous/organic and non-aqueous media (Gomes and Steiner, 2004).

Soda lakes represent a specific type of salt lakes. Like most of the other inland salt lakes, soda lakes are located in areas with a dry climate that facilitates salt accumulation in depressions (Sorokin and Kuenen, 2005). Soda lakes, particularly the more dilute soda lakes, are the most productive aquatic environments in the world, with productivities, on average, an order of magnitude greater than the average for terrestrial aquatic environments (Grant *et al.*, 1990). Dense populations of alkaliphilic and organotrophic bacteria are supported by the primary productivity, and there are active sulphur and nitrogen cycles in the lakes (Grant and Jones, 2000). Since the microorganisms in the soda lakes are mostly alkaliphilic, it is to be expected that extracellular enzymes produced by these microorganisms would be active under alkaline conditions, moreover, active in the virtual absence of significant levels of $Mg^{2+}$ and $Ca^{2+}$. This is indeed the case, and such enzymes are of biotechnological interest, particularly as detergent additives, since detergents used in domestic and industrial washing processes are alkaline and contain sequestering agents to remove $Ca^{2+}$ (which adversely affects the water hardness and foam characteristics).

Lake Van is by volume the fourth largest closed body of water (volume 607 $km^3$, area 3570 $km^2$, maximum depth

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450 m, lake level 1648 m above sea level) and harbours the largest known microbialites on Earth (Kempe and Degens, 1984). It is also known as the largest soda lake on earth with a pH of 9.7-9.8 and a salinity of 21.7 (Berber and Yenidünya, 2005). The surface of these huge carbonate pinnacles is covered by coccoid cyanobacteria whereas their central axis is occupied by a channel through which neutral, relatively Ca enriched, groundwater flows into highly alkaline (pH 9.7) Ca-poor lake water (Lopez-Garcia *et al.*, 2005). Because of winter convection, chemical composition of the lake water is homogeneous throughout the lake. Though they bring in waters of varying compositions, the rivers discharge a larger quantity of bicarbonate than alkaline earth metals. This soda-chemistry may be attributed to postvolcanic $CO_2$-activity (Degens *et al.*, 1984).

Microbial alkaline proteases constitute one of the most important groups of hydrolytic enzymes, accounting for about 50% of the total industrial enzyme market, since industrial applications of alkaline proteases such as detergents, food, leather and silk has increased remarkably (Gessesse, 1997; Horikoshi, 1999; Kumar and Takagi, 1999; Kim *et al*., 2001; Ghorbel *et al.*, 2003; Manczinger *et al*., 2003; Joo and Chang, 2005; Patel *et al.*, 2005; Amoozegar *et al*., 2006; Hadj-Ali *et al.*, 2007).

*Bacillus* strains have considerable importance because of their ability to produce large amount of alkaline protease (Mabrouk et al., 1999; Joo *et al*., 2002, 2003; Manczinger *et al*., 2003; Denizci *et al*., 2004; Joo and Chang, 2005; Patel *et al*., 2005; Folmsbee *et al*., 2006; Kamoun *et al*., 2006; Öner *et al*., 2006; Sılva *et al*., 2006; Hadj-Ali *et al*., 2007). While, there are a lot of work related with the proteases from alkaliphiles and thermophiles (Mabrouk *et al*., 1999; Joo and Chang, 2005; Kazan *et al*., 2005), proteases from halophiles and moderate halophiles have not been investigated extensively. A few proteases from halophilic bacteria have been studied (Manachini *et al*., 1998; Ventosa *et al*., 1998; Gimènez *et al*., 2000; Joo and Chang, 2005; Amoozegar *et al*., 2006).

In this paper, we are reporting the halotolerant alkaline protease producer *Bacillus licheniformis* BA17 strain that was isolated from Van Lake in Turkey.

## MATERIALS AND METHODS

**Chemicals.** Chemicals used in the bacterial cell cultivation and taxonomic studies were supplied by Merck AG (Darmstadt, Germany). All other chemicals were analytical grade and supplied by Sigma Chem. Ltd. (USA).

**Screening of alkaline protease producing strains and phenotypic characterisation.** The soil samples were collected from the sediment of the Van Lake, a soda lake in the east part of Turkey. Soil samples (1 g) were inoculated in 5 ml growth medium (DSMZ 625: HP101 Halophile Medium containing, g/l: peptone10, yeast extract 1, $MgSO_4 \cdot 7H_2O$ 4.3, $NaNO_3$ 2, NaCl 100, agar 20) in 25 ml glass bottles and incubated for overnight at 37 $^oC$, after treatment for 10 min at 80 $^oC$. After enrichment, the broth was diluted serially with sterile distilled water and the diluted samples were plated onto agar plates containing (g/l) peptone 20, $NaNO_3$ 2.5, $(NH_4)NO_3$ 2.5, lactose 5, $ZnSO_4$ 1.25, $NaH_2PO_4$ 2, and skim milk 10 (Kumar and Takagi, 1999). The pH of the medium was adjusted to 9.6 with $Na_2CO_3$ after sterilisation. Plates were incubated at 37 °C for 24-48 h. A clear zone around the colonies indicated the protease producing microorganisms. Microorganisms which exhibited the largest clear zone around their colonies were purified and alkaline protease activity were determined in liquid medium containing (g/l) yeast extract 5, starch 10, $MgSO_4 \cdot 7H_2O$ 0.2, and $K_2HPO_4$ 1. The liquid medium was also used as a basal medium for alkaline protease production. The BA17 strain which produced maximum alkaline protease activity in liquid medium was selected and used in all further investigations. The morphological, cultural, physiological and biochemical properties of the strain BA17 have been determined as suggested by Bergey's Manual of Determinative Bacteriology (Claus and Berkeley, 1986). Carbon source utilisation experiments were performed using API 50 CHB gallery (Biomérieux) according to the supplier's instructions. Phenotypic assays for strain BA17 were performed as duplicate.

**Determination of cellular fatty acids.** The cellular fatty acid analyses of microorganisms were carried out by the Sherlock-MIDI Automated Microbial Identification System version 4.0 (MIDI Inc., Newark, DE) according to the procedures described in the User Manual of MIDI.

**Genomic DNA extraction and 16S rDNA sequence analysis.** Genomic DNA of strain BA17 was extracted from cultures using the Genomic DNA Purification Kit (Fermentas, USA) as described by the manufacturer.

Amplification of 16S rDNA gene was accomplished using the Bacteria-specific oligonucleotide primers f27(5'-AGAGTTTGATCATGGCTCAG-3', Escherichia coli position 8-27) (Britschgi and Giovannoni, 1991) and U1492r (5'-ACCTTGTTACGACTT-3', *Escherichia coli* position 1507-1492) (Edgcomb *et al*., 2002). The amplification was done by initial denaturation at 94 °C for 5 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C, and extension at 72 °C for 3 min, ending with 10 min of incubation at 72 °C. The amplification of the four PCR reactions were combined and purified with Wizard SV Gel and PCR Clean-UP System (Promega, USA) as described by the manufacturer.

DNA sequencing of purified PCR product was performed in both directions using an automated sequence by ABI-310 (Applied Biosystems). Primers used for sequencing were f27, U1492r and U517f (5'-GTGCCAGCMGCCGCGG-3', *E. coli* position 514-529). Counting building was carried out by using SeqMan from the DNA Star package. Homology search (GenBank/EMBL/DDBJ) was carried out by using the Basic Local Alignment Search Tools (BLAST) program (Altschul *et al*., 1990) and 16S rDNA sequences were aligned by the Clustal multiple alignment program (Clustal W 1.8) (Thompson *et al*., 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al*., 2004). The 16S rRNA sequence of *Bacillus cereus* and *Bacillus lentus* was chosen as the outgroup sequence.16S rDNA sequence of the strain BA17 has been deposited in the NCBI databases under the accession number DQ176435.

**Alkaline protease production.** For alkaline protease production, the strain BA17 were inoculated with 24-h-old inoculum culture at 1% v/v ratio in 250 ml Erlenmeyer flask containing 50 ml basal medium containing (g/l) yeast

extract 5, starch 10, $MgSO_4 \cdot 7H_2O$ 0.2, and $K_2HPO_4$ 1. The medium pH was adjusted to 9.0 by carbonate buffer solution (4.2% w/v $NaHCO_3$ and 5.3% w/v $Na_2CO_3$) after sterilisation. The flasks were incubated in an orbital shaker (180 rpm) at 37 °C and cultivated for 30 h. Different carbon and nitrogen sources were used for the production of alkaline protease enzyme. Protease production was first tested in basal medium containing different organic and inorganic nitrogen sources ($NH_4NO_3$, $NaNO_3$, $(NH_4)_2SO_4$, yeast extract, peptone, 1:1 mixture of peptone and yeast extract). The amount of inorganic and organic nitrogen sources in the growth medium were kept at 1% (w/v) and 0.5% (w/v), respectively (Mehrotra *et al*., 1999). The effect of various carbon sources on protease synthesis in media containing (w/v) sodium nitrate 1% and yeast extract 0.5% as nitrogen source, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% $K_2HPO_4$ and 1% starch was also investigated by replacing starch with 1% (w/v) xylose, glucose, arabinose, maltose, rhamnose and lactose. In order to determine the extent of improvement $Ca^{2+}$ ions would bring to the enzyme production, the medium was supplemented with 0-0.5% (w/v) $CaCl_2$. At the end of the incubation period, the cultures were centrifuged and supernatants were used for enzyme activity. The growth of the microorganism was determined by measuring absorbance at 600 nm.

**Determination of alkaline protease activity.** Protease activity was measured by the method of Takami *et al*. (1989) using casein as substrate. 0.5 ml aliquot of the culture supernatant and 2.5 ml of 0.6% casein in 50 mM glycine-NaOH-NaCl buffer pH 10.5 were incubated at 30 °C for 20 min. The reaction was stopped by the addition of 2.5 ml TCA solution and subsequently incubated at 30 °C for 30 min. The precipitates were removed via filtration and 2.5 ml 0.5 M $Na_2CO_3$ followed by 0.5 ml Folin & Ciocalteu's Color Solution were then added to 0.5 ml of clear filtrate. After 30 min incubation at room temperature, supernatant was estimated spectrophotometrically at 660 nm using sterile medium treated in the same way as a blank.

One unit of alkaline protease activity was defined as the amount of the enzyme able to produce 1 μg tyrosine in one min under the above assay conditions.

**Temperature and pH profile of crude enzyme.** The effect of temperature on the enzyme activity was investigated at temperatures between 30 and 80 °C at pH 10.5. Optimum pH of the crude preparation was studied in the range of 8-13 using 50 mM glycine-NaOH buffer for pH 8-12 and 200 mM glycine-NaOH buffer for pH 12.5 and 13. Reaction mixtures were incubated at 30 °C for 20 min. Alkaline protease activity at different temperature and pH values were measured as described above. The temperature and pH values at which the highest activity was obtained were considered as 100 and other activities are expressed as relative activity. Results are given as arithmetic averages of multiple experiments with less than 5% of standard error.

**Effect of phenylmethylsulphonyl fluoride (PMSF)**. In order to investigate the effect of PMSF on enzyme activity, bulk enzyme was incubated with 5 mM PMSF for 2 h. Serine specific active site directed irreversible inhibitor PMSF was freshly prepared in absolute ethanol as stock solution and diluted to appropriate concentration with 50 mM NaOH-Glycine-NaCl buffer, pH 10.5. Enzyme solution (235 U/mg specific activity, 0.118 mg/ml protein) was mixed with PMSF at a concentration of 5 mM, and incubated at 30°C for 2 h.At the end of incubation, residual activity was determined.

## RESULTS

### Morphological and physiological characteristics

Bacterial isolate BA17 was identified as member of *Bacillus* species. Isolate BA17 is a Gram positive, aerobic, motile, sporulating rod-shaped bacterium. Spores are ellipsoidal and positioned central in nonswollen sporangium. The cells were able to grow well in nutrient broth at a pH range of 5.7-10, and at temperatures of 30 and 40 °C. The optimal growth temperature was 37 °C and no growth was observed at 55 and 65 °C. Bacterial growth at a wide range of NaCl concentration (from 0 to 20%) showed that the isolate BA17 is a halotolerant strain. Isolate BA17 and the type strain of *Bacillus licheniformis* ATCC 14580$^T$ (according to Bergey's) showed nearly identical phenotypes except the growth at temperature 50 and 55 °C and gelatin hydrolysis. Isolate BA17 was able to utilise erythritol, ribose, D-xylose, L-xylose, glucose, fructose, mannose, sorbose, mannitol, sorbitol, N-acetyl glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, starch, D-lyxose, D-lucose. However, BA17 strain was not able to utilise glycerol, D-arabinose, L-arabinose, adonitol, β-methyl-D-xyloside, galactose, rhamnose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, melezitose, raffinose, glycogen, xylitol, gentibiose, D-tagatose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, 5-keto-gluconate.

Main fatty acid composition of isolate BA17 was found to be 44.75% and 18.31% anteiso-C 15:0 and iso-C 15:0, respectively. The major fatty acid component of BA17, type species of *B. licheniformis* CCTCC AB94036$^T$, *B. licheniformis* 170513 and *B. licheniformis* 170514 are predominant fatty acids in the genus *Bacillus* strains examined (Song *et al*., 2000).

### 16S rDNA sequences analysis and DNA base composition

A phylogenetic tree based on comparison of 16S rDNA sequences of reference *Bacillus* strains (Fig. 1) was constructed in order to understand the phylogenetic position of our strain. BLAST analyses of the 1483 bases of the 16S rDNA sequence of the halotolerant bacterium *Bacillus* strain BA17 revealed that it had closest match with *Bacillus licheniformis* ATCC 14580$^T$ (X68416). The overall biochemical, physiological and chemotaxonomical traits also suggest that isolate BA17 is placed among the member of *Bacillus licheniformis* group.

### Protease production

The protease production was first investigated in basal medium containing different organic and inorganic nitrogen sources. As shown in Table 1, *B. licheniformis* BA17 exhibited higher productivity of alkaline protease in basal medium containing 0.5% yeast extract and 1% sodium nitrate as nitrogen sources. Protease production was reduced in the presence of yeast extract and peptone at a concentra-

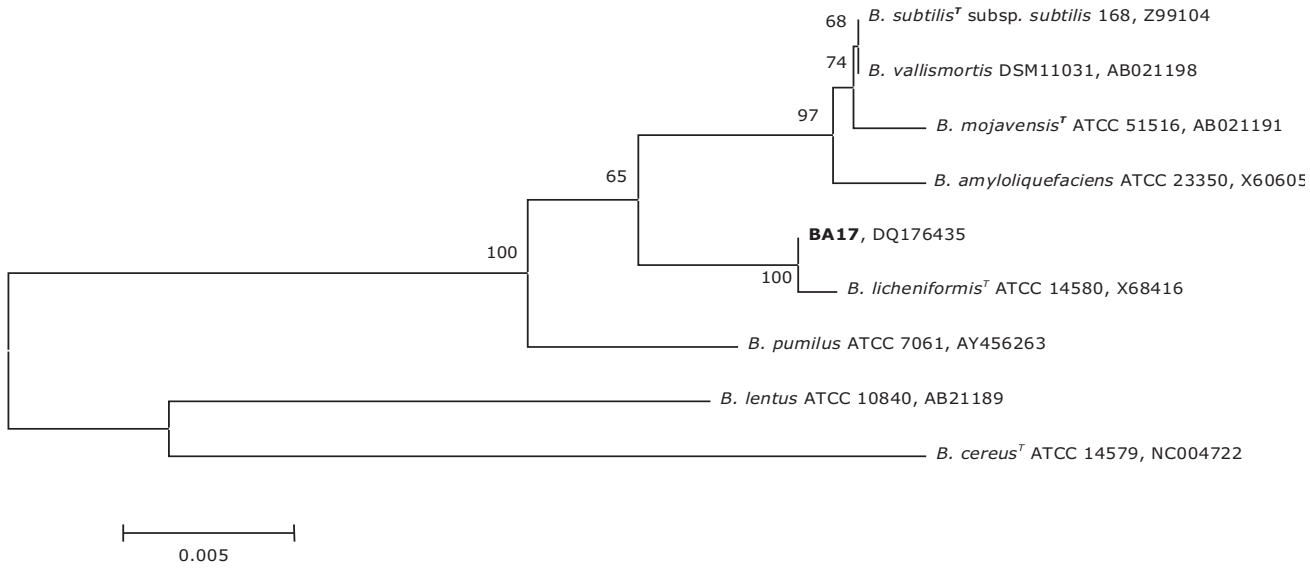


FIG. 1 - Neighbour-joining tree showing the position of isolate BA17 with the other members of the *Bacillus subtilis* group. Numbers show the level of bootstrap support out of 1000 resembling. The bar indicates 0.005 nucleotide substitutions per nucleotide.

tion of 0.25%, although the highest growth was obtained. The lowest protease production (0.81 U/ml) was obtained when yeast extract at a concentration of 0.5% was used alone. No extracellular protease activity was determined when sodium sulphate was added to the medium including 0.5% yeast extract.

The highest alkaline protease production was obtained in the presence of maltose as carbon source (Table 2). Although glucose promoted the bacterial growth, it also reduced the enzyme activity to 16.60 U/ml. The lowest enzyme production was obtained when the microorganism was grown in the presence of xylose. Since maltose was the most favourable carbon source, the effect of maltose concentration between 0.5 and 150 g/l was also investigated. As shown in Table 3, alkaline protease activity and growth were increased with increasing maltose concentration up to 50 g/l. At higher maltose concentrations, both protease activity and growth were decreased. Although maximum protease productivity (141 U/ml) was observed at 50 g/l maltose concentration, product yield as 28440 U/g maltose (estimated as protease production per gram of carbon source added) reached a maximum value at 0.5 g/l of maltose. In the presence of $Ca^{2+}$ ions, no significant improvement in enzyme production was observed.

TABLE 1 - Growth and protease production with different nitrogen source

| Nitrogen Source | Protease activity (U/ml) | Biomass ($A_{660}$) |
|---|---|---|
| Yeast extract 0.5% | 0.81 | 1.72 |
| Yeast extract 0.25% + Peptone 0.25% | 3.02 | 2.11 |
| Peptone 0.5% | 1.95 | 1.64 |
| Yeast extract 0.5% + Ammonium nitrate 1% | 14.39 | 1.49 |
| Yeast extract 0.5% + Sodium nitrate 1% | 28.53 | 1.61 |
| Yeast extract 0.5% + Ammonium sulphate 1% | 0 | 1.49 |

### Effect of temperature, pH and PMSF on enzyme activity

The effects of temperature and pH on protease activity are shown in figure 2A and 2B. The optimum temperature and pH of the alkaline protease of strain BA17 were found to be 60 ºC and pH 11, respectively.

Complete inhibition of enzyme activity in the presence of PMSF indicated that the crude enzyme preparation contained serine-protease(s).

## DISCUSSION

Halotolerant and halophilic microorganisms offer a multitude of actual or potential applications in various fields of biotechnology because of their adaptation and survival abilities in a wide range of salinity (Margesin and Schinner, 2001). During the last decade, the extensive studies on hypersaline environments that have been carried out in many geographical areas have permitted the isolation and taxonomic characterisation of a large number of halophilic species (Ventosa *et al.,* 1998). Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially (Patel *et al.*, 2005). In this work, 24 protease-producing bacterial species were isolated from Van Lake in Turkey. Among these, *Bacillus licheniformis* BA17 identified on the basis of a 16S rDNA sequence is the best producer of extracellular protease was selected for further studies.

*Bacillus licheniformis* is ubiquitous in nature, existing predominately in soil as spores. Although the strain BA17 shared the main phenotypic features of type strain *B. licheniformis* except growth at 50-55 ºC and gelatin hydrol-

TABLE 2 - Effect of different carbon source on alkaline protease production by *Bacillus licheniformis* BA17

| Carbon source | Starch | Xylose | Glucose | Arabinose | Maltose | Rhamnose | Lactose |
|---|---|---|---|---|---|---|---|
| Protease activity (U/ml) | 28.53 | 5.83 | 16.60 | 17.08 | 107 | 10.03 | 20.46 |
| Biomass (A660) | 1.61 | 2.50 | 5.56 | 1.10 | 4.77 | 1.65 | 2.00 |

TABLE 3 - Effect of maltose concentration on protease production by *Bacillus licheniformis* BA17

| | Maltose concentration (g/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.5 | 5 | 10 | 20 | 50 | 75 | 100 | 150 |
| Protease activity (U/ml) | 14.22 | 67.21 | 107 | 103 | 141 | 41.55 | 50.24 | 21.24 |
| Biomass (A660) | 2.25 | 4.38 | 4.77 | 4.31 | 4.25 | 1.98 | 1.97 | 1.93 |
| Yield (U/g maltose) | 28440 | 13442 | 10734 | 5153 | 2828 | 554 | 502 | 142 |

yses were different from type strain of *B. licheniformis* ATCC 14580$^T$ (according to Bergey's).

Song *et al*. (2000) reported that the highest cellular fatty acid composition of *B. licheniformis* CCTCC AB 94036$^T$, *B. licheniformis* 170513 and *B. licheniformis* 170514 were anteiso-C15:0 which is very close to our finding. Silva *et al*. (2006) obtained similar results with our findings that nine *B. licheniformis* strains were close and highest content of anteiso-C15:0 was determined in those strains. The high similarity on the fatty acid composition between isolate BA17 and *B. licheniformis* strains (Song *et al*., 2000; Silva *et al*., 2006) indicates that isolate BA17 could be considered as *B. licheniformis* in terms of its cellular fatty acid composition.

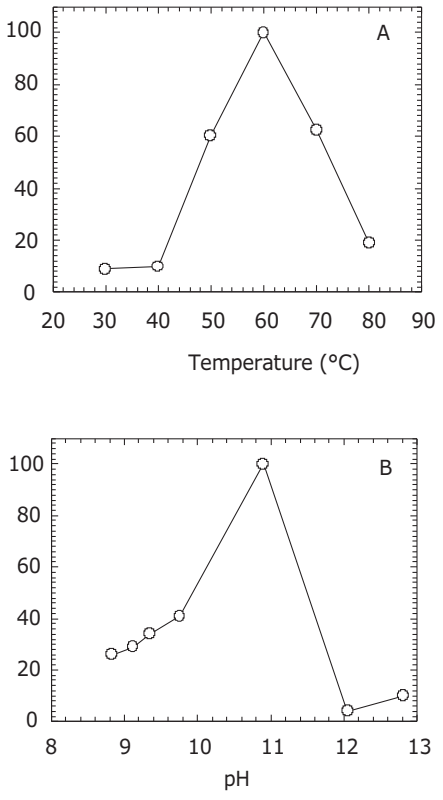


FIG. 2 - Temperature (A) and pH (B) profile of the alkaline protease of *Bacillus licheniformis* BA17.

According to carbon utilisation, there were some differences between isolate BA17 and 98 different *B. licheniformis* strains studied by Manachini *et al*. (1998). *Bacillus licheniformis* BA17 was able to utilise 25 carbohydrates out of 58 carbon sources examined. Manachini *et al*. (1998) explained the variation in the assimilation of carbon source with the difference in geographical areas in which isolations were carried out. Thus, the isolate BA17 from Van Lake differed in the assimilation of glycerol, L-xylose, L-arabinose, sorbose, galactose, rhamnose, inositol, α-methyl-D-gentibiose, D-turanose, melibiose, inulin, D-lyxose and D-fucose from those studied by Manachini *et al*. (1998).

According to 16S rDNA analyses of isolate BA17, the isolate BA17 showed 98.8% homology with *Bacillus licheniformis* ATCC 14580$^T$. Construction of phylogenetic tree placed the isolate BA17 in a phylogenetic position related to cluster of type species of *B. licheniformis* ATCC 14580$^T$ (Fig. 1). Considering all phenotypic, chemotaxonomic and molecular analysis results, isolate BA 17 was presumptively identified as *Bacillus licheniformis* BA 17.

Alkaline protease production depends greatly on the availability of both carbon and nitrogen sources in the medium (Moon and Parulekar, 1991). Both inorganic and organic nitrogen sources are used efficiently by *Bacillus* sp. for alkaline protease production (Mehrotra *et al.*, 1999). Although complex nitrogen sources are usually used for alkaline protease production, the requirement for nitrogen supplement show differences from organism to organism (Kumar and Takagi, 1999). In this work, inorganic ($NH_4NO_3$, $NaNO_3$ and $(NH_4)_2SO_4$) and organic nitrogen sources (yeast extract and peptone) were used for the production of alkaline protease. Enzyme production from *B. licheniformis* BA17 in the presence of yeast extract, peptone and peptone plus yeast extract was sufficiently low, although Prakasham *et al.* (2005) have found that yeast extract showed maximum influence in enhancement of enzyme production, followed by peptone. Hadj-Ali *et al*. (2007) obtained the similar results with Prakasham *et al*. (2005) that alkaline protease production from *B. licheniformis* NH1 was low in the absence of yeast extract. Patel *et al.* (2005) obtained maximum haloalkaliphilic *Bacillus* sp. protease activity (110 U/ml) with casamino acids followed by gelatin, peptone, peptone plus yeast extract and yeast extract. Chauhan and Gupta (2004) reported that complex organic nitrogen sources such as soybean meal, casamino acid and peptone were observed to induce high protease

production while simple inorganic sources supported poor alkaline protease production. However, we obtained the highest protease production when yeast extract and $NaNO_3$ were used together as organic and inorganic nitrogen sources, respectively. Stimulatory effect of $NaNO_3$ on alkaline protease production has also been reported by Kumar and Takagi (1999). In accordance with literature, in this work, mixture of inorganic and organic nitrogen sources were observed to induce high protease production.

*Bacillus licheniformis* BA17 exhibited higher productivity of alkaline protease in culture media containing maltose as a carbon source. Similar to our results, increased alkaline protease production were reported by several workers who used different sugars such as lactose and maltose (Kumar and Takagi, 1999). Mabrouk *et al.* (1999) studied the alkaline protease production by *Bacillus licheniformis* ATCC 21415 and obtained the maximum production of alkaline protease using a mixture of lactose (4%) and glucose (1.5%) as carbon source. On the other hand, Puri *et al.* (2002) reported that starch used as carbon source resulted in the highest enzyme activity produced by a *Bacillus* sp. Chauhan and Gupta (2004) studied the production of alkaline protease enzyme by *Bacillus* sp. RGR-14 which produced maximum alkaline protease in the presence of starch followed by mannitol, maltose and glycerol. Although Ferrero *et al*. (1996) and Mehrotra *et al*. (1999) reported better protease synthesis in the presence of glucose, the protease production from *B. licheniformis* BA17 in the presence of glucose was six times lower than that of maltose used as a carbon source. Similar to our results, repression effect of glucose on protease synthesis was reported by Hadj-Ali *et al.* (2007).

Divalent metal ions such as calcium, cobalt, copper, iron, magnesium are required in the fermentation medium for optimum production of alkaline protease (Kumar and Takagi, 1999). There are also studies reporting improved enzyme yields by the addition $Ca^{2+}$ ions to the growth medium (Mabrouk *et al*., 1999; Nascimento and Martins, 2004). Mabrouk *et al.* (1999) reported a 26.6% increase in enzyme activity by supplementing the medium with $CaCl_2$ which was attributed to the stabilizing effect of $Ca^{2+}$ ion on the alkaline protease enzyme. Nascimento and Martins (2004) also observed that protease activity was stimulated by $Ca^{2+}$. However, in this work, supplementing the medium with $CaCl_2$ did not result in any improvement of enzyme production.

Considerable amount of reports appeared in the literature about the alkaline proteases produced by *Bacillus* sp. The optimum temperature and pH of BA17 alkaline protease as 60 $^{o}$C and 11 were similar those reported by Joo *et al*. (2003) and Kazan *et al*. (2005). In addition, the pH and temperature profile of alkaline proteases studied by Gessesse (1997), Banerjee *et al*. (1999), Joo *et al*. (2002), Banik and Prakash (2004) and Al-Shehri and Mostafa (2004) were lower than our findings. Proteases can be classified according to their sensitivity to inhibitors (North, 1982). The inhibition of *B. licheniformis* BA17 protease(s) indicates that the preparation contains serine protease(s).

The above studies showed that *Bacillus licheniformis* BA17 is used for the production of alkaline protease which is of biological interest, particularly as detergent additive with its optimum temperature and pH values. The maximum protease production at lab-scale was obtainable using yeast extract and $NaNO_3$ as nitrogen source. In addition, the production of enzyme in the absence of $Ca^{2+}$ ions make the enzyme preparations more suitable for washing processes, since detergents used in domestic and industrial washing processes are alkaline and contain sequestering agents to remove $Ca^{2+}$ (which adversely affects the water hardness and foam characteristics).The scale up studies for enzyme production and genetic manipulation to improve enzyme production and properties are in progress.

**Acknowledgements**

We would like to acknowledge Prof. Fikrettin Sahin for his valuable help for FAME analyses. This work was supported financially by The Scientific and Technological Research Council of Turkey (TUBITAK) with the project number TBAG 2321-103T069.

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