

# Newly isolated *Bacillus clausii* GMBAE 42: an alkaline protease producer capable to grow under highly alkaline conditions

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2003/0710: received 15 August 2003, revised 7 October 2003 and accepted 11 October 2003

## ABSTRACT

A.A. DENIZCI, D. KAZAN, E.C.A. ABELN AND A. ERARSLAN. 2003.

**Aims:** The isolation and identification of new *Bacillus* sp. capable of growing under highly alkaline conditions as alkaline protease producers.

**Methods and Results:** A *Bacillus* strain capable of growing under highly alkaline conditions was isolated from compost. The strain is a Gram-positive, spore-forming, motile, aerobic, catalase- and oxidase-positive, alkaliphilic bacterium and designated as GMBAE 42. Good growth of the strain was observed at pH 10. The strain was identified as *Bacillus clausii* according to the physiological properties, cellular fatty acid composition, G + C content of genomic DNA and 16S rRNA gene sequence analyses. The result of 16S rRNA sequence analyses placed this bacterium in a cluster with *B. clausii*. The G + C content of the genomic DNA of the isolate GMBAE 42 was found to be 49 mol%. The crude extracellular alkaline protease produced by the isolate showed maximal activity at pH 11.0 and 60°C.

**Conclusions:** The results suggest that isolated strain GMBAE 42 is a new type of *B. clausii* capable of growing at pH 10.0 and produce extracellular alkaline protease very active at pH 11.0.

**Significance and Impact of the Study:** Isolated strain could be used in commercial alkaline protease production and its enzyme can be considered as a candidate as an additive for commercial detergents.

**Keywords:** alkaline protease, *Bacillus clausii*, G + C content, phenotypic characterization, phylogeny 16S rRNA.

## INTRODUCTION

Alkaliphilic micro-organisms are widely distributed in nature and can be found in almost all environments. Among them alkaliphilic *Bacillus* strains are of considerable importance in biotechnological applications (Fritze *et al.* 1990; Kumar and Takagi 1999). Nielsen *et al.* (1995) and Fritze *et al.* (1990) have made considerable revisions within the past two decades on the classification of alkaliphilic *Bacillus*

species according to phylogenetic and phenotypic characterizations, and they described nine new species with the following names: *B. clausii*, *B. gibsonii*, *B. horikoshii*, *B. pseudoalkaliphilus*, *B. pseudofirmus*, *B. agaradhaerens*, *B. clarkii*, *B. halmopalus* and *B. halodurans* in addition to previously described species *B. alcalophilus* and *B. cohnii*. Enzymes such as alkaline protease from alkaliphilic *Bacillus* strains have found great industrial application potential (Yumoto *et al.* 1998; Jasvir *et al.* 1999; Takami *et al.* 1999; Takami and Horikoshi 2000; Takami and Krulwich 2000; Gupta *et al.* 2002; Saeki *et al.* 2002).

Alkaline proteases are one of the most important groups of industrial enzymes widely used in detergent, food and

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leather tanning industries. Alkaline proteases can also be used on the hydrolysis of fibrous proteins such as horn, feather and hair for converting them to useful biomass. Other potential industrial applications of alkaline protease include the utilization in peptide synthesis, in the resolution of the racemic mixture of amino acids, in the hydrolysis of gelatine layers of X-ray films and the recovery of silver (Anvar and Saleemuddin 1998; Kumar and Takagi 1999). Among the alkaliphilic micro-organisms exhibiting protease activity the members of the genus *Bacillus* were found to be abundantly present (Kumar and Takagi 1999). Therefore, alkaliphilic *Bacillus* strains are quite important and interesting both for academic and industrial reasons. In this paper, we are reporting the identification of a newly isolated alkaline protease producer *B. clausii* strain designated as GMBAE 42 capable of growing under highly alkaline conditions.

## MATERIALS AND METHODS

### Chemicals

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

### Strain isolation

The samples collected from the composts were diluted in sterile 0.9% saline solution and then plated onto skim-milk agar plates including 0.1% glucose, 0.2% peptone, 0.5% yeast extract, 0.1%  $K_2HPO_4$ , 0.02%  $MgSO_4 \cdot 7H_2O$ , 0.5% skim-milk (skim-milk was sterilized separately). The pH of the medium was adjusted to 10.0 by addition of 10%  $Na_2CO_3$  solution after sterilization. Inoculated plates were incubated for 72 h at 30°C in order to obtain colonial growth. The colonies with clear zones formed by the hydrolysis of skim milk were evaluated as alkaline protease producers. Depending on the zone diameter and clearance isolate GMBAE 42 was selected as good alkaline protease producer and used in all further investigations. The strain was submitted to the Netherland Culture Collection of Bacteria under the number NCCB 100039.

### Culture conditions for alkaline protease production

The medium containing 1.0% starch, 0.5% yeast extract, 0.1%  $K_2HPO_4$  and 0.02%  $MgSO_4 \cdot 7H_2O$  was used for the alkaline protease production of the isolate GMBAE 42. The medium pH was adjusted to 10.0 by aseptic addition of 10%  $Na_2CO_3$  solution after sterilization. Medium (100 ml)

containing 500 ml Erlenmeyer flasks were inoculated by 18-h-old inoculum culture at 1% v/v ratio and cultivated at 37°C by shaking at 150 rev min<sup>-1</sup> for 3 days.

### Determination of alkaline protease activity

The same procedures and substrate (0.6% Hammersten casein) described by Takami *et al.* (1989) were used for the determination of alkaline protease activity. One unit of alkaline protease activity was defined as the amount of the enzyme able to produce 1 µg tyrosine in 1 min under assay conditions. All the enzyme assays were performed independently by repeating five times and the results were calculated as mean values. The standard errors of mean values were lower than 3%.

### Phenotypic characterization

Eighty-seven of the 88 different tests shown in Tables 1 and 2 for estimation of the morphological, cultural, physiological and biochemical properties of the strain GMBAE 42 were carried out according to methods described by Sneath *et al.* (1986) and Nielsen *et al.* (1995). The phenylalanine test was carried out with the method described by Fritze *et al.* (1990). API 50 CH gallery (Biomérieux, Marcy L'Etoile, France) was used for carbohydrate utilization tests. Whole of the phenotypic assays for strain GMBAE 42 and *B. clausii* DSM 8716<sup>T</sup> were performed as triplicate.

### Analysis of cellular fatty acids

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

### DNA base composition

DNA was isolated using a genomic DNA isolation column (Qiagen, Westburg, Leusden, The Netherlands) following the prescriptions. The DNA was melted in 0.1x saline citrate buffer pH 7.0 (SSC) and thermal melting point ( $T_m$ ) of DNA was determined by melting curve analysis obtained by spectrophotometer at 260 nm according to method of Marmur and Doty (1962). The G + C content was calculated from the following equation given by Owen *et al.* (1969):  $(GC \text{ mol}\%) = 2.08T_m - 106.4$ .

### Sequence determination of 16S rRNA gene

Sequence analysis was performed on an 1800-bp PCR product using eight different primers (16S500R, BSF8/20,

**Table 1** Phenotypic properties of the isolate GMBAE 42 and reference strain *Bacillus clausii* DSM 8716<sup>T</sup>

Property	GMBAE 42	* <i>Bacillus clausii</i> DSM 8716 <sup>T</sup>
Form	Rod	Rod
Mobility	+	+
Spore	+	+
	(subterminal)	(subterminal to paracentral)
Gram stain	+	+
Catalase	+	+
Oxidase	+	+
NO <sub>3</sub> to NO <sub>2</sub>	+	+
Growth at		
5°C	-	-
30°C	+	+
40°C	+	+
50°C	w	+
55°C	-	-
pH 6.8	-	-
pH 7	+	+
pH 8	+	+
pH 9	+	+
pH 10	+	+
pH 11	+	+
pH 12	+	-
pH 12.5	-	-
Growth in		
% 2 NaCl	+	+
% 5 NaCl	+	+
% 7 NaCl	+	+
% 10 NaCl	+	+
Hydrolysis of		
Casein	+	+
Gelatine	+	+
Starch	+	+
Tween 20, 40, 60 and 80	-	-
Arginine hydrolase	-	-
Lysine hydrolase	-	-
Urease	-	-
Deamination of phenylalanin	-	-
Utilization of citrate	+	-
Indol production	-	-
Degradation of tyrosine	-	-
Growth with lysozyme	-	-

w, weakly.

\*Results of Nielsen *et al.* (1995).

(†)Results obtained in RIGEB Laboratory.

16S500F, BSR798/15, BSF1099/16, BSR1114/16, 16S1500R and 16S1500F) for sequencing reactions. The sequence analyses were performed in NCCB by using ABI 3700 96 capillaries sequencer. Contig building was carried out by using SeqMan from the DNA Star package (DNASTAR, Madison, WI, USA). Homology search (GenBank/EMBL/DDBJ) was carried out by using the Basic Local Alignment

**Table 2** Carbohydrate utilization patterns of the isolate GMBAE 42 and reference strain *Bacillus clausii* DSM 8716<sup>T</sup>

Property	GMBAE 42	<i>Bacillus clausii</i> DSM 8716 <sup>T</sup>
Glycerol	+	+
Erythritol	-	-
D-arabinose	-	-
L-arabinose	-	+
Ribose	+	+
D-xylose	+	+
L-xylose	-	-
Adonitol	-	-
β-Methyl-D-xyloside	-	-
Galactose	+	+
Glucose	+	+
Fructose	+	+
Mannose	+	+
Sorbose	+	-
Rhamnose	+	+
Dulcitol	+	+
Inositol	+	+
Mannitol	+	+
Sorbitol	+	+
α-Methyl-D-mannoside	-	+
α-Methyl-D-glucoside	+	+
N-acetyl glucosamine	+	+
Amygdalin	+	+
Arbutin	+	+
Esculin	+	+
Salicin	+	+
Cellobiose	+	+
Maltose	-	+
Lactose	-	-
Melibiose	-	+
Saccharose	+	+
Trehalose	+	+
Inulin	-	-
Melezitose	-	+
Raffinose	-	+
Starch	+	+
Glycogen	-	+
Xylitol	+	+
Gentiobiose	+	+
D-turanose	+	+
D-lyxose	-	+
D-tagatose	+	+
D-fucose	-	-
L-fucose	-	-
D-arabitol	+	-
L-arabitol	-	-
Gluconate	-	-
2-Keto-gluconate	-	-
5-Keto-gluconate	-	-

(\*)Results of Nielsen *et al.* (1995).

(†)Results obtained in RIGEB Laboratory.

Search Tools (BLAST) program (Altschul *et al.* 1990) in the reference database. 16S rDNA sequences were aligned by using the Clustal multiple alignment program (Clustal W 1.8) (Thompson *et al.* 1994), and nucleotide substitution rates ( $K_{nuc}$ ) were calculated. Sites involving gaps were excluded from all analyses. A phylogenetic tree was inferred by the neighbour-joining method (Saitou and Nei 1987).

### Nucleotide accession number

16S rRNA sequence data of the isolate GMBAE 42 reported in this article have been submitted to NCBI, NLM, NIH and GenBank nucleotide sequence databases under the accession number AY152839.

### Temperature and pH profiling

Temperature and pH profiles of the alkaline protease of *B. clausii* GMBAE 45 were estimated at 30–65°C temperature and 8–13 pH ranges, respectively. Every reaction mixture contains the same amount of enzyme (923 U mg<sup>-1</sup> specific activity and 0.207 mg ml<sup>-1</sup> protein) and reacts under the same conditions except for the pH values and temperatures. In the case of temperature profile estimation; 0.5 ml enzyme solution was mixed with 2.5 ml 0.6% Hammersten casein solution prepared at 50 mM pH 10.5 glycine–NaOH buffer and incubated for 20 min at different temperatures. Buffer pH was stable at the temperatures studied. In the case of pH profile estimation; 0.5 ml enzyme solution in 50 mM pH 10.5 glycine–NaOH buffer was mixed with 2.5 ml 0.6% Hammersten casein solutions prepared in 50 mM glycine–NaOH buffer at pH values between 8.0 and 12.0. Substrate was dissolved in 200 mM glycine–NaOH buffer for the measurements at 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.

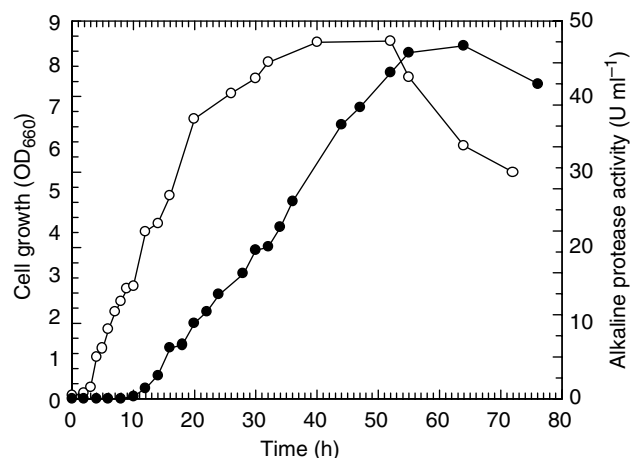
## RESULTS

### Alkaline protease production

The enzyme production started after a 10-h lag phase, then exponentially increased equal to the increase on cellular growth and reached the maximum after depletion of starch in culture medium within 60–70 h of cultivation. Time course of the growth and alkaline protease production of *B. clausii* GMBAE 42 is shown in Fig. 1.

### Morphological and physiological characteristics

Morphological and physiological characteristics of bacterial isolate GMBAE 42 were investigated according to the



**Fig. 1** Time course of the growth and alkaline protease production of *Bacillus clausii* GMBAE 42. (○) Cell growth, (●) enzyme activity

methods described in *Bergey's Manual of Determinative Bacteriology* (Sneath *et al.* 1986). It was identified as the member of *Bacillus* species. The colonial appearance of the strain GMBAE 42 on agar plates was creamy yellow-orange colour with of 1–4 mm diameters. The phenotypic characteristics and carbohydrate utilization pattern of the isolate GMBAE 42 are summarized in Tables 1 and 2, respectively, in comparison with the reference strain *B. clausii* DSM 8716<sup>T</sup> (Nielsen *et al.* 1995). Isolate GMBAE 42 is Gram-positive, aerobic, catalase- and oxidase-positive, motile, spore-forming (ellipsoidal spores located subterminally), and rod-shaped bacterium. The cells were able to grow at pH values between 7 and 12, and in the presence of 2–10% of NaCl at pH 7. The cells were able to grow at 20–50°C temperature interval and optimal growth temperature was 37°C. No growth was observed at 55°C. Isolate GMBAE 42 and reference strain *B. clausii* DSM 8716<sup>T</sup> showed nearly identical phenotypes according to the 39 tested characteristics except the growth at pH 12 and citrate utilization (Table 1). The carbohydrate utilization tests showed similar results in 39 of the 49 tests. (Table 2). Differences were observed on L-arabinose, sorbose, maltose, melibiose, melezitose, raffinose, glycogen, D-lyxose, D-arabitol and  $\alpha$ -methyl-D-mannoside utilizations.

### Cellular fatty acids

The taxonomic similarity between isolate GMBAE 42 and reference strain *B. clausii* DSM 8716<sup>T</sup> was estimated by the analysis of their cellular fatty acid compositions. The whole-cell fatty acid composition of both strains is shown in Table 3. The fatty acid composition of the two strains was found to be highly similar except for two saturated fatty acids C<sub>10:0</sub> and C<sub>11:0</sub> (iso 3OH). Isolate GMBAE 42 and

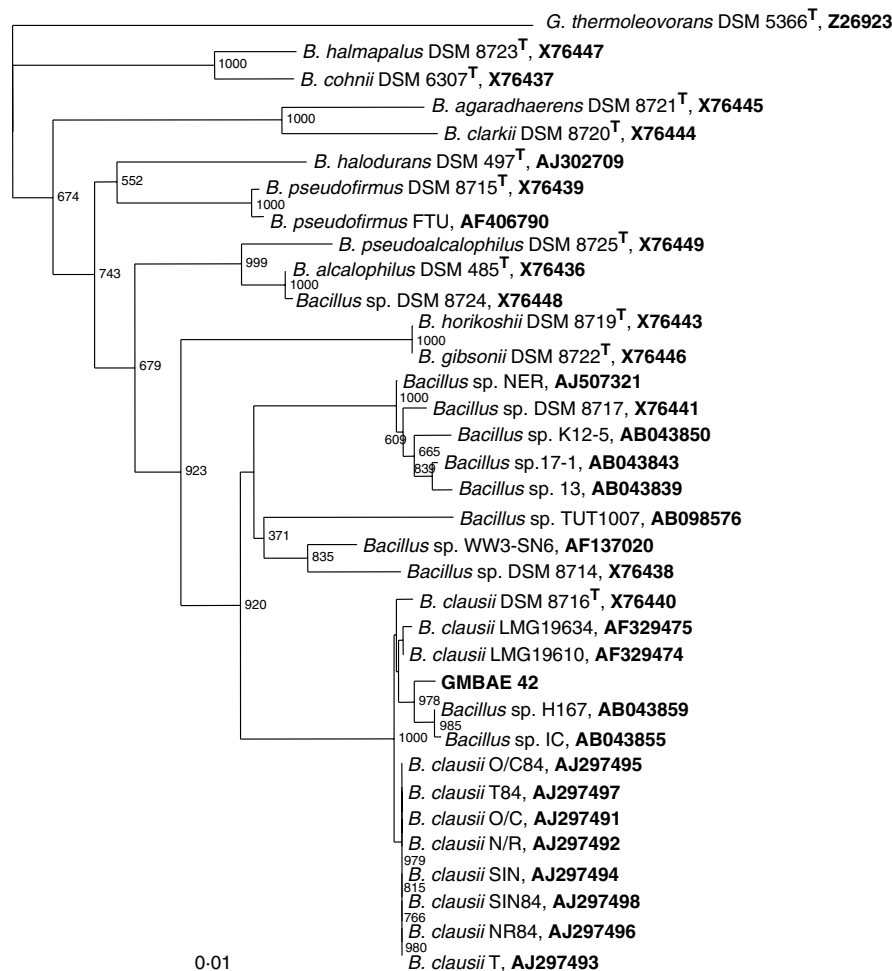
**Table 3** Cellular fatty acid compositions of the strain GMBAE 42 and *Bacillus clausii* DSM 8716<sup>T</sup>

Fatty acid	% of Total fatty acids	
	Isolate GMBAE 42	<i>Bacillus clausii</i> DSM 8716 <sup>T</sup>
10 : 0	–	0.29
11 : 0 iso 3OH	1.55	–
iso-C 14 : 0	1.86	1.70
14 : 0	0.55	0.60
iso-C 15 : 0	36.53	38.81
anteiso-C 15 : 0	14.90	17.76
16 : 1 ω7c alkol	3.59	3.32
iso-C 16 : 0	2.52	2.24
16 : 1 ω1 Ic	2.31	2.72
C 16 : 0	2.10	2.06
iso 17 : 1 ω10c	1.94	1.94
iso-C 17 : 0	15.47	16.35
anteiso-C 17 : 0	11.68	9.95

*B. clausii* DSM 8716<sup>T</sup> does not contain C<sub>10:0</sub> and C<sub>11:0</sub> saturated fatty acids, respectively.

### 16S rDNA (rRNA) sequences analysis and DNA base composition

A number of new taxa have been proposed for alkaliphilic *Bacillus* strains (Nielsen *et al.* 1995; Fritze 1996; Yumoto *et al.* 1998; Saeki *et al.* 2002). We constructed a phylogenetic tree based on comparison of 16S rDNA sequences of reference alkaliphilic *Bacillus* strains (Fig. 2) in order to understand the phylogenetic position of our strain. The sequence analysis for the 1666 bases of 16S rDNA gene of the isolate GMBAE 42 was determined and compared with those of reference *Bacillus* spp. The sequence of isolate GMBAE 42 showed a 99% homology with the alkaliphilic *B. clausii* strains. However, the G + C content of the genomic DNA of isolate GMBAE 42 was found to be 49 mol%.



**Fig. 2** Phylogenetic tree of *Bacillus clausii* GMBAE 42 associated with the other members of the *Bacillus* genus. Distance matrix was calculated on the basis of Jukes and Cantor (1969), and topology was inferred using the 'neighbour-joining' method (Saitou and Nei 1987) based on bootstrap analyses of 1000 trees. The 16S rRNA sequence of *Geobacillus thermoleovorans* was chosen arbitrarily as the outgroup sequence

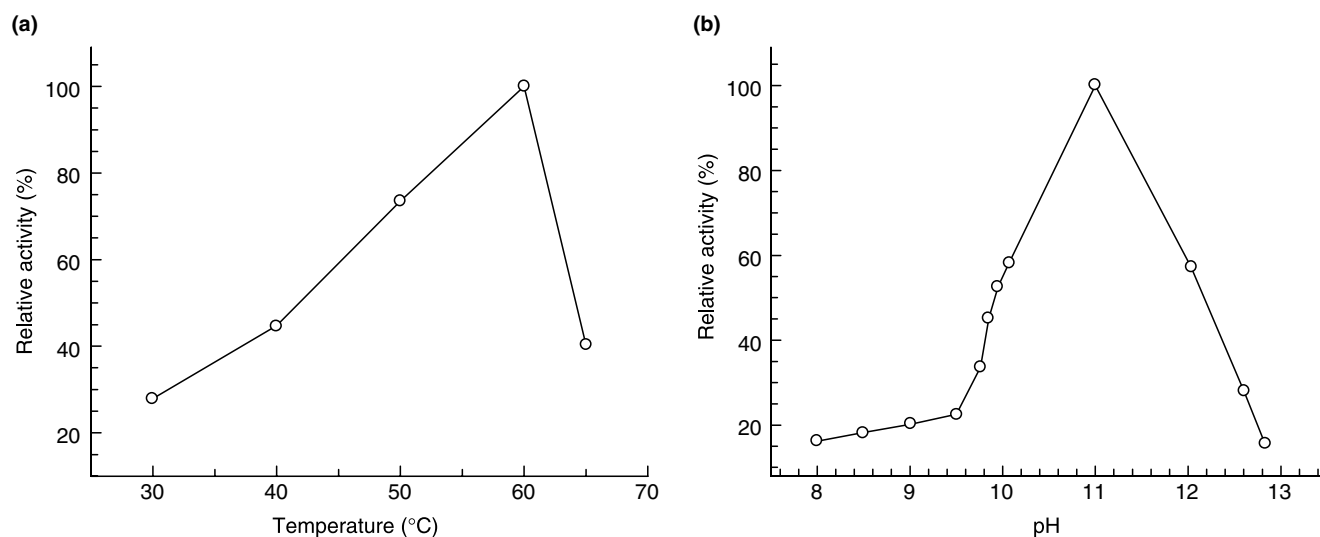


Fig. 3 (a) Temperature and (b) pH activity profiles of the alkaline protease of *Bacillus clausii* GMBAE 42

### Temperature and pH activity profiles of the alkaline protease of strain GMBAE 42

The temperature and pH activity profiles of the alkaline protease of strain GMBAE 42 were determined to be within 30–65°C temperature and 8–13 pH ranges, respectively (Fig. 3). The optimal temperature and pH values of enzyme were found to be 60°C and pH 11.0, respectively.

### DISCUSSION

The few extensive studies undertaken to classify the truly alkaliphilic *Bacillus* strains (strains that grow at or above pH 9) and alkali tolerant *Bacillus* strains (strains that also grow at pH 7) have led to the conclusion that these bacteria may be clustered in 11 different phenospecies and 13 different genospecies which are, with the exception of *B. alcalophilus* and *B. cohnii*, phylogenetically distinct from all validly described *Bacillus* species (Fritze *et al.* 1990; Nielsen *et al.* 1994; Nielsen *et al.* 1995). However, some heterogeneity in phenotypic characteristic has been found in strains putatively aligned with given phenon, particularly strains assigned to the phenon encompassing the species *B. clausii* (Nielsen *et al.* 1995), which indicates that there are intrinsic difficulties in identifying the alkaliphilic strains at the species level (Senesi *et al.* 2001). The differences on some phenotypic properties between *B. clausii* DSM8716<sup>T</sup> and isolate GMBAE 42 should be considered on these aspects.

The major fatty acid component of GMBAE 42 and *B. clausii* DSM 8716<sup>T</sup> was found to be iso-C<sub>15:0</sub>. Iso-C<sub>15:0</sub> and anteiso-C<sub>15:0</sub> are predominant fatty acids in the alkaliphilic micro-organisms examined (Yumoto *et al.* 1998). The high similarity on the fatty acid composition

between isolate GMBAE 42 and *B. clausii* DSM 8716<sup>T</sup> indicates that isolate GMBAE 42 can be considered as a *B. clausii* in terms of cellular fatty acid composition. *Bacillus clausii* LMG 19610, *B. clausii* LMG 19634, Enterogermina strains studied by Senesi *et al.* (2001) and *B. clausii* DSM 8716<sup>T</sup> studied by Nielsen *et al.* (1995) showed 99% homology with the strain GMBAE 42. Construction of phylogenetic tree placed the isolate GMBAE 42 in a phylogenetic position related to cluster of type species *B. clausii* DSM 8716<sup>T</sup> and other *B. clausii* strains (Fig. 2). The chromosomal DNA composition of *B. clausii* cluster 6 given by Nielsen *et al.* (1995) are between 42.8 and 45.5% mol G + C. The G + C content of genomic DNA of isolate GMBAE 42 was 49 mol%. This alkaliphilic strain can be considered in the group of low G + C containing bacteria. Although there are a few differences between strain GMBAE 42 and *B. clausii* DSM 8716<sup>T</sup>, according to the results of 16S rRNA sequence and FAME analyses the strain GMBAE 42 was identified as *B. clausii*.

Considerable amount of reports appeared in the literature about the alkaline proteases produced by alkaliphilic bacilli. Jasvir *et al.* (1999) obtained similar results for the optimal temperature and pH values of the extracellular alkaline protease of *Bacillus* sp. NG312. Oberoi *et al.* (2001) reported sodium dodecyl sulfate (SDS)-stable alkaline protease from *Bacillus* sp. RGR14, optimally active at pH 11.0 and 60°C. Beg and Gupta (2003) reported the temperature and pH optimum of the commercial alkaline protease obtained from *B. mojavensis* as 60°C and 10.5, respectively. Singh *et al.* (2001) reported a serine alkaline protease produced by a newly isolated *Bacillus* sp. SSR1 having optimal activity at pH 10.0 and 40°C. Huang *et al.* (2003) reported the extracellular alkaline serine protease of *B. pumilus* showing

maximal activity at pH 10.0 and 55°C. The thermostable alkaline proteases from various alkaliphilic and thermophilic *Bacillus* sp. having maximum activity at pH 11.0 and 70°C were reported by Johnvesly and Naik (2001) and Takami *et al.* (1989). Kaur *et al.* (2001) reported a highly thermo stable alkaline protease of the obligate alkaliphilic *Bacillus* sp. P-2 maximally active at pH 9.6 and 90°C. The optimal pH and temperature values of alkaline protease purified from *B. licheniformis* MIR29 were found to be 12.0 and 60°C by Ferrero *et al.* (1996). The protease of *B. horicoshii* exhibited the highest activity at 45°C and pH 9.0 (Joo *et al.* 2001). The thermostable alkaline protease of the facultatively thermophilic and alkaliphilic *B. brevis* strain were found to be most active at pH 10.5 and 37°C (Banerjee *et al.* 1999). However, little or no work relating to the alkaline protease of *B. clausii* has appeared in the literature (Christiansen and Nielsen 2002a,b). According to a report of Beg and Gupta (2003) the temperature and pH optimum of the commercial alkaline proteases used in detergents stay within 15–65°C and 7–12 pH intervals. Consequently, alkaline protease of the strain GMBAE 42 can be considered as a suitable candidate as an additive for commercial detergents.

## ACKNOWLEDGEMENTS

We would like to kindly acknowledge Assoc. Prof. Dr Fikrettin Şahin for his valuable assistance for FAME analyses and Nesrin Karahan, Ing. M. F. Figge, Ing. G. Poot and A. Lugtenberg for their valuable technical assistance. This research was supported by The Scientific and Technical Research Council of Turkey (TUBITAK) by the project number TBAG 2129–102T003.

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