www.biodicon.com Biological Diversity and Conservation

ISSN 1308-8084 Online; ISSN 1308-5301 Print 9/2 (2016) 169-179

Research article/Araştırma makalesi

Isolation and identification of a novel thermo-alkalophilic *Anoxybacillus* sp. strain KB4 from Kuşburnu hot spring in Turkey

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Abstract

Thermophiles are able to grow at temperatures up to 110°C and at different pH (thermo-acidophiles and thermo-alkalophiles) and they are an interesting source of biotechnological applications. The aim of this study was to isolate thermophilic bacterium from Kuşburnu hot water spring in the town of Diyadin in Turkey. The thermophilic strain KB4 was identified by morphological, physiological, biochemical tests and 16S rRNA gene sequencing**.** Strain KB4 was found to be an aerobic, Gram-positive, rod shaped, motile and yellow-pigmented. Optimal growth was obtained at pH 9.0–10.0, at 55-60 ºC, and at 3% (w/v) NaCl. Potantial of different carbon and nitrogen sources utilization were examined. The strain KB4 was able to utilize some carbon and nitrogen sources, including glucose, galactose, sucrose, maltose, arabinose, xylose, yeast extract, peptone, tryptone and casamino acids. Phylogenetic analysis based on 16S rRNA gene sequences showed that isolate KB4 was most closely related to *Anoxybacillus pushchinoensis* K1 (T) with a 98.78% sequence similarity. In addition, this study will be a guiding study in determining the diversity of thermophilic bacteria from geothermal waters of Turkey.

Key words: *Anoxybacillus*, isolation, identification, thermophilic, 16S rRNA gene

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Kuşburnu sıcak su kaynağından yeni termo-alkalofilik *Anoxybacillus* sp. KB4 şusunun izolasyonu ve tanımlanması

Özet

Termofiller 110 ºC'ye kadar sıcaklıklarda ve farklı pH'larda (termo-asidofil ve termo-alkalofil) büyüyebilme yeteneğine sahiptirler ve biyoteknolojik uygulamaların ilgi çeken bir kaynağıdırlar. Bu çalışmanın amacı, Türkiye'deki Diyadin ilçesi Kuşburnu sıcak su kaynağından termofilik bakteri izole etmektir. KB4 suşu, morfolojik, fizyolojik, biyokimyasal testler ve 16S rRNA gen sekanslamayla tanımlanmıştır. KB4 suşu, aerobik, Gram-pozitif, çubuk şeklinde, hareketli ve sarı pigmentli olarak bulunmuştur. Optimum üreme pH 9.0-10.0'da, 55-60 ºC'de ve %3 (w/v) NaCl konsantrasyonunda elde edilmiştir. Farklı karbon ve azot kaynaklarının kullanımı denendi. KB4 suşu, glukoz, galaktoz, sükroz, maltoz, arabinoz, ksiloz, yeast ekstrakt, pepton, tripton ve casamino asit gibi bazı karbon ve azot kaynaklarını kullanabilmektedir. 16S rRNA gen sekansına bağlı filogenetik analizler KB4 suşunun %98.78 sekans benzerliği ile *Anoxybacillus pushchinoensis* K1(T)'e oldukça yakın olduğu göstermektedir. Ek olarak, bu çalışma Türkiyede'ki jeothermal sulardan termofilik bakterilerin çeşitliliğini belirlemede rehber olacaktır.

Anahtar kelimeler: *Anoxybacillus*, izolasyon, tanımlama, termofilik, 16S rRNA geni

1. Introduction

Extreme environments, which are extreme conditions of heat or cold, pH, salinity, pressure, and radiation, are inhabited by diverse populations of microorganisms (Rothschild and Mancinelli, 2001). Extremophilic microorganisms include members of all three domains of life, the *Archaea*, *Bacteria* and *Eukarya*. It is possible to isolate thermophilic microorganisms from environments with interval heat, such as soil and compost. Geothermal areas are favourable

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habitats for a diversity of thermophile. Among the geothermally heated habitats are the alkaline, mainly carbonate-containing hot springs around a neutral pH, and acidic areas including some mud-holes (Canganella and Wiegel, 2014). Turkey is rich in geothermal sources and there are 140 geothermal sources (Inan et al., 2011). Thermophilic microorganisms have gained more importance in biotechnology, primarily in industry (enzyme production, secondary metabolites, bioremediation, etc.). In addition, they have been used for production of renewable energy as well as for removing heavy metals from waste. Recently, with the latest developments in biotechnology and molecular biology, many studies aiming at the discovery of new thermophilic bacteria have been conducted.

Termophilic bacteria of the species *Anoxybacillus* was first described in 2000 by Pikuta et al. To date, there are 26 species and 3 subspecies of *Anoxybacillus* that have been recognized. These are as follows: *Anoxybacillus pushchinensis* DSM 12423^T and *Anoxybacillus flavithermus* DSM 2641 (Pikuta et al., 2000) *Anoxybacillus flavithermus* subsp. *flavithermus* (Dai et al., 2011; Pikuta et al., 2000), *Anoxybacillus gonensis* NCIMB 13933^T (Belduz et al., 2003), *Anoxybacillus ayderensis* NCIMB 13972^T and *Anoxybacillus kestanbolensis* NCIMB 13071^T (Dulger et al., 2004), *Anoxybacillus contaminans* DSM 15866^T (De Clerck et al., 2004), *Anoxybacillus voinovskiensis* NCIMB 13956^T (Yumoto et al., 2004) *Anoxybacillus amylolyticus* DSM 15939^T (Poli et al., 2006), *Anoxybacillus kamchatkensis* DSM 14988 (Kevbrin et al., 2006), *Anoxybacillus tunisiense* (Sayeh et al., 2007), *Anoxybacillus kamchatkensis* subsp. *asacchardens* subsp. nov. (Gül-Güven et al., 2008), *Anoxybacillus bogrovensis* DSM 17956^T (Atanassova et al., 2008) , *Anoxybacillus rupiensis* DSM 17127 (Derekova et al., 2008) , *Anoxybacillus kualawohkensis* (Bradley, 2009), *Anoxybacillus eryuanensis* E-112^T (Zhang et al., 2011), *Anoxybacillus flavithermus* subsp. *yunnanensis* (Dai et al., 2011), *Anoxybacillus mongoliensis* DSM 19169 (Namsaraev et al., 2011), *Anoxybacillus salavatliensis* DSM 22626^T (Cihan et al., 2011), *Anoxybacillus tengchongensis* T-11^T (Zhang et al., 2011), *Anoxybacillus thermarum* DSM 17141 (Poli et al., 2011), *Anoxybacillus tepidamans* DSM 16325^T and *Anoxybacillus caldiproteolyticus* DSM 15730^T (Coorevits et al., 2012), *Anoxybacillus beppuensis* TSSC-1 (Kikani and Sing, 2012) *Anoxybacillus vitaminiphilus* 3nP4^T (Zhang et al., 2013), *Anoxybacillus kaynarcensis* DSM 217065 (Inan et al., 2013), *Anoxybacillus suryakundensis* DSM 27374^T (Deep et al., 2013) *Anoxybacillus calidus* DSM 25520^T (Cihan et al., 2014) and *Anoxybacillus geothermalis* (Filippidou et al., 2015).

This is the first study regarding the identification of thermophile *Anoxybacillus* isolated from Kuşburnu hot spring, in the town of Diyadin in Turkey. Bacterial identification are based on 16S rRNA gene which is evolutionary unchanged region throughout prokaryotic organisms (Meintanis et al., 2008; Patel, 2001; Woese et al., 1990). Therefore, we carried out physio-biochemical analysis and 16S rRNA gene sequencing for identification of thermophilic *Anoxybacillus.* In addition, this study attaches great importance as Kuşburnu hot water was a source not studied before with regard to bacteria isolation; this will thus be a guiding study in determining the diversity of bacteria of thermophilic characteristics from geothermal waters of Turkey.

2. Materials and methods

Chemicals

All chemicals were purchased from Sigma (Sigma–Aldrich, St Louis, USA) and were of the highest quality available unless otherwise stated. All chemicals used were of analytical grade.

Location and sampling

The water samples were obtained from Kuşburnu hot spring (39° 26' 46.86" N, 43° 41' 11.2626" E), Ağrı, North-Eastern Turkey. The temperature and pH of hot spring water were 70 °C and 7.5, respectively. The water samples were serially diluted with 1% sterilized water and were streaked and inoculated onto Nutrient Broth (NB) agar plates. Before the inoculation, all of the samples were treated with heat at 80 °C for 10 min. Heat-resistant spores were able to survive, while non-spore-forming bacteria died from the heat. After heat treated, all plates were incubated at 55-60 °C for 2 days. After incubation, different colonies developed in the media were selected and purified by subculturing. Subsequently, single colonies grown on the agar medium were taken out and inoculated in a new medium, and this step was repeated at least three times for purification.

Morphological, physiological and biochemical characterizations of isolates: Selected colonies were phenotypically characterized on the basis of shape, size, colour, surface, aspect, elevation, cell wall, light and consistency, etc. The cell morphology and motility were studied by the light microscopy of native preparations.

Gram staining was carried out according to Dussault (1955) under light microscopy. The formation of spores was tested by microscopic observations with malachite green staining. The temperature and pH range for growth were determined by incubating the isolates in NB liquid medium at different temperature (20 to 80 °C) and different pH (4.0-11.0). The effect of NaCl on the bacterial growth was examined in NB medium containing 0.5 to 5% (w/v) NaCl.

Different biochemical properties of the selected isolates such as catalase, oxidase, indole, citritase and urease activity, Methyl red test, Voges–Proskauer test, motility were examined according to Bergey et al. (1989) and Claus and Berkeley (1986). Utilization of different carbon sources (glucose, galactose, lactose, fructose, maltose, arabinose), and gelatine, starch, caseine and lipid were tested according to Dulger et al. (2004). The utilization of organic compounds such as sole carbon and nitrogen sources was tested in basal medium (5 ml) supplemented with 0.5% (w/v) concentrations of the compounds and incubated at 55 ºC. The antibiotic sensitivity of the isolated bacteria was

determined using different standard antibiotic discs such as, Ampicillin (AMP), Amoxycilin Clawlanic Acide (AMC), Bacitracin, Chloramphenicol (CAP), Gentamicin (GM), Imipenem (IPM), Netilmicin (NET), Nystatin, Ofloxacin (OFX) and Rifampicin ®.

DNA isolation: Genomic DNA was isolated using the phenol – chloroform method according to Marmur (1961).

Amplification of 16S rRNA gene: 16S rDNA sequence was amplified from isolated genomic DNA with the upstream primer: 5'-ATTCTAGAGTTTGATCATGGCTTCA-3' and the downstream primer: 5'-ATGGTACCGTGTGACGGGCGGTGTTGTA-3'. Amplification of the 16S rDNA gene, and purification of the PCR product were carried out as described previously (Reiney et al., 1994).

Bioinformatics analysis and phylogenetic tree construction: Purified PCR products were sequenced and electrophoresed by GATC Biotech (gatc.biotech.com). Obtained sequences were compared using a BLAST search tool (http://www.ncbi.nlm.nih.gov/blast/) database on NCBI [National Centre of Biotechnology (http://www.ncbi.nlm.nih.gov)]. The 16S rRNA sequence of isolated strain was aligned with reference sequences showing sequence homology from the NCBI database using the multiple sequence alignment programme. The isolate was identified using the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012) on the basis of 16S rRNA sequence data. The results were then presented as a phylogenetic tree. Phylogenetic tree was constructed by the neighbour-joining method. The stability of the tree obtained from the above cluster analyses was assessed by using BOOTSTRAP programme in sets of 1,000 resampling (Felsenstein, 1993).

3. Results

In our country, which is quite rich in geothermal sources, there are 140 geothermal sites that are officially recorded (Ercan Akkaya and Kıvanç, 2008). In spite of having lots of thermal sources, there are few studies which are related to the microbial characteristics and biotechnologically important thermophilic microorganism profiles of these sources. There have been 26 identified species of *Anoxybacillus* since the recognition of *Anoxybacillus* by Pikuta et al. since 2000. Most of species of this genus have been isolated from hot springs. *Geobacillus, Anoxybacillus* and *Bacillus* spp. were isolated from hot springs in Pamukçu, Ilıca, Akdağ, Sorgun by Adıgüzel et al. (2009); *Anoxybacillus* in Aydın, Denizli, İzmir, Nevşehir by Cihan (2013) and in Balçova by Yavuz et al. (2004) in Turkey. Genc et al. (2015) and Baltacı et al. (2016) isolated thermophilic bacteria from Diyadin hot water springs and consequently found out that they were close to *A.gonensis* ve *A. kaynarcensis.* Researchers involoved in identification of microorganisms obtained in thermal sources in Turkey have contributed to literature by getting 6 new species and 1 new sub-species recognized.These are *Anoxybacillus ayderensis* NCIMB 13972T (Dulger et al., 2004), *Anoxybacillus kestanbolensis* NCIMB 13971T (Dulger et al., 2004), *Anoxybacillus calidus* DSM 25520T (Cihan et al., 2014), *Anoxybacillus gonensis* NCIMB 13933T (Belduz et al., 2003), *Anoxybacillus kaynarcensis* DSM 217065 (Inan et al., 2013), *Anoxybacillus salavatliensis* 22626T (Cihan et al., 2011) and *Anoxybacillus kamchatkensis* subsp. *asacchardens* subsp. nov. (Gül-Güven et al., 2008). In this study, the most dominant isolate was isolated and characterized from Kuşburnu hot spring. The obtained isolate was given KB4 code and stored at -80 ºC in cryotubes containing glycerol in the stocks of the Molecular Biology Research Laboratory of Faculty of Sciences at Dicle University. Selected strain, KB4, was identified by morphological, physiological, biochemical and 16S rRNA gene sequencing. Colony morphology of this strain was small, circular, smooth, matt and yellow pigmented. The cells of the KB4 appeared to be rod shaped, Gram-positive, motile and to possess sub-terminal endospores (Figure 1).

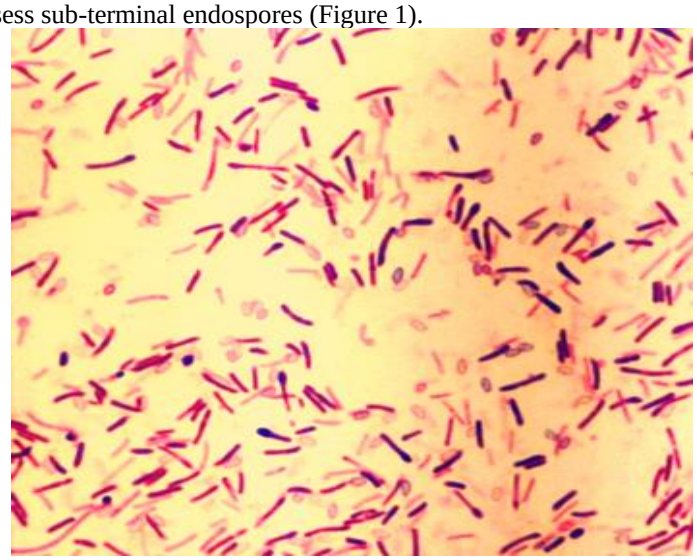


Figure 1. Cells and vegatative sporulation of strain KB4 grown at 55 ºC on nutrient broth

In recent studies, the genus *Anoxybacillus* have represented neutrophilic, obligately thermophilic, motile, endospore-forming and aerobic (Coorevits et al., 2012; Derekova et al., 2008; Dulger, 2004; Inan et al., 2013; Pikuta et al., 2000; Poli et al., 2011) or facultatively anaerobic (Atanassova et al., 2008; Cihan et al., 2011; Cihan et al., 2014; Kevbrin et al., 2006; Namsaraev et al., 2011; Poli et al., 2006; Zhang et al., 2011) bacteria.

Table 1 summarizes data of the species of *Anoxybacillus* genus, which are most similar in their physiological and biochemical characteristic to strain KB4, despite some remarkable differences existing between them.

The pH growth range of the strain we obtained was from 6.0 to 11.0 with an optimum at pH 9.0-10.0 (Figure 2). Many members of *Anoxybacillus* genus are alkalophilic such as *A. puschionensis* (Pikuta et al. (2003), *A. ayderensis* and *A. kestanbolensis* (Dulger et al., 2004), *A. calidus* (Cihan et al., 2014), *A. gonensis* (Beldüz et al., 2003), *A. salavatliensis* (Cihan et al., 2011) and *Anoxybacillus tengchongensis* (Zhang et al., 2011), but not all of them obligate alkalophiles.

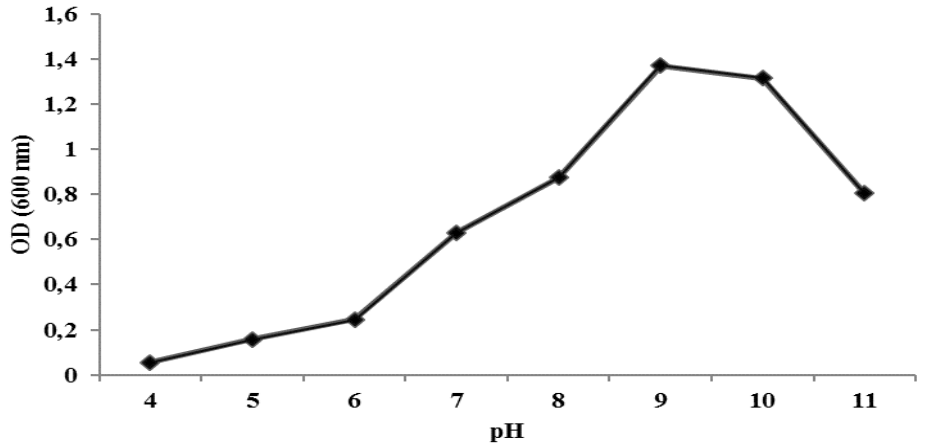


Figure 2. Effects of pH on growth of the strain KB4. The cells were incubated at 160 rpm, at different pH, at 55 °C for 24 hours.

Strain KB4 grew well at 40 °C to 70 °C and the optimum temperature was 55-60 °C (Figure 3). As it is known, the true thermophiles show no growth, or show only very feeble growth from below 40 to 45 °C. Their development requires temperatures above 50 °C, and some are able to develop at a temperature of 80 °C, though most abundant growth is shown at 60 to 70 °C (Bergey, 1919). In addition, all of the identified *Anoxbacillus* species as seen in Table 1 have been found to be thermophilic and with optimum temperatures between 50 and 65 °C. Today, thermophilic bacteria are vitally important because of their high-temperature resistant enzymes. Therefore, it is industrially of great importance to reveal the microbial characteristics of thermal sources and study the characteristics of thermophilic bacillus. The optimum growth time of KB4 was determined to be the 24th h (Figure 4).

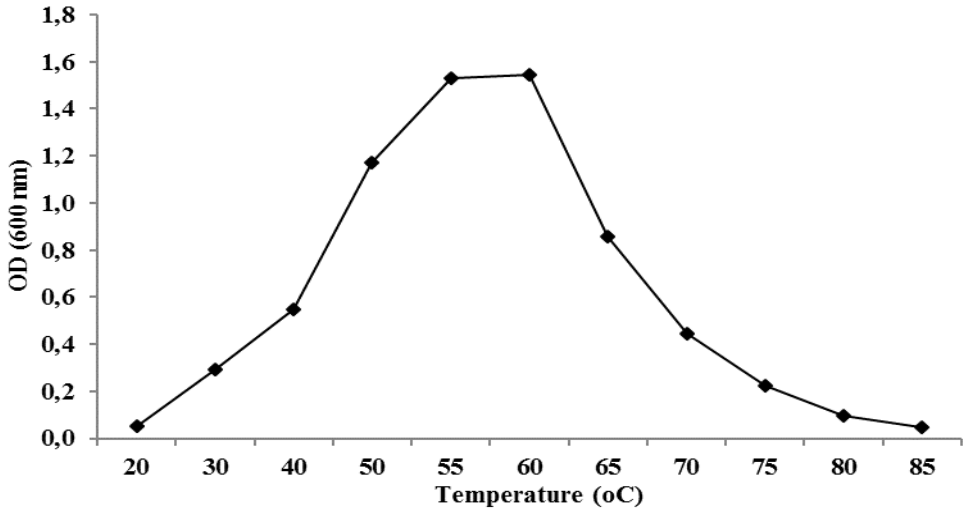


Figure 3. Effects of temperature on growth of the strain KB4. The cells were incubated at 160 rpm, pH 9.0 at different temperature for 24 hours.

Table 1. Comparison of strain KB4 with other *Anoxybacillus* species

Characteristic	**Strain KB4**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Shape of cells	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram staining	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	v	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Motility	(-)	(-)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
Spore formation	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	ND	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Temperature (°C)	55-60	62	61	50	65	50-55	60	55	50	55	60-65	55-60	57-62	60	60	55	60	50	55	65	57-60	54
pH	9.0-10	9.5-9.7	5.6	7.5-8.5	8.0	7.5-8.5	7.0	8.0-8.5	7.0	8.0	7.0	7.5-8.5	6.8-8.5	7.0	8.0	6.0-6.5	8.0-9.0	8.5	6.0	7.2	7.0-7.5	7.0-8.0
Tolerance to NaCl (3.0%)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)
Relation to O_2	A	An	A/FAn	FAn	FAn	An	A	FAn	FA	FA	A/FAn	FAn	FAn	A	FAn	SA	FAn	FA	A	SA	SA	FAn
Catalase activity	(-)	(-)	(+)	(+)	(+)	ND	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	ND	(+)
Oxidase	(-)	(-)	(-)	(-)	ND	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(+)
Urease	(-)	ND	ND	(-)	(-)	(-)	ND	ND	(-)	ND	(-)	(-)	ND	ND	(-)	ND	ND	ND	ND	ND	ND	ND
Citritase	(-)	ND	(-)	ND	ND	ND	ND	ND	(-)	ND	ND	ND	ND	ND	ND	(-)	ND	ND	(+)	ND	(-)	(+)
Indol Production	(-)	ND	(-)	(-)	(-)	(-)	ND	ND	(-)	ND	(-)	(-)	ND	ND	(-)	(-)	ND	ND	ND	ND	ND	ND(-)
Nitrate reduction	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(-)	(+)	(+)	(-)	(-)	(-)	(+)/(-)
Methyl red test/ Voges-Proskauer test	(+)/(-)	ND	(+)/(-)	(-)/(-)	ND	ND	(+)/(+)	(+)/(-)	(+)/(-)	ND	(+)/(-)	ND	(+)/(-)	ND	ND	(-)/(-)	(-)/(-)	ND	(+)/(-)	ND	(-)/(-)	(+)
Hydrolysis of gelatin	(-)	(-)	(-)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(+)	(+)	(+)	(-)	(-)	(-)

Hydrolysis of casein	(-)	(-)	(+)	(-)	(-)	ND	(+)	(-)	ND	(+)	(-)	ND	(-)	(-)	(+)	(+)	(-)	(+)	(+)w	(-)	(-)	(-)
Hydrolysis of starch	(+)	(+)	(+)	(+)	(+)	(+)	(+)w	(+)	(+)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(-)
Utilization of carbon sources																						
Glucose	(+)	(+)	(+)	ND	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(-)	(+)
Sucrose	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	ND	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(-)	(-)	(+)
Galactose	(+)	(-)	(+)	ND	(-)	ND	(+)	(-)	(+)	ND	(+)	ND	(+)	ND	(-)	(-)	(+)	ND	(+)	ND	(-)	(-)
Lactose	(+)	(-)	(-)	(+)	(-)	(-)	(+)	(+)	(-)	ND	ND	(-)	(-)	ND	(-)	(-)	(-)	ND	(+)	ND	(-)	(-)
Maltose	(+)	ND	(+)	(-)	(+)	(+)	ND	ND	(+)	ND	(+)	ND	(+)	ND	(-)	(+)	ND	ND	ND	ND	ND	(+)
Arabinose	(+)	(-)	(-)	(+)	(+)	(-)	(-)	(-)	(-)	ND	(+)	(-)	(-)	ND	(+)	(+)	(+)	ND	(+)	ND	(-)	(+)
Xylose	(+)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(+)	ND	(-)	(+)	(-)	ND	(+)	(+)	(-)	ND	(+)	ND	w	(+)

Strains: KB4; 1, *Anoxybacillus pushchinensis* DSM 12423^T (Pikuta et al., 2000); 2, *Anoxybacillus amylolyticus* DSM 15939^T (Poli et al., 2006); 3, *Anoxybacillus ayderensis* NCIMB 13972^T (Dulger et al., 2004); 4, *Anoxybacillus bogrovensis* DSM 17956^T (Atanassova et al., 2008); 5, *Anoxybacillus kestanbolensis* NCIMB 13971^T (Dulger et al., 2004); 6, *Anoxybacillus caldiproteolyticus* DSM 15730^T (Coorevits et al., 2012); 7, *Anoxybacillus calidus* DSM 25520^T (Cihan et al., 2014); 8, *Anoxybacillus contaminans* DSM 15866^T (De Clerck et al., 2004); 9, *Anoxybacillus eryuanensis* E-112^T (Zhang et al., 2011); 10, *Anoxybacillus flavithermus* DSM 2641 (Pikuta et al., 2000); 11, *Anoxybacillus gonensis* NCIMB 13933^T (Belduz et al., 2003); 12, *Anoxybacillus kamchatkensis* DSM 14988 (Kevbrin et al., 2006); 13, *Anoxybacillus kaynarcensis* DSM 217065 (Inan *et al.*, 2013); 14, *Anoxybacillus mongoliensis* DSM 19169 (Namsaraev et al., 2011); 15, *Anoxybacillus rupiensis* DSM 17127 (Derekova et al., 2008); 16, *Anoxybacillus salavatliensis* 22626^T (Cihan et al., 2011); 17, *Anoxybacillus tengchongensis* T-11^T (Zhang et al., 2011); 18, *Anoxybacillus tepidamans* DSM 16325^T (Coorevits et al., 2012); 19, *Anoxybacillus thermarum* DSM 17141 (Poli et al., 2011); 20, *Anoxybacillus vitaminiphilus* 3nP4^T (Zhang et al., 2013); 21, *Anoxybacillus voinovskiensis* NCIMB 13956^T (Yumoto et al., 2004). (+), Positive; w, weakly positive; (-), negative; A, aerobe; AN, anaerobe; FA, facultative aerobe; FAn, facultative anaerobe; SA, sitrict aerobe; ND, not determined.

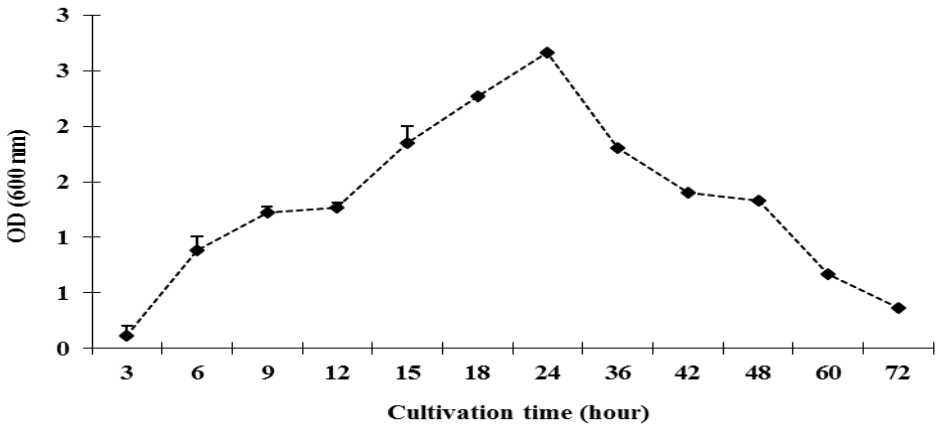
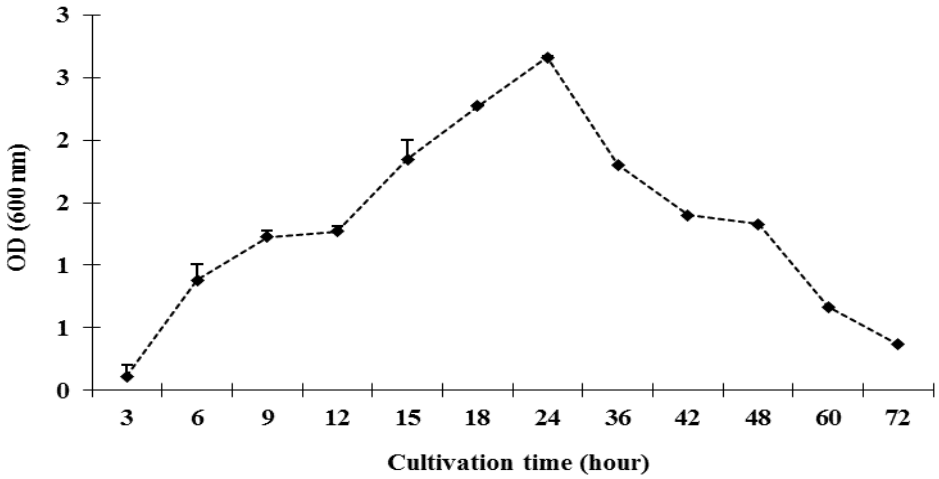


Figure 4. Effects of cultivation time on growth of the strain KB4. The cells were incubated at 160 rpm, pH 9.0, at 55 °C for 72 hours. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.



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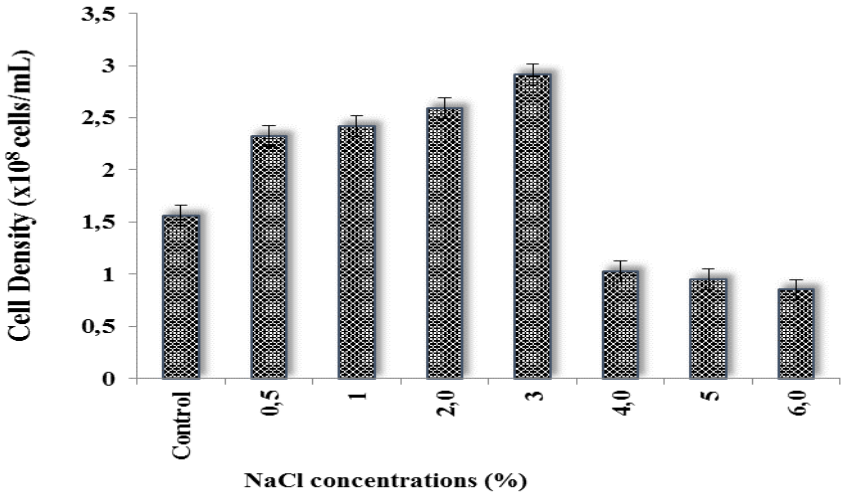

Figure 5. Effects of NaCl concentration on growth of the strain KB4. For this experiment, different concentrations of NaCl were added into the medium and incubated at 160 rpm, pH 9.0, at 55 °C for 24 hours. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

Growth was observed up to 3% NaCl (w/v) (Figure 5). As it can be seen in Table 1, the identified *A. puschionensis, A. kestanbolensis, A. contaminans, A. eryuanensis, A. kaynarcensis, A. tengchongensis and A. vitaminopilus* species of *Anoxbacillus* grow in salt concentration up to 3% and have halotolerant characteristics. Halotolerant bacteria are nonhalophilic microorganisms that can thrive in the absence of salts and can tolerate relatively high salt concentrations. But, nonhalophilics generally need 100-200 mM NaCl, the slight halophiles are able to grow up to 1.25 M NaCl, moderately halophilic bacteria can grow in the presence of 3.0 M NaCI, and extreme halophiles can grow in media containing up to 20–25% salt (Arahal and Ventosa, 2002). KB4 strain, because of these characteristics, has both thermophilic and moderately halophilic characteristics.

The isolate KB4 was positive for nitrate reduction and starch hydrolysis Methyl red test, while it was negative for catalase activity, oxidase, urease, indole production, Voges–Proskauer test, starch and casein hydrolysis (Table 1). Among carbon and nitrogen sources, the strain KB4 utilized glucose, galactose, sucrose, maltose, arabinose, xylose, yeast extract, peptone, tryptone and casamino acids (Figure 6a and 6b). Kevbrin et al. (2006) and Namsaraev et al. (2011) have reported that *Anoxybacillus kamchatkensis* DSM 14988 and *Anoxybacillus mongoliensis* DSM 19169 were able to utilize yeast extract, peptone, tryptone and casamino acids as nitrogen sources.

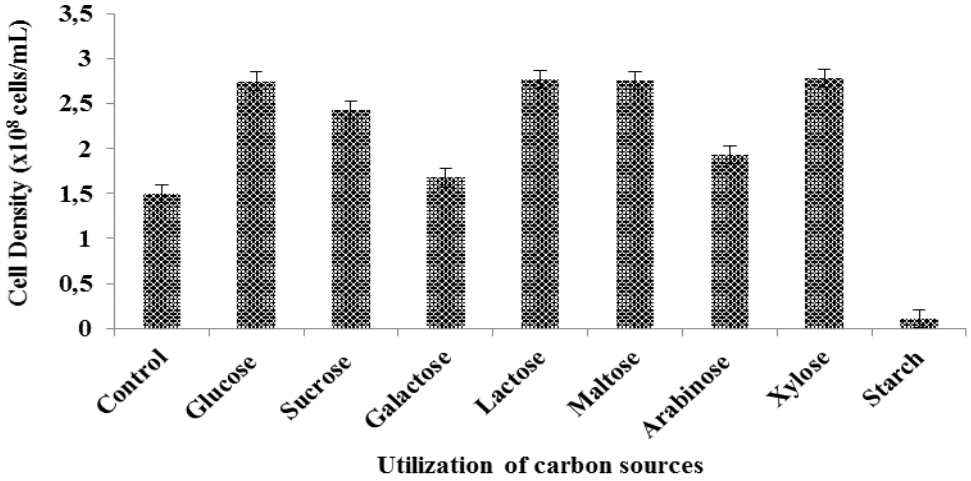

Figure 6a. Utilization of carbon sources. For this experiment, different carbon sources were added into the medium at 2.0% (w/v) and incubated at 160 rpm, pH 9.0, at 50 °C for 24 hours. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

Figure 6b.

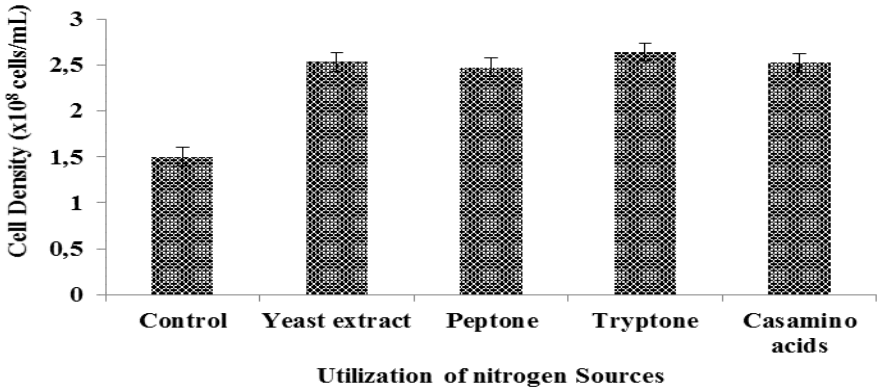

Figure 6b. Utilization of nitrogen sources. For this experiment, different nitrogen sources were added into the medium at 2.0% (w/v) and incubated at 160 rpm, pH 9.0, at 55 °C for 28 hours. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

The strain KB4 was resistant to Nystatin and Bacitracin, while it was sensitive to AMP, AMC, CAP, GM, IPM, NET, OFX and R (Table 2).

Anoxybacillus pushchinensis (Pikuta et al., 2000) is sensitive to Bacidracin, but not to Peniciline, AMP and CAP. With this aspect, the strain KB4 that we isolated differs from *Anoxybacillus pushchinensis*. The growth of *A. gonensis* (Belduz et al., 2003), *A. kestanbolensis* (Dulger et al., 2004), *A. ayderensis* (Dulger et al., 2004), *A. amylolyticus* (Poli et al., 2006) and *A. rupiensis* (Derekova et al., 2008) are inhibited by CAP, AMP, GM, Streptomycin.

Table 2. Antibiotic test results of strain KB4

Antibiotics	Test result
Ampicillin (AMP)	s
Amoxycilin Clawlanic Acide (AMC)	s
Chloramphenicol (CAP)	s
Gentamicin (GM)	s
Imipenem (IPM)	s
Netilmicin (NET)	s
Ofloxacin (OFX)	s
Rifampicin (R)	s
Bacidracin	R
Nystatin	R

S, sensitive; R, resistant

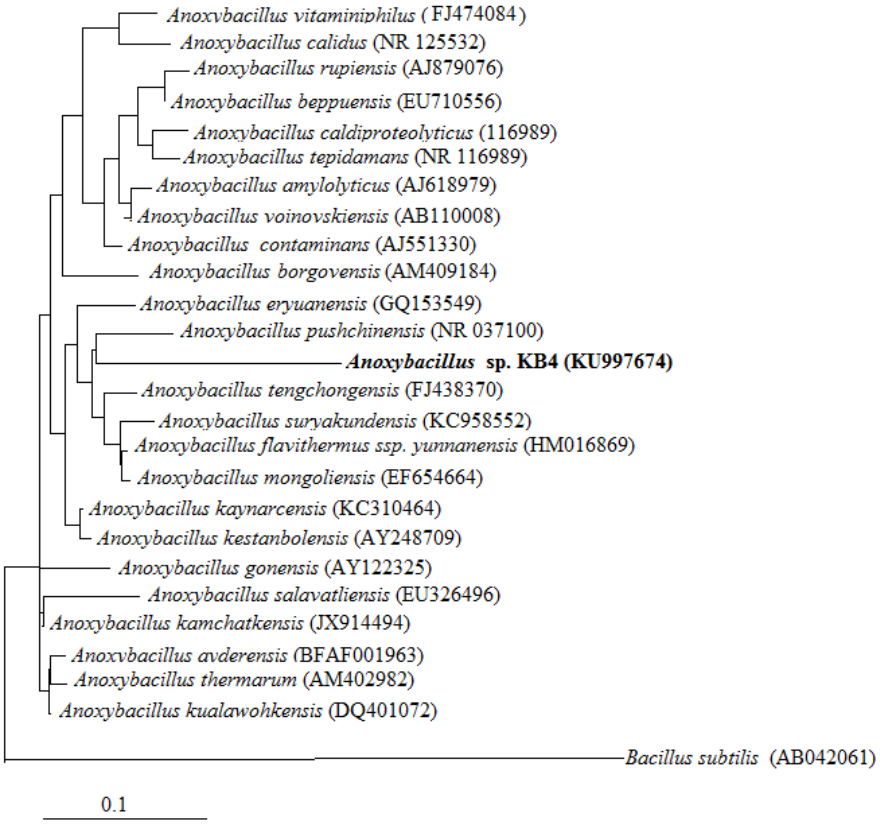

Figure 7. Neighbour-joining evolutionary distance phylogenetic relationships on the basis of 16S rRNA gene sequence data of the thermophilic strain KB4 isolated from Kuşburnu hot springs in Turkey and all the representative members of the genus *Anoxybacillus*. The accession numbers are given in parentheses. The bar shows 0.1 nucleotide substitutions per position.

As with other groups of bacteria, studies of 16S rDNA and of DNA have very valuable applications in the classification of aerobic endospore-formers (Logan, 2002). The 16S rRNA gene is known to be a good molecular clock as its primary structure is highly conserved, and thus 16S rRNA gene sequencing is one of the widely used standard techniques in modern bacterial taxonomy by forming the basis of the bacterial phylogeny (Rosselló-Mora, 2005). The total 16S rRNA gene sequence of the strain KB4 (GenBank accession number is KU997674) falls within the genus *Anoxybacillus* and shows high similarity to *Anoxybacillus pushchinoensis* K1(T) (98.78%). A phylogenetic tree was constructed using the neighbor-joining method showing the position of the novel isolate KB4 with respect to other related species of genus *Anoxybacillus* (Figure 7). As a result, although methods that are not based on culture provide us with the most accurate information regarding species, the isolation and sustaining of the cultures through classical methods carry great importance, and the combined use of phenotopic, chemotaxonomic and genetic methods in identification of microorganisms is thought to be the most appropriate approach (Yücel Şengün and Kılıç, 2016). A

DNA–DNA hybridization study in the future needs to be performed to confirm the identity and taxonomic group of KB4 strain isolated in our study

4. Conclusions and discussion

In this study, termo-alkalophilic *Anoxybacillus* sp. KB4 obtained from the Kuşburnu Hot Spring was isolated and identified according to morphological, physiological, biochemical, and 16S rRNA analyses. The *Anoxybacillus* sp. KB4 strain that was identified is usable in biotechnology due to its termo-alkalophilic characteristics and its ability to release some enzymes. This study is significant in identification and in getting information about the biodiversity of the microorganisms obtained from the hot water springs.

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(Received for publication 17 May 2016; The date of publication 15 August 2016)