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ORIGINAL PAPER

Molecular characterisation and numerical analysis of novel moderately thermophile *Anoxybacillus* sp. FMB1

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FATMA MATPAN BEKLER[1*], SEÇİL YALAZ[2], KEMAL GÜVEN[3]
[1]Dicle University, Science Faculty, Department of Biology, 21280 Diyarbakır/TURKEY
[2] Dicle University, Science Faculty, Mathematics Department, 21280 Diyarbakır, TURKEY
[3] Dicle University, Science Faculty, Department of Molecular Biology and Genetics, 21280 Diyarbakır/TURKEY
**Address for correspondence to: fatmatpan@hotmail.com*

Abstract

A moderately thermophilic bacteria was isolated from the water sample collected from Yozgat, in the Central Anatolia Region of Turkey. Morphological, physiological and biochemical characteristics of thermophilic isolate were determined in comparison to standard type strains, and they were all identified and classified into homogenous groups by using fundamental principles of numerical taxonomy. The UPGMA method was used for numerical taxonomy and the phenogram was drawn with MATLAB. Phylogenetic analyses were also performed by the neighbour-joining and UPGMA methods. According to the numerical definition result and 16S rRNA analysis, the strain FMB1 was most closely related to Anoxybacillus ayderensis AB04[T]. The results showed that MATLAB computer program using UPGMA method can be used as an alternative or additional method in bacterial classification, before 16S rRNA analysis performed.

Keywords: *Anoxybacillus*, characterization, 16S rRNA, numerical analysis

1. Introduction

The members of genus *Anoxybacillus*, which are known as rod-shaped, Gram positive, endospore-forming, anaerobes or facultative anaerobes and moderately thermophilic bacteria, are widely isolated from geothermal areas. Recently, many studies have been carried out on the members of this genus with increasing biotechnological and industrial interest for their thermostable products. Thus, the isolation of these bacteria and the determination of their phylogenetic relationship are of importance.

Prokaryotes can be classified by using phenotypic characterization such as cell morphology, staining behaviour, ultrastructural characteristics, biochemical reactions, utilising carbon and nitrogen sources or by phylogenetic analysis. Phylogenetic analysis means *inferring* or estimating the evolutionary relationships (BRINKMAN & LEIPE [1]). The routine strategy for phylogeny is based on the correlation of a precise morphologic and phenotypic description of the isolate to be recognized. Generally, *Bergey's Manual of Systematic Bacteriology* or the *Manual of Clinical Microbiology* are used as a standard reference for morphologic and phenotypic identification by microbiologists. However, different computer programs and methods were designed for the evaluation and identification could differ among laboratories (CLARRIDGE [2]). Phylogenetic studies are carried out using molecular phylogeny in cases where morphological and phenotypic characterization is insufficient.

Molecular phylogenies are based on the information about the evolution of the organisms. Deducing phylogenies from molecular information might be helped out through the point analysis of similarities and differences in the studied sequences (ALBRECHT & BORGES [3]). The part of the DNA, which has been by far the most common housekeeping

genetic marker for the phylogeny and taxonomy of bacteria, is the 16S rRNA gene (BOTTGER [4], JANDA & ABBOTT [5]). The 16S rRNA gene sequence, which is about 1,550 bp has been determined for a large number of strains because of some important reasons, including (i) to be common in almost all bacteria (ii) is a useful evolutionary chronometer, and (iii) the size of 16S rRNA gene is large enough to be used in bioinformatics (JANDA & ABBOTT [5]). Universal primers, which usually chosen as complementary to the conserved regions, are used for comparative taxonomy. GenBank, the largest databank of genetic sequence, has previously deposited sequences, helping out to compare the sequence of an unknown strain (CLARRIDGE [2]).

In this study, a thermophilic bacterial strain designated as FMB1 was isolated from Yozgat, in the Central Anatolia Region of Turkey. The identification of the strain was carried out by numerical taxonomy utilising the phenotypic characteristics in comparison to 16S rRNA sequence analysis.

2. Materials and Methods

2.1 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.

2.1 Location and sampling

The water samples were obtained from Sorgun hot spring (39° 48' 14.0718" N, 35° 12' 31.0752" E), Yozgat, in the Central Anatolia Region of Turkey. The temperature and pH of hot spring water were 60 °C and 7.5, respectively. The water samples were inoculated into the modified liquid Thermus medium (TM) [Tryptone, 3 g/L; Yeast extract, 1 g/L; containing Nitsch's trace element (0.5 ml/L) and Castenholz salts (100 ml/L)] and incubated at 50 °C for 24 hours in a shaker. After cultivation, samples were streaked on TM agar (TMA) and incubated at 50 °C for 2 days. Subsequently, different colonies developed in the media were selected and purified by subculturing.

2.3 Morphological, physiological and biochemical characterization

Selected colonies were phenotypically characterized on the basis of shape, size, colour, surface, aspect, elevation and consistency, etc. The cell morphology was studied by the light microscopy of 24 hours old bacterial cultures growing on TMA plates. Gram staining to confirm the Gram reaction and spore position was investigated using light microscope according to the Dussault method (DUSSAULT [6]). The formation of spores was carried out by malachite green staining under light microscopy. Motility was determined by the hanging drop method. For the presumptive identification, data of phenotypic characteristics from the conventional methods such as catalase, indole, oxidase, citritase and urease activity, Methyl red test/Voges–Proskauer test, utilization of different carbon and nitrogen sources (glucose, galactose, lactose, fructose, maltose, arabinose, gelatine, starch, caseine and lipid) were used.

The optimum growth temperature and pH were determined by incubating the isolate in TM liquid medium at different temperature (20 to 90 °C) and different pH range (4.0-11.0). The effect of NaCl on growth was examined in 100 mL TM liquid medium which was prepared in a phosphate buffer (50 mM) with final salt (NaCl) concentrations of 0.5%, 1.0%,

2.0%, 3.0% and 5.0% in a 300 mL Erlenmayer flask and 1mL of 18h old bacterial culture was inoculated in to this medium. Then, all flasks were incubated at 50 °C for 2 days in the shaker. The cell growth was measured hourly by spectrophotometer at 600 nm (OD_{600}). All experiments were performed at least in duplicate.

2.4 Numerical taxonomy and phenogram

Numerical taxonomy was first developed and elaborated by Sokal and Sneath in 1963. In biological systematics, this system is useful to generate a taxonomy using numeric algorithms like cluster analysis rather than using subjective evaluation of their properties (SOKAL & SNEATH [7], SNEATH & SOKAL [8]). Phenetics is a field of numerical taxonomy. Classifications are formed based on the patterns of overall similarities. Clustering was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) which is based on the model sum of squares coefficient. UPGMA is a preferred as it is a useful tree-construction method for distance matrices (NEI & KUMAR [9]). The fundamental thought for UPGMA is to repeatedly combine a pair of operational taxonomic units (OTUs) with the smallest distance (or highest similarity) into a new OTU upto only one OTU is left and the distance between two OTUs is calculated by the arithmetic mean of all distances between their inter-pairs of sequences (or other kinds of data), where an inter-pair of sequences (x, y) means that x is in the one OTU and y is in the other (TAKEZAKI & NEI[10]). The UPGMA algorithm constructs a rooted tree called dendrogram that reflects the structure present in a pairwise similarity or dissimilarity matrix. The UPGMA algorithm produces rooted dendrograms and requires a constant-rate assumption, distances from the root to every branch tip are equal. In biological systematics, dendogram is called as phenogram when the phenetics are used for classification.

Numerical identification of the strain as *Anoxybacillus* species was performed with MATLAB computer program. The program uses UPGMA method to produce numerical taxonomy and phenogram and compares the test results of an unknown isolate with the known test result scales in these tests for a collection of related bacterial species, using established numerical methods. Nearly all of the characters existed in one or two mutually exclusive states and scored positive (+) or negative (-). Some characters could not be determined and scored as ND (not determined). Qualitative multistate characters, such as some of pigmentation and morphological tests, were coded as several independent characters and were scored present (1) for the character state, absent (0) and ND (0.5) for other alternatives (Table 1).

2.5 DNA isolation, amplification of 16S rRNA gene, bioinformatics analysis and phylogenetic tree construction

The genomic DNA was extracted from strain FMB1 using Master Pure TM Gram Positive DNA Purification Kits (Epicentre Biotechnologies, Germany) according to the manufacturer's instructions. 16S rDNA sequence was amplified from isolated genomic DNA with the upstream primer: 5'-ATTCTAGAGTTTGATCATGGCTTCA-3' and the downstream primer: 5'-ATGGTACCGTGTGACGGGCGGTGTTGTA-3'. PCR mediated amplification of the 16S rDNA and purification of the PCR product was carried out according to Wilson et al. (WILSON & al. [27]). Purified PCR products were sequenced by BigDye Terminator v1.1 Cycle Sequencing and reactions were electrophoresed according to manufacturer's protocol. After sequencing, multiple alignments were performed using the CLUSTAL X program and all sequences were compared using a BLAST search tool database on NCBI [National Centre of Biotechnology (http://www.ncbi.nlm.nih.gov)]. Phylogenetic

analyses were performed by the neighbour-joining and UPGMA method. Phylogenetic tree was constructed using CLC Sequence Viewer 7.7.1 (QIAGEN Aarhus/Denmark).

Table 1: Codes used in numerical analysis of phenotypic characters of *Anoxybacillus* species

Characteristic	Strain FMB1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Shape of cells	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Gram staining	1	1	1	1	1	1	1	1	0.5	1	1	1	1	1	1	1	1	1	1	1	1	1
Motility	1	0	1	1	0	1	1	1	0.5	1	1	1	1	1	1	1	1	1	1	1	1	0
Spore formation	1	1	1	1	1	1	1	1	0.5	1	1	1	1	1	1	1	1	1	1	1	1	1
Temperature (50 °C)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
pH (7)	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	1	0	1
Tolerance to NaCl (3.0%)	0	1	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	1	0	0	1	0
Relation to O_2	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	1
Catalase activity	1	0	1	1	1	0.5	1	0	1	1	1	1	0	0	1	1	1	1	1	1	0.5	1
Oxidase	0	0	0	0	0.5	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	1
Urease	0	0.5	0.5	0	0	0	0.5	0.5	0	0.5	0	0	0.5	0.5	0	0.5	0.5	0.5	0.5	0	0.5	0.5
Citritase	0	0.5	0	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5	1	0.5	0	1
I0.5ol Production	0	0.5	0	0	0	0	0.5	0.5	0	0.5	0	0	0.5	0.5	0	0	0.5	0.5	0.5	0.5	0.5	0.5
Nitrate reduction	0	1	0	0	0	1	0	0	1	0	1	0	1	1	0	0	1	1	0	0	0	1
Voges-Proskauer test	0	0.5	0	0	0.5	0.5	1	0	0	0.5	0	0.5	0	0.5	0.5	0	0	0.5	0	0.5	0	0
Methyl red test	0	0.5	1	0	0.5	0.5	1	1	1	0.5	1	0.5	1	0.5	0.5	0	0	0.5	1	0.5	0	1
Hydrolysis of gelatin	1	0	0	1	1	0	1	0	1	1	0	1	0	1	1	0	1	1	1	1	0	0
Hydrolysis of casein	0	0	1	0	0	0.5	1	0	0.5	1	0	0.5	0	0	1	1	0	1	1	0	0	0
Hydrolysis of starch	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0
Utilization of carbon sources																						
Glucose	0	1	1	0.5	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1
Sucrose	0	1	1	1	1	1	1	1	0.5	1	1	1	1	1	1	0	1	1	1	0	0	1
Galactose	0	0	1	0.5	0	0.5	1	0	1	0.5	1	0.5	1	0.5	0	0	1	0.5	1	0.5	0	0
Lactose	1	0	0	1	0	0	1	1	0	0.5	0.5	0	0	0.5	0	0	0	0.5	1	0.5	0	0
Maltose	0	0.5	1	0	1	1	0.5	0.5	1	0.5	1	0.5	1	0.5	0	1	0.5	0.5	0.5	0.5	0.5	1
Arabinose	1	0	0	1	1	0	0	0	0	0.5	1	0	0	0.5	1	1	1	0.5	1	0.5	0	1
Xylose	1	0	0	1	0	0	0	1	1	0.5	0	1	0	0.5	1	1	0	0.5	1	0.5	0.5	1

1, *Anoxybacillus pushchinensis* DSM 12423T (PIKUTA & al. [11]); 2, *Anoxybacillus amylolyticus* DSM 15939T (POLI & al. [12]); 3, *Anoxybacillus ayderensis* NCIMB 13972T (DULGER & al. [13]); 4, *Anoxybacillus bogrovensis* DSM 17956T (ATANASSOVA & al. [14]); 5, *Anoxybacillus kestanbolensis* NCIMB 13971T (DULGER & al. [13]); 6, *Anoxybacillus caldiproteolyticus* DSM 15730T (COOREVITS & al. [15]); 7, *Anoxybacillus calidus* DSM 25520T (CİHAN & al. [16]); 8, *Anoxybacillus contaminans* DSM 15866T (DE CLERCK & al. [17]); 9, *Anoxybacillus eryuanensis* E-112T (ZHANG & al. [18]); 10, *Anoxybacillus flavithermus* DSM 2641(PIKUTA & al. [11]); 11, *Anoxybacillus gonensis* NCIMB 13933T (BELDUZ & al. [19]); 12, *Anoxybacillus kamchatkensis* DSM 14988 (KEVBRIN & al. [20]); 13, *Anoxybacillus kaynarcensis* DSM 217065 (INAN & al. [21]); 14, *Anoxybacillus mongoliensis* DSM 19169 (NAMSARAEV & al. [22]); 15, *Anoxybacillus rupiensis* DSM 17127(DEREKOVA & al. [23]); 16, *Anoxybacillus salavatliensis* 22626T (CIHAN & al. [24]); 17, *Anoxybacillus tengchongensis* T-11T (ZHANG & al. & al. [18]); 18, *Anoxybacillus tepidamans* DSM 16325T (COOREVITS & al. [15]); 19, *Anoxybacillus thermarum* DSM 17141 (POLI & al. [12]); 20, *Anoxybacillus vitaminiphilus* 3nP4T (ZHANG & al. & al. [25]); 21, *Anoxybacillus voinovskiensis* NCIMB 13956T (YUMOTO & al. [26]).

3. Results and discussion

3.1 Determination of phenetics, numerical taxonomy and phenogram

Isolation steps performed with water samples from Sorgun hot spring allowed the isolation of a moderately termophilic isolate. The strain, namely FMB1, was characterized by morphological and physio-biochemical characteristics, as well as by genetic analysis. It was deposited in Molecular Biology Research Laboratory in Science Faculty at Dicle University.

The strain FMB1 is rod-shaped (0.3–0.6 µm in diameter and 2.5–4.0 µm long.), Gram positive, motile, edospore forming (sub-terminal) and anaerobe bacterium. Although the genus *Anoxybacillus* contains the endospore-forming, thermophilic rod shaped bacteria which are close to the genus *Bacillus*, termophilic members of *Anoxybacillus* were first described by Pikuta et al. in 2000. To date, 26 species and 3 subspecies belonging to *Anoxybacillus* genus have been recognized.

The strain FMB1 was grown between 6-60 hours (optimum of 24 hours; Fig. 1). 24 hr of incubation time was used for further studies.

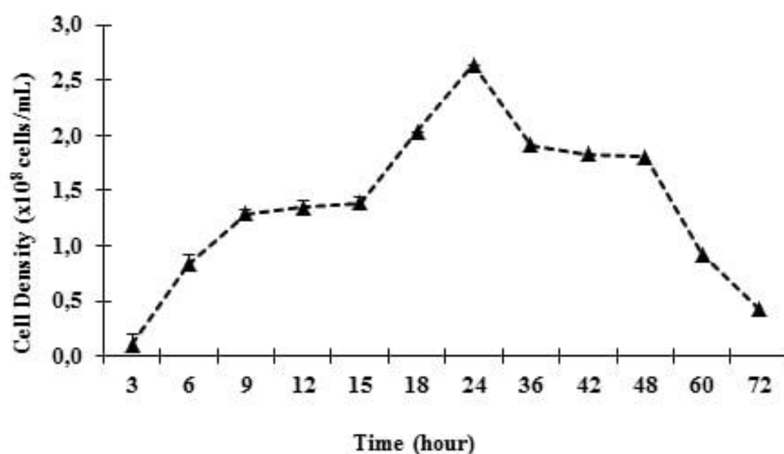

Figure 1. Effects of cultivation time on the growth of strain FMB1. The cells were incubated at 160 rpm, pH 7.0, at 50 °C for 72 hours. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

The optimum growth pH was 7.0 (Fig. 2) for strain FMB1. Some species require specific carbonate ions in growth medium. Although *Anoxybacillus* cells are alkaliphilic or alkali tolerant, previous studies show that many members of *Anoxybacillus* genus optimally grow at neutral pH (7.0), such as *A. caldiproteolyticus* DSM 15730T (COOREVITS & al. [15]), *A.contaminans* DSM 15866T (DE CLERCK & al. [17]), *A. flavithermus* DSM 2641 (PIKUTA & al. [11]), *A. kaynarcensis* DSM 217065 (INAN & al. [21]), *A. thermarum* DSM 17141 (POLI & al. [28]), *A. vitaminiphilus* 3nP4^T (ZHANG & al. & al. [25]) and *A. voinovskiensis* NCIMB 13956^T (YUMOTO & al. [26]).

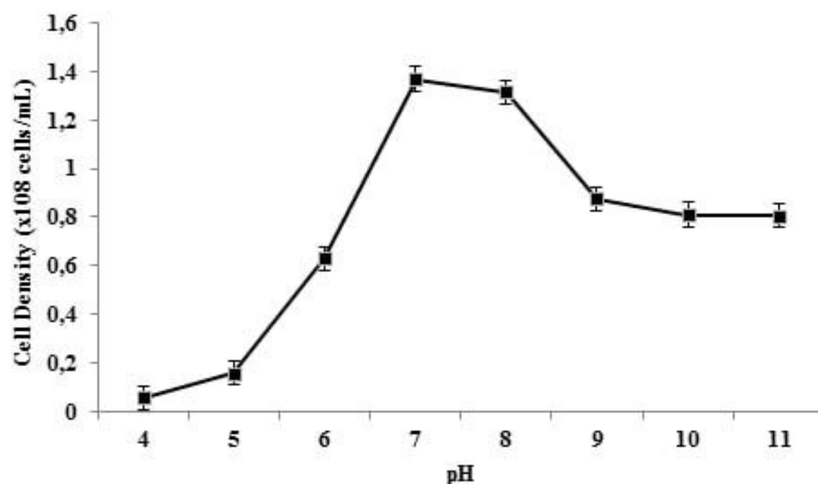

Figure 2. Effects of pH on the growth of strain FMB1. The cells were incubated at 160 rpm, at different pH, at 50 °C for 24 hours.

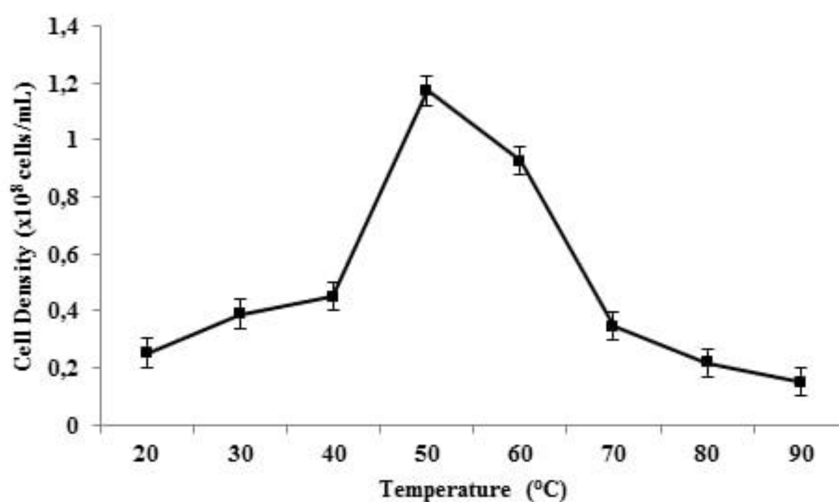

Figure 3. Effects of temperature on the growth of strain FMB1. The cells were incubated at 160 rpm, pH 7.0, at different temperature for 24 hours.

Thermophiles (literally heat lovers) are microorganisms that live and grow at temperatures above the mesophilic range (25-40 °C) which characterizes the mainstream of life (ROBB & al. [29]). The optimum growth temperature was 50 ^{o}C (Fig. 3) for strain FMB1 such as in other *Anoxybacillus* species *A. ayderensis* NCIMB 13972^{T} (DULGER & al. [13]), *A. contaminans* DSM 15866^{T} (DE CLERCK & al. [17]) and *A. tengchongensis* T-11^{T} (ZHANG & al. [18]). From these results, the strain FMB1 seems to have considerable moderately thermophile.

As mentioned in the *Bergey's Manual of Systematic Bacteriology*, the common characteristic of all *Anoxybacillus* species is independence from NaCl and comparatively low resistant to salt. FMB strain was found to grow at a NaCl concentrations between 0.5 and 2% (optimum 1%; Fig. 4).

Figure 4. Effects of NaCl concentration on the growth of strain FMB1. For this experiment, different concentrations of NaCl were added into the medium and incubated at 160 rpm, pH 7.0, at 50 °C for 24 hours.

Physio-biochemical characters of FMB1 were determined and compared with related *Anoxybacillus* species (Table 2).

Table 2: Physological and biochemical properties of strain FMB1 and related *Anoxybacillus* type strains.

Characteristic	Strain FMB1	1	2	3	4	5	6	7
Shape of cells	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram staining	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Motility	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Spore formation	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Temperature	50	50	55-60	57-62	60	60	60	65
pH	7.0	7.5-8.5	7.5-8.5	6.8-8.5	7.0	8.0	8.0-9.0	7.2
Tolerance to NaCl (3.0%)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Relation to O_2	An	FAn	FAn	FAn	A	FAn	FAn	SA
Catalase activity	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(+)
Oxidase	(-)	(-)	(+)	(-)	(-)	(+)	(+)	(-)
Urease	(-)	(-)	(-)	ND	ND	(-)	ND	(-)
Citritase	(-)	ND	ND	ND	ND	ND	ND	ND
I0.5ol Production	(-)	(-)	(-)	ND	ND	(-)	ND	ND
Nitrate reduction	(-)	(-)	(-)	(+)	(+)	(-)	(+)	(-)
Voges-Proskauer test	(-)	(-)	ND	(-)	ND	ND	(-)	ND
Methyl red test	(-)	(-)	ND	(+)	ND	ND	(-)	ND
Hydrolysis of gelatin	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(-)
Hydrolysis of casein	(-)	(-)	ND	(-)	(-)	(+)	(-)	(-)
Hydrolysis of starch	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(-)
Utilization of carbon sources								

Glucose	(-)	ND	(+)	(+)	(+)	(+)	(+)	(-)
Sucrose	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
Galactose	(-)	ND	ND	(+)	ND	(-)	(+)	ND
Lactose	(+)	(+)	(-)	(-)	ND	(-)	(-)	ND
Maltose	(-)	(-)	ND	(+)	ND	(-)	ND	ND
Arabinose	(+)	(+)	(-)	(-)	ND	(+)	(+)	ND
Xylose	(+)	(+)	(+)	(-)	ND	(+)	(-)	ND

1, *A. ayderensis* NCIMB 13972^T (DULGER & al. [13]); 2, *A. gonensis* NCIMB 13933T (BELDUZ & al. [19]); 3, *A. kamchatkensis* DSM 14988 (KEVBRIN & al. [20]); 4, *A. kaynarcensis* DSM 217065 (INAN & al. [21]); 5, *A. mongoliensis* DSM 19169 (NAMSARAEV & al. [22]); 6, *A. salavatliensis* 22626T (CIHAN & al. [24]); 7, *A. thermarum* DSM 17141 (POLI & al. [12]). (+), Positive; (-), negative; A, aerobe; AN, anaerobe; FAn, facultative anaerobe; SA, sitrict aerobe; ND, not determined.

The tests results showed that catalase, gelatine and starch activity were positive, while oxidase, urease, citritase, indol production, nitrate reduction, Voges-Proskauer test and Methyl red test were negative. In addition, the strain FMB1 is able to utilize lactose, arabinose and xylose (Fig. 5a). *A. ayderensis* NCIMB 13972^T (DULGER & al. [13]), *A. caldiproteolyticus* DSM 15730^T (COOREVITS & al. [15]), *A. calidus* DSM 25520^T (CİHAN & al. [16]) and *A. tepidamans* DSM 16325^T (COOREVITS & al. [15]) were also able to utilize lactose.

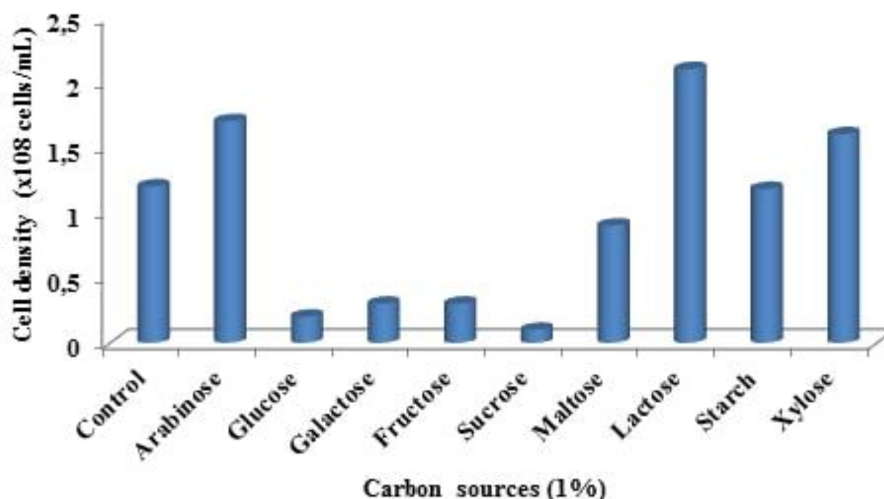

Figure 5a. Utilization of carbon sources. For this experiment, different carbon sources were added into the medium at 1.0% (w/v) and incubated at 160 rpm, pH 7.0, at 50 °C for 24 hours.

Figure 5b also represents the utilization of nitrogen sources, showing that caseine, casamino acide, peptone, yeast extract, glycine, urea, ammonium sulphate and ammonium chloride can not be utilized by this strain. Physiological and biochemical test results clearly showed that strain FMB1 possesed some differences compared to other *Anoxybacillus* species.

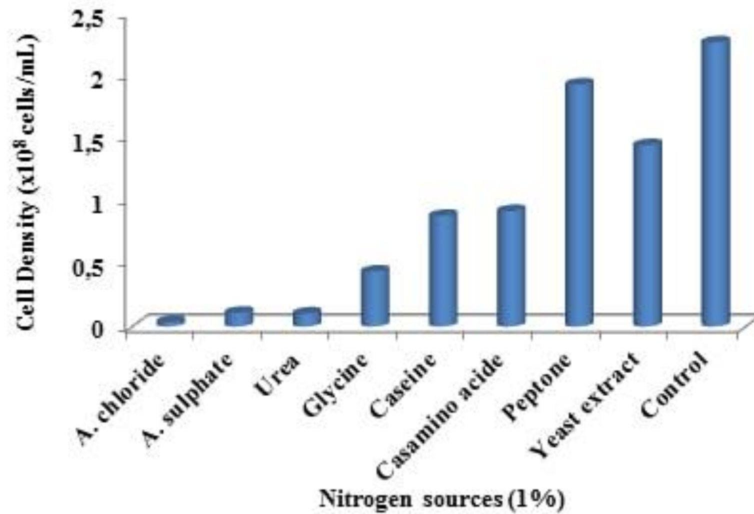

Figure 5b. Utilization of nitrogen sources. For this experiment, different nitrogen sources were added into the medium at 1.0% (w/v) and incubated at 160 rpm, pH 7.0, at 50 °C for 24 hours.

Phenogram tree, which is formed by phenetics, is obtained by MATLAB computer programme using UPGMA method. In UPGMA method, all the nodes thought have equal distance from the root. This means that UPGMA is "ultrametric". The process uses the nearest two clusters to combine them into a higher-level cluster.

In numerical classification of strain FMB1, 21 different *Anoxybacillus* strains and 30 characters were used. Numerical classification was carried out following the published studies of thermophilic *Anoxybacillus* species recorded in GenBank (www.ncbi.nlm.nih.gov) as new species. In this program, a maximum number of nodes can be given to generate a phenogram or a phenogram containing all possible nodes can be generated without giving the number of nodes. In the present study, all possible nodes were obtained without giving the number of nodes (Fig. 6).

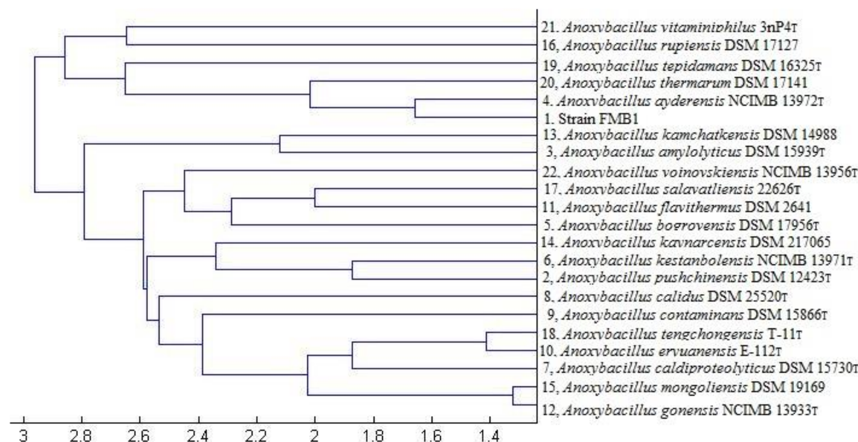

Figure 6. Phenogram tree showing the similarity and relationship of the phenetic characters of FMB1 strain and 21 other *Anoxybacillus* species.

In Figure 6, the columns give distances between species. It can be seen that FMB1 strain exhibits a similar phenetic characteristic to *A. ayderensis* AB04T indicated as number 4.

3.2 16S rRNA sequence analysis and phylogenetic relationship

The 16S rRNA sequence is a biomarker composed of specific and conserved sequences used for identification of unknown bacteria and statistical comparisons of a wide range of organisms (DEMİREL [30]). Although 16S rRNA gene sequencing is very useful for the bacterial classification, it is not generally powerful to identify at the species level but at least related to the genus level. 16S rRNA sequencing results should be used with standard identification methods and especially new species should be reported carefully. The 16S rRNA gene sequence of the strain FMB1 showed a high similarity (99.7%) to *A. ayderensis* AB04^T, (99.6%) to *A. thermarum* DSM 17141^T, (99.5%) to *A. kamchatkensis* JW-VK-KG4^T, (99.5%) to *A. salavatliensis* A343^T, (99.0%) to *A. gonensis* G2^T, (98.8%) to *A. kaynarcensis* D1021^T, (98.7%) to *A.mongoliensis* T4^T (Table 3).

Table 3: 16S rRNA gene similarity values of strain FMB1 and related taxa based on partial sequence comparison

Microorganisms	**% similarity of 16S rRNA gene sequences**
Anoxybacillus ayderensis AB04^T	99.7
Anoxybacillus thermarum DSM 17141^T	99.6
Anoxybacillus kamchatkensis JW-VK-KG4^T	99.5
Anoxybacillus salavatliensis A343^T	99.5
Anoxybacillus mongoliensis T4^T	98.7
Anoxybacillus gonensis G2^T	99.0
Anoxybacillus kaynarcensis D1021^T	98.8

Evolutionary relationship of strain FMB1 and related 26 thermophilic *Anoxybacillus* species were determined by using the Neighbor-Joining method. The *p*-distance of nucleotide difference was used to construct the tree according to Saitou and Nei (SAITOU and NEI [31]) and the stability of the tree obtained from cluster analyses described above was assessed by using BOOTSTRAP programme in sets of 1,000 resampling (FELSENSTEIN & CHURCHILL [32]) (Fig. 7).

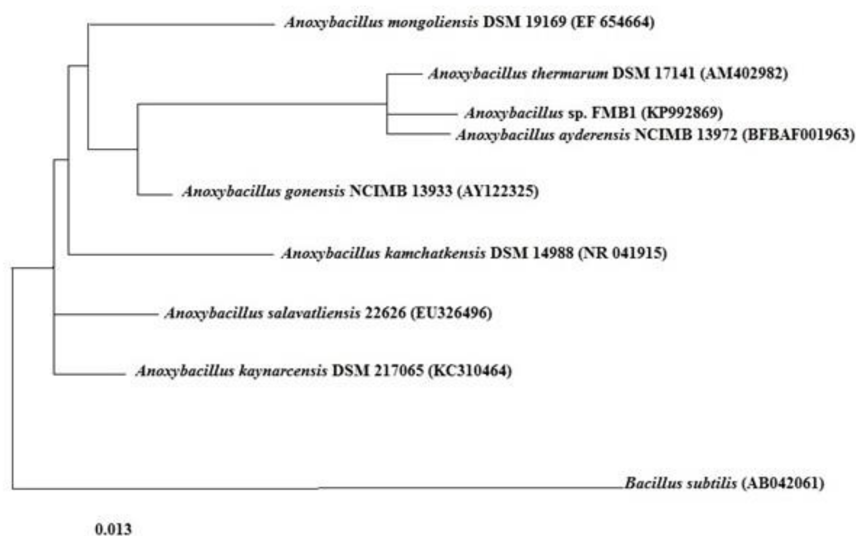

Figure 7. Neighbour-joining evolutionary distance phylogenetic relationships on the basis of 16S rRNA gene sequence data of the thermophilic strain FMB1 and all the representative members of the genus *Anoxybacillus*. The accession numbers are given in parentheses. The bar shows 0.1 nucleotide substitutions per position.

The tree is drawn to scale with branch lengths in the same units since those evolutionary distances helped to build the phylogenetic tree. In the present study, the phenetic and the phylogenetic results were compared. On the basis of phenotypic characteristics and phylogenetic data, it was proposed that the strain FMB1 (= DSMZ ID 16-25) should be placed in the genus *Anoxybacillus.* The GenBank accession number for the 16S rRNA sequence is KP992869. 16S rRNA sequences analysis may be insufficient for comparison and the identification of closely related species (VANDAMME & al.[33]). Thus, sequence data needs to be supported by another genomic method, DNA–DNA hybridization, is expected to confirm the identity and taxonomy group of strain FMB1.

4. Conclusion

In this study, a moderately thermophilic FMB1 strain was isolated and classified separately by numerical analysis and molecular techniques. Both numerical classification and 16S rRNA analysis revealed that the strain FMB1 was close to *A. ayderensis* AB04^{T}. The results obtained from both methods showed high compatiblity. It can be clearly seen that MATLAB computer program using UPGMA method may well be used as an alternative or additional method in bacterial classification, before 16S rRNA analysis.

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