

Phenotypic-Genotypic Characterization and Enzyme Activity of *Bacillus* Spp. Isolated from Saricay Stream

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Abstract

63 bacteria consist of *Bacillus* genus were isolated from water samples of Saricay stream (Canakkale). Isolates were characterized for phenotypic properties, antimicrobial and heavy metal resistance and evaluated for some extracellular enzyme activities. 13 bacterial colonies were selected according to their different phenotypic-genotypic characteristics and described by 16S rDNA sequence analysis methods.

There was a high incidence of resistance to Cefoxitin (CN30 µg/mL), while all of isolates sensitive against to Kanamycin (K30 µg/mL) and Chloramphenicol (C30 µg/mL). The trends in heavy metal resistance: Chromium (Cr) > (Zinc) Zn > Copper (Cu) > Manganese (Mn). Totally 55 protease, 49 amylase, 30 cellulase and 59 lipase activities were determined from all of isolated *Bacillus* sp. None of the isolates showed the esterase activity. As a result of molecular identification six isolates were showed the highest similarity to different strain of *Bacillus amyloliquefaciens* and others similar to *Bacillus* sp. B22(2008), *B. subtilis* SD-45, *Sporolactobacillus dextrus*, *Bacillus* sp. IHBB3375, *B. cereus* RJ1, Uncultured organism clone ELU0176-T465-S-NIPCRAMgANa_000604, Uncultured organism clone ELU0110-T273-S-NI_000292.

This study is first of its kind to explore the bacterial enzyme resource from Saricay stream. This work forms the foundation for future research to enzyme characterization and assessed

using in industrial fields explores the biotechnological potential of these *Bacillus* sp. isolates.

Keywords: *Bacillus* sp., Enzyme activity, 16s rDNA sequencing, Saricay stream

1. Introduction

In modern times, the products of biological origin, particularly enzymes, are attracting the attention of researchers. Their role in several biological and commercial processes has been duly emphasized. To meet the current increased demand, studies on the cost-effective production of industrially important enzymes have become necessary. Therefore, selection of the right organism plays a key role in high yield of desirable enzymes (Singh et al., 2010).

The potential contribution of marine and fresh water organisms to the discovery of new bioactive molecules is increasingly challenging (Parida et al., 2010). Bacterial organisms present in the fresh waters represent a potential source for enzyme production. *Bacillus* species have been major workhorse industrial microorganisms with roles in applied microbiology, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular medium, and the GRAS (generally regarded as safe) status with the Food and Drug Administration for species (Schallmeyer et al., 2004). These spore-forming bacteria represent also a major micro flora in many natural biotopes, where they play an important role in ecosystem development; they are able to transform many chemical compounds (Mohanty et al., 2011).

In this study, we performed an investigation for *Bacillus* strains isolated from Saricay stream that are biotechnologically interesting for industrial application, by isolating and identifying thirteen strains that produced have better extracellular enzymes activities, and identified them with 16S rDNA method.

2. Materials and Methods

2.1 Study Area

The Saricay stream is located in the southwest region of Marmara, latitude 39°, 40'-40° 45' N and longitude 25°, 37'-27° 45' E, in Canakkale, Turkey. This water resource is used for agriculture as on irrigation water and drinking water for animals (Hacioglu & Dulger, 2010).

2.2 Isolation of *Bacillus* sp. and Phenotypic Characterization

All water samples were collected from Saricay stream, kept at 4 °C and brought under cold chain to laboratory. For isolation of *Bacillus* sp. and to destroy all the vegetative microbial cells water samples were incubated for 10 min at 80 °C. Isolation and purification were done by dilution series method on nutrient agar (Oxoid) plates at 37 °C for 24 h. After incubation, the colonies with different morphological appearances selecting in order to phenotypic description and determine the enzyme activities (Topuz & Kiran, 2007). Isolates were stained with Gram staining for purification control and determination of morphological features.

For taxonomic identification, the isolates were subjected to series of biochemical tests as described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994), which included catalase, oxidase, indole, Voges-Proskauer, citrate utilization, H₂S production, motility, acid production from mannitol, sucrose, glucose, growth at different NaCl concentrations (2-5-7-10%), temperature (30-40-50 and 55 °C) and pH ranges (5 to 12). 13 bacterial colonies were selected according to their different morphological, physiological and biochemical characteristics.

2.3 Test of Antibiotic Sensitivity

The inhibition of *Bacillus* strains by various antibiotics was tested by standard disc diffusion technique (Bauer et al., 1966). The following antibiotics were used; Kanamycin (K30 µg/mL), Oxytetracycline (O30 µg/mL), Cefmetazole (CMZ30 µg/mL), Furazolidone (FR50 µg/mL), Erythromycin (E15 µg/mL), Cefoxitin (CN30 µg/mL), Ampicillin (A10 µg/mL), Cefotaxime (CE30 µg/mL) and Chloramphenicol (C30 µg/mL) (CLSI, 2009).

2.4 Determination of the Minimal Inhibitory Concentration (MIC) of Heavy Metals

MIC for each bacterial isolate for four heavy metals was determined using Mueller – Hinton agar (Merck) containing Zn^{+2} , Cu^{+2} , Cr^{+3} , and Mn^{+2} at concentrations ranging from 12.5 µg/mL to 3200 µg/mL. The metals were added as $ZnSO_4$, $CuSO_4 \cdot 5H_2O$, $K_2Cr_2O_7$ and $MnCl_2 \cdot 2H_2O$ (Merck). The isolates were considered resistant if the MIC values exceeded that of the *E. coli* K-12 strain which was used as the control (Matyar et al., 2008).

2.5 Determination of Enzyme Activity of Isolates

All of the isolates were screened for their protease, amylase, lipase, cellulase and esterase activities qualitatively on agar plates. Protease, amylolytic, lipase, esterase, cellulose activity were tested on Skim Milk Agar, M9-starch Agar, Tributyrin Agar, Tween 80 Medium, CMC agar, incubated at 37 °C for 48 h, respectively (Topuz & Kiran, 2007; Ertugrul et al., 2007; Prabhavathy et al., 2013).

2.6 Genomic DNA Isolation, 16S rDNA Gene Amplification and Sequencing

Genomic DNA was extracted from the cultures growing on nutrient agar for 18 h at 37 °C by using genomic DNA purification kit (Fermentas). The 16S rDNA gene was amplified by using the bacterial universal primers 27 F (5'-AGA GTT TGA TCC TGG CTC AG-3') -1492 R (5'-GGT TAC CTT GTT ACG ACT T-3') as described previously [13]. PCR was carried out by Thermocycler (Techne, TC-512) in 0.2 µL reaction tubes with 50 µL final mixture volume. Each reaction mixture contained 20 pmol/µL concentration of F and R primers, 2 mM dNTP mix (Fermentas), 10 µL of 10 × PCR buffer, 25 mM $MgCl_2$, 5U/µL Taq DNA polymerase (Fermentas) and 3 µL of genomic DNA. Amplification was performed according to the following program: preheating for 5 min at 94 °C; 30 cycles of denaturation for 1 min at 94 °C; annealing for 1min at 54 °C; extension for 1 min at 72 °C and a final terminal extension for 10 min at 72 °C (Deng et al., 2010). PCR products were examined by 1% agarose gel electrophoresis in 1 × TAE buffer at 100 V. 1 kb DNA ladder (Fermentas) was used as a molecular size standard.

2.7 Phylogenetic Analysis

The sequences of 16S rDNA genes were compared with those from the GenBank database using the NCBI Blast program. Sequences were aligned using the ClustalW program. Phylogenetic analyses were performed using the maximum-likelihood and neighbour-joining methods with bootstrap values based on 1000 replications. The phylogenetic tree (Saitou & Nei, 1987) was constructed with the MEGA package version 4 (Tamura et al., 2007). As the phylogenetic analyses derived from neighbour-joining method were in congruence with those obtained using the maximum-likelihood algorithm, only the data obtained from the neighbour-joining method is presented in the study.

3. Results

In this study, sixty three bacteria were isolated from Saricay stream and some biochemical tests, pH, NaCl and temperature tolerance tests were performed for the determination of phenotypic characteristics of all isolates. All the strains were found to be Gram, catalase, motility, and oxidase positive, H₂S negative spore-forming *Bacillus* sp. rods. The isolates revealed variation in citrate (57%) and VP (25%) tests, indole utilization (9.5%) and acid production from mannitol (17%), sucrose (52%), and glucose (92%). The physiological studies showed the growth of all of isolates at 30-55 °C. 36 isolates tolerate to 2-10% and 27 isolates tolerate to 2-7% NaCl concentration; 56 isolates growth at pH 5-10, 7 isolates growth at pH 8-11. Optimum growth temperature of all isolated bacteria was determined at 37 °C. Moreover all isolates were divided into 13 different groups according to phenotypic characteristics.

3.1 Antimicrobial resistance of isolated bacteria

The highest rates of resistance were against CN30 was the most commonly reported antimicrobial agent (85.71%), followed by CMZ30 (15.87%), O30 (14.28%). Also resistant to other antibiotics observed in varying proportions. But all isolates sensitivity only two antibiotics - C30 and K30 - except B61, B36, respectively. However B7, B24, B35, B38, B43, B60 isolates have sensitive against all studied antibiotics (Table 1).

Table 1. Antibiotic resistance profile of isolated bacteria

Code	Antibiotics								
	CN30 (n=54)	O30 (n=9)	FR50 (n=6)	A10 (n=8)	CMZ30 (n=10)	E15 (n=7)	CE30 (n=5)	C30 (n=1)	K30 (n=1)
B1	+	+	-	-	-	-	-	-	-
B2	+	-	-	-	-	-	-	-	-
B3	+	-	-	-	-	-	-	-	-
B4	+	-	-	-	-	-	-	-	-
B5	+	-	-	-	-	-	-	-	-
B6	+	+	-	-	-	-	-	-	-
B7	-	-	-	-	-	-	-	-	-
B8	+	-	+	-	-	+	-	-	-
B9	+	-	-	-	-	-	-	-	-
B10	+	-	-	-	-	-	-	-	-
B11	+	+	-	-	-	-	-	-	-
B12	+	-	-	-	-	-	-	-	-
B13	+	-	-	-	-	-	-	-	-
B14	+	-	-	-	-	-	-	-	-
B15	+	-	-	-	+	+	-	-	-
B16	+	-	-	-	-	-	-	-	-
B17	+	+	+	-	-	+	-	-	-
B18	+	+	-	-	-	-	-	-	-
B19	+	-	-	-	-	-	-	-	-
B20	+	-	-	-	-	-	+	-	-

B21	+	-	-	+	+	-	+	-	-
B22	+	-	-	-	-	-	-	-	-
B23	+	-	+	-	-	+	-	-	-
B24	-	-	-	-	-	-	-	-	-

Table 1 (continued)

Code	Antibiotics								
	CN30 (n=54)	O30 (n=9)	FR50 (n=6)	A10 (n=8)	CMZ30 (n=10)	E15 (n=7)	CE30 (n=5)	C30 (n=1)	K30 (n=1)
B25	+	-	-	-	-	-	-	-	-
B26	+	+	-	-	-	-	-	-	-
B27	+	+	-	-	-	-	-	-	-
B28	+	-	-	-	-	-	-	-	-
B29	+	-	-	-	-	+	-	-	-
B30	+	-	+	-	-	-	-	-	-
B31	+	+	-	-	-	-	-	-	-
B32	+	-	-	-	-	-	-	-	-
B33	+	-	-	-	-	-	-	-	-
B34	+	-	-	-	-	+	-	-	-
B35	-	-	-	-	-	-	-	-	-
B36	+	-	-	+	-	-	-	-	+
B37	+	-	-	-	-	-	-	-	-
B38	-	-	-	-	-	-	-	-	-
B39	+	+	-	-	-	-	-	-	-
B40	+	-	-	-	-	-	-	-	-
B41	+	-	-	-	+	-	-	-	-
B42	+	-	-	-	-	-	-	-	-
B43	-	-	-	-	-	-	-	-	-
B44	+	-	-	-	-	-	-	-	-
B45	+	-	-	-	-	-	-	-	-
B46	+	-	-	-	-	-	-	-	-
B47	+	-	-	-	-	-	-	-	-
B48	+	-	-	-	-	-	-	-	-
B49	+	-	-	-	-	-	-	-	-
B50	+	-	-	-	-	-	-	-	-
B51	+	-	-	+	+	-	-	-	-
B52	+	-	+	+	+	-	-	-	-
B53	+	-	-	-	-	-	-	-	-
B54	+	-	+	+	+	-	+	-	-
B55	+	-	-	-	+	-	-	-	-

Table 1 (continued)

Code	Antibiotics								
	CN30 (n=54)	O30 (n=9)	FR50 (n=6)	A10 (n=8)	CMZ30 (n=10)	E15 (n=7)	CE30 (n=5)	C30 (n=1)	K30 (n=1)

B56	+	-	-	+	+	-	+	-	-
B57	+	-	-	+	+	-	-	-	-
B58	+	-	-	+	+	-	+	-	-
B59	+	-	-	-	-	-	-	-	-
B60	-	-	-	-	-	-	-	-	-
B61	-	-	-	-	-	+	-	+	-
B62	+	-	-	-	-	-	-	-	-
B63	+	-	-	-	-	-	-	-	-

3.2 Heavy Metal Resistance

In the present study, resistance to four heavy metals (Mn^{+2} , Cr^{+3} , Zn^{+2} and Cu^{+2}) was investigated for all isolates. The trends in heavy metal resistance are shown in Table 2; Cr (100.00%) > Zn (57.14%) > Cu (55.55%) > Mn (28.57%) (Table 2).

Table 2. Heavy metal tolerance of isolated bacteria

Heavy metal	Mn > 1600 (n = 18) 28.57%	Cr > 100 (n = 63) 100%	Zn > 400 (n = 36) 57.14%	Cu > 400 (n = 35) 55.55%
Code of resistant bacteria	B3,B4,B5,B6,B10,B11,B13, B14,B15,B18, B24,B26,B2,7B28,B30,B33, B37, B56	B1-B63 (All isolated bacteria)	B1,B2,B3,B4,B5,B6 B7,B9,B10,B11,B17, B18, B19,B20,B21, B22, B26,B27,B28, B30,B31, B33,B35, B36,B43,B44,B45, B46,B48,B49, B51, B52,B53,B54,B55, B56,B57,B58, B60, B61	B1,B2,B3,B4,B5,B6,B7,B10,B11, B12, B13,B15,B16,B18,B19, B20,B21, B27, B28,B29, B31, B33, B37, B38, B43,B44,B46,B47, B50, B53,B54,B56B59, B60, B63

3.3 Enzyme Capacity of Isolated Bacteria

Extracellular enzyme activities of isolated bacteria were given in Table 3. Isolates showed protease (n=55; 87%), amylase (n=49; 78%), cellulose (n=30; 48%) and lipase (n=59; 94%) activity. But esterase enzyme potential is determined to be insufficient.

3.4 16S rDNA Sequencing and Analysis

According to phenotypic characteristics, isolates were divided into thirteen groups and molecular identification of selected isolates from each group was carried out. Molecular data and comparative phylogenetic tree of isolates were given in Table 4 and Figure 1, respectively.

Table 3. Extracellular enzyme activity of isolated bacteria

Code	Enzyme					Code	Enzyme				
	Protease	Amylase	Cellulase	Esterase	Lipase		Protease	Amylase	Cellulase	Esterase	Lipase
B1	+	+	-	-	+	B33	+	+	+	-	+
B2	+	+	-	-	+	B34	-	-	-	-	-
B3	+	+	-	-	+	B35	+	+	-	-	+
B4	+	+	+	-	+	B36	+	+	+	-	+
B5	+	+	-	-	+	B37	+	+	-	-	+
B6	+	+	-	-	+	B38	+	+	-	-	+
B7	+	+	+	-	+	B39	+	+	-	-	+
B8	-	-	-	-	-	B40	+	+	+	-	+
B9	-	+	-	-	+	B41	+	-	-	-	+
B10	+	-	+	-	+	B42	+	+	-	-	+
B11	+	+	+	-	+	B43	+	+	-	-	+
B12	+	+	-	-	+	B44	+	+	+	-	+
B13	+	+	+	-	+	B45	+	+	+	-	+
B14	+	+	-	-	-	B46	+	+	+	-	+
B15	+	-	+	-	+	B47	+	+	+	-	+
B16	+	+	+	-	+	B48	+	+	+	-	+
B17	+	+	-	-	+	B49	+	+	+	-	+
B18	+	+	+	-	+	B50	+	+	+	-	+
B19	+	-	-	-	+	B51	+	-	+	-	+
B20	+	-	-	-	+	B52	+	-	+	-	+
B21	+	+	-	-	+	B53	+	+	+	-	+
B22	+	-	-	-	+	B54	+	+	+	-	+
B23	+	-	-	-	+	B55	+	-	+	-	+
B24	+	+	+	-	+	B56	-	+	+	-	+
B25	+	-	+	-	+	B57	+	+	+	-	+
B26	+	+	-	-	+	B58	+	+	+	-	+
B27	+	+	-	-	+	B59	+	+	-	-	+
B28	+	+	-	-	+	B60	-	+	-	-	+
B29	+	+	-	-	+	B61	-	-	-	-	-
B30	+	+	+	-	+	B62	-	+	-	-	+
B31	+	+	-	-	+	B63	-	+	-	-	+
B32	+	+	+	-	+						

Table 4. The 16S rDNA gene sequence similarities of the 13 Bacillus isolates to their closest relative(s)

Bacterial Code	Base Number	Reference database Their similar species	from NCBI Name of Strain	Similarity Percentage (%)
B1	1434	<i>Bacillus amyloliquefaciens</i>	subsp. <i>plantarum</i> UCMB5113	100

B3	1511	<i>Bacillus</i> sp.	B22 (2008)	99
B4	1471	<i>Bacillus subtilis</i>	SD-45	99
B6	952	<i>Sporolactobacillus dextrus</i>	EH21	94
B8	1472	<i>Bacillus amyloliquefaciens</i>	Aec23	99
B9	887	<i>Bacillus amyloliquefaciens</i>	Zy25	99
B15	1469	<i>Bacillus amyloliquefaciens</i>	WY13	99
B19	935	<i>Bacillus</i> sp.	IHB B 3375	99
B23	1431	<i>Bacillus amyloliquefaciens</i>	Aec23	96
B41	1446	Uncultured organism clone	ELU0176-T465-S-NIPCRAMgANa_000604	99
B46	1512	<i>Bacillus amyloliquefaciens</i>	subsp. <i>plantarum</i> UCMB5113	97
B48	1456	Uncultured organism clone	ELU0110-T273-S-NI_000292	99
B54	1447	<i>Bacillus cereus</i>	RJ1	100

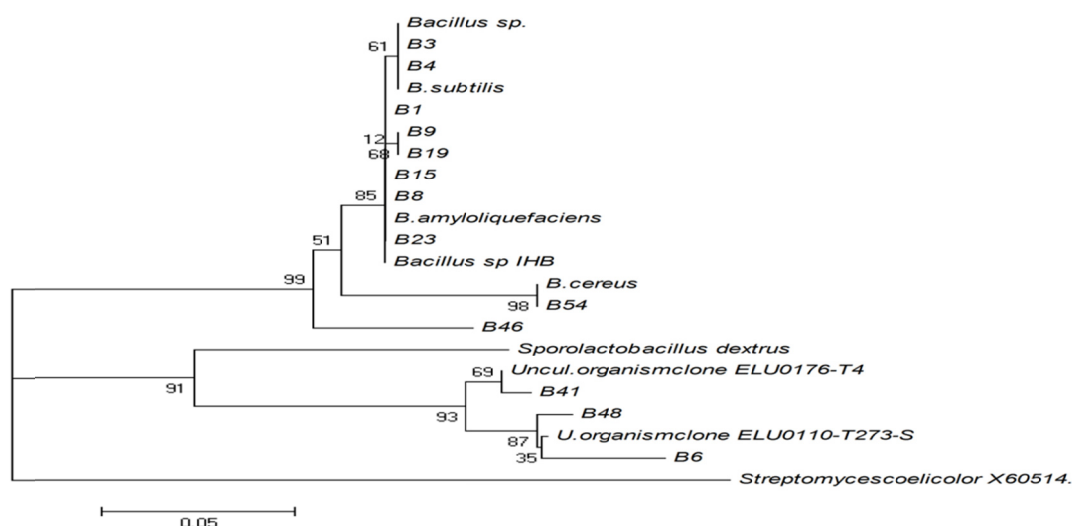


Figure 1. Phylogenetic tree showing the relative positions of identified isolates by the neighbor-joining method of partial 16S rDNA sequences

4. Discussion

This study on identification of *Bacillus* sp. isolated from Saricay stream (Canakkale) by biochemical tests, antimicrobial and heavy metal analysis and characterization of enzyme activity of isolated bacteria. Members of this genus are attractive industrial organisms in terms of important antibiotic and enzyme resources as well as their high growth rates and short fermentation cycles. Therefore, investigations concerning *Bacillus* sp. identification and biological products are very common (Singh et al., 2010; Schallmeyer et al., 2004; Ertugrul et al., 2007; Prabhavathy et al., 2013). On the contrary, research on freshwater *Bacillus* diversity and industrial potential are very limited (Mohanty et al., 2011). In addition to, although there were so many researches about microbial quality of Saricay stream (Hacioglu & Dulger, 2010), this report will be first investigation on the bacterial diversity and their potential enzymatic activity.

In our study obtained phenotypic traits showed that 7 out of 63 isolates were found to be alkaliphilic and others alkalotolerant *Bacillus* sp. All of the isolates also were determined as the mesophilic halo tolerant bacteria which is tolerant to different temperatures. In addition, all bacteria were divided into different 13 groups by the phenotypic properties of bacteria and 16S rDNA sequences of a bacterium from each group are analyzed for species identification. As a result of molecular identification six isolates were showed the highest similarity to different strain of *B. amyloliquefaciens* and others similar to *Bacillus* sp. B22(2008), *B. subtilis* SD-45, *Sporolactobacillus dextrus*, *Bacillus* sp. IHBB3375, *B. cereus* RJ1, Uncultured organism clone ELU0176-T465-S-NIPCRAMgANa_000604, Uncultured organism clone ELU0110-T273-S-NI_000292.

Because of the increasing enzyme technology and the variety usage of the products, industrial enzymes branch of biotechnology are gaining more and more importance. Therefore, quantitatively presence of industrially important protease, amylase, cellulase, lipase enzyme activity of isolated bacteria has been demonstrated. However, esterase enzyme potential of the isolates was found to be inadequate. It was determined that some of isolates also may be indicative more than one enzyme activity. Thus, researchers expressed that there are more than ten enzyme widely used in industry which is synthesized by *Bacillus* species and subspecies (Kumar & Takagi, 1999). Our findings have a correlation of these reports.

When the antibiotic resistance of strains examined it has revealed that all strains have the high degree of resistance to different group antibiotics. There was a high incidence of resistance to CN30, while all of isolates sensitive against to K30 and C30 (except B36, B61, respectively). Antibiotics, for which the isolate is resistant, may be supplemented to fermentation medium during enzyme production process so as to check the contamination by other sensitive isolates (Singh et al., 2010). Antibiotic CN30 could be used for this purpose in enzyme production process from these isolated strains.

The isolated organisms also exhibited a high degree of tolerance to elevated concentrations ($\mu\text{g/mL}$) of Cr (100), Zn (400) and Cu (400). In leather industries, this Cr tolerate isolates may be useful for dehairing process as the use of chromium is very common in the tanning process. Further, the protease producing organisms displaying heavy metal tolerance may be of potential use for the treatment of multi metal contaminated sludge generated during wastewater treatment (Singh et al., 2010). This case reveals that strains which has only resistance to antibiotics but also heavy metal resistant would be an important source of enzymes for many industrial processes.

It can be concluded that all isolated bacterial species are determined that they belong to the *Bacillus* genus with phenotypic and phylogenetic characteristics. The result is encouraging as bacteria with useful enzyme production potential are found in the fresh water of Saricay stream. The present study is a preliminary screening report of diversity of *Bacillus* species and their enzyme producing potential from Saricay stream. *B. amyloliquefaciens* can be expressed as a dominant species. Also in this study have not previously been cultured two *Bacillus* sp. species were isolated and cultured.

Isolation of *Bacillus* sp. and other industrial microorganisms from fresh water environment would also provide ample scope to assess their biotechnological potential (production of biotechnologically valuable compounds such as enzymes and antimicrobial substances).

Attempt should be made for proper evaluation and exploration of these microbes for the biotechnological applications.

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