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Isolation and Molecular Characterization of *Bacillus* spp. Isolated from Soil for Production of Industrial Enzymes

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Abstract: The main objective of our study was to investigate the morphologic and phenotypic properties, antibiotic resistance, random amplified polymorphic DNA (RAPD), enterobacterial repetitive intragenic consensus (ERIC) and Single-stranded conformation polymorphism (SSCP) profiles of the *Bacillus* spp. isolated from soil of Sinop environs. The aim of the investigation was also to check their ability for protease, amylase and cellulose enzymes production. A total of thirty-nine isolates were identified as *Bacillus* spp. based on morphological and physiological properties. The isolates were resistant on the percentage of 100% to penicillin; 66.6% to rifampicine; 23.7% to novobiocin; 48.7% to cefepime; 87.1% to ceftazidime; 89.7% to oxacillin; 2.5% to streptomycin; 30.7% to clindamycin; 7.6% to tetracycline; 12.8% to ampicillin/sulbactam; 10.2% to gentamicin; 56.4% to ceftriaxone; 84.6% to polymyxin B and 12.8% to amikacin were determined. In addition, all isolates were susceptible to imipenem, ciprofloxacin, meropenem and ofloxacin. All of the isolates produced at least two of the three enzymes, and 53.8% of strains were able to produce all three enzymes. The dendrograms generated by using M13-10, OPL3 RAPD primers, ERIC primers and P11/P13 SSCP primers revealed that the strains were separated to two major groups at similarity levels of 5%, 2%, 38% and 15% or above, respectively. As a result, SSCP, ERIC and RAPD analysis revealed 100% polymorphism indicating the strength of these methods in the differentiation of *Bacillus* isolates. In addition, it appears that *Bacillus* strains associated with soil are the source of industrial enzymes and may play an important role in enzyme catalyzed organic matter cycling in soil environments.

Keywords: *Bacillus* spp., SSCP, RAPD, ERIC-PCR, Bacterial enzymes.

1. Introduction

The genus *Bacillus* is a large and heterogeneous group of Gram-positive, rod-shaped, aerobic and facultative anaerobic, and endospore forming bacteria (Slepecky and Hemphill, 1992). *Bacillus* isolates have variety of roles in ecology, biotechnology, industry as well as in clinical microbiology. However, some *Bacillus* species shows same morphological and biochemical properties due to this it is still very difficult to characterize new strains, which makes it hard to separate them. Thus, increasingly molecular methods are used for quick species or strains identification (Kumar *et al*., 2014). Traditional microbiological classification is rooted in organisms' morphology, physiology and biochemical properties and first requires that species are isolated by growth in vitro. On the other hand, 16S ribosomal RNA (rRNA) gene sequencing is a popular alternative to traditional methods and provides several advantages (Salipante *et al*., 2013). However, 16S rRNA sequencing is time-consuming and expensive. Also, the 16S rRNA gene does not discriminate sufficiently at the interspecies level (Mauchline *et al*., 2011). To overcome this problem,

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researchers evaluated the use of PCR for discrimination of the subspecies using various forms primers specific for random amplified polymorphic DNA (RAPD), repetitive extragenic palindromic sequences (REP) and enterobacterial repetitive intragenic consensus (ERIC) sequences (Johansson *et al*., 2000). In addition, Single-stranded conformation polymorphism (SSCP) offers a rapid, simple and inexpensive method for detecting whether or not DNA fragment are identical in sequence, and so can greatly reduce the amount of sequencing necessary (Omri *et al*., 2014). In conventional SSCP, the amplified PCR product was denatured to two single-stranded DNAs (ssDNAs) and subjected to non-denaturing polyacrylamide gel electrophoresis. Under non-denaturing conditions, ssDNA has a secondary structure that could be determined by the nucleotide sequence. The different positions of the bands of ssDNA on the gel indicate different sequences (Widjojoatmodja *et al*., 1995; Srinivasa *et al*., 2012). A number of studies found that the SSCP analysis allow discrimination between bacterial species and strains (Widjojoatmodja *et al*., 1994; Olivares-Fuster *et al*., 2007; Kanlisch *et al*., 2010; Lesnik *et al*., 2015).

Enzymes such as proteases, celluases and amylases are widely used in the industry. The majority of the enzymes used in the industry are of microbial origin because microbial enzymes are relatively more stable than the corresponding enzymes derived from plants and animals (Mohapatra *et al*., 2003). The easily decomposable proteins, starches and sugars are quick for bacteria. Most of the microbes living in the soil can secrete the enzymes needed to break up these simple chemical compounds (Bot and Benites, 2005). Hereby, bacteria are capable of producing enzymes that play an important role in organic matter decomposition and nutrient cycling. Especially, the capacity of selected Bacillus strains to produce and secrete large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers (Schallmey *et al*., 2004).

The aim of this study was to evaluate whether Sinop soil may be potential reservoir of protease, amylase and cellulase enzymes producing *Bacillus* strains. Beside this, we investigated (*i*) the morphologic and biochemical properties of the *Bacillus* spp. isolated from soil environs; (*ii*) distribution of these isolates, in terms of their genetic diversity; and (*iii*) antibiotic resistance of these strains.

2. Material and Methods

Isolation of *Bacillus* spp. from Soil

Twenty-seven soil samples from different towns and villages of Sinop peninsula Turkey were collected and 39 bacteria were isolated. The samples were collected at depth of 5-10 cm below the soil surface. The samples were transported in cooling bags until processing at the laboratory. One gram of each soil sample was homogenized in 9 mL of 0.85% NaCl in sterile test tubes. A 0.1 ml aliquot of the dilution was spread aseptically on Nutrient Agar (NA - Merck) medium and incubated at 30 ^{o}C for 24 hour. After incubation, representative colonies were selected on the basis of distinct morphological characteristics. The selected bacteria are identified by their morphological, physiological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Brenner *et al*., 2005).

Enzyme tests

(i) Protease

The screened isolates were spot inoculated on skim milk agar. After that the plates were incubated at 30^{o}C for 24-96 h. The isolate showing clear zone around the colony was considered as enzyme producer and measured in millimeter (mm) as the difference between the diameter of the halo and colony.

(ii) Cellulase

The screened bacteria were grown on carboxymethyl cellulose (CMC) agar medium. The CMC agar plates were incubated at 37^{o}C for 5 days to allow for the secretion of cellulase. After the incubation, the plate was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 min. The formation of a clear zone of hydrolysis indicated cellulose degradation (Ariffin *et al*., 2006), and measured in millimeter (mm) as the difference between the diameter of the halo and colony.

(iii) Amylase

The isolated strains were observed for amylase production on M9 synthetic medium composed of Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), NH_4Cl (1 g), $MgSO_4$ (240 mg), $CaCl_2$ (10 mg), peptone 3 g, agar 15 g and 1% (w/v) soluble starch (Merck) per 1 liter of distilled water. The pH was adjusted to 7.4 with 10% Na_2CO_3. The fresh bacteria cell (10^6 – 10^9 per plate) inoculated on M9 medium and then incubated at 37^{o}C for 4 or 5 days, and subsequently visualizing starch degradation halos by staining with iodine vapors (Shibuya *et al*., 1986; Hols *et al*., 1994), and measured in millimeter (mm) as the difference between the diameter of the halo and colony.

Antibiotic Susceptibility Testing

Antibiotic susceptibility of all isolates was performed by Kirby-Bauer (1966) disc diffusion technique on Mueller-Hinton agar using 18 antibiotic discs. A panel of antimicrobial agents comprising 10 classes of antibiotics were tested (Table 1). Interpretations of the response of isolates to antibiotics were susceptible, intermediate and resistant was based on the standards of the Clinical and Laboratory Standards Institute (CLSI, 2010). *Staphylococcus aureus* ATCC 25923 was included as a control.

Table 1: Antibiotic agents used in experiments

Antibiotic Classes	**Antibiotic (µg)**
Tetracycline	Tetracycline (30)
Carbapenems	Imipenem (10) Meropenem (10)
Cephalosporins	Ceftazidime (30) Cefepime (30) Ceftriaxone (30)
β-Lactamases	Ampicillin/Sulbactam (20) Penicillin G (10) Oxacillin (1)
Aminoglycosides	Streptomycin (10) Gentamicin (10) Amikacine (30)
Glycopeptides	Novobiocin (5)
Macrolides	Erythromycin (15)

Miscellaneous	Rifampicin (5)
Lincosamide	Clindamycin (2)
Fluoroquinolones	Ciprofloxacin (5) Ofloxacin (5)

AK: Amikacine, CAZ: Ceftazidime, CIP: Ciprofloxacin, CN: Gentamicin, CRO: Ceftriaxone, DA: Clindamycin, FEP: Cefepime, IPM: Imipenem, NV: Novobiocin, OX: Oxacillin, P: Penicillin G, PB: Polymyxin B, RD: Rifampicin, S: Streptomycin, SAM: Ampicillin / Sulbactam, TE: Tetracycline, MEM: Meropenem, OFX: Ofloxacin

Genomic DNA Isolation, RAPD and ERIC PCR Analyses

Thirty-nine *Bacillus* isolates were activated with incubation at 37^{o}C for 24 h. Recovered bacteria were centrifuged at 3,000 rpm for 5 min and cell pellets were resuspended in 500 µLTE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Pellets were then incubated at 55^{o}C for 30 min following the addition of 50 µl SDS (10%) and 25 µl proteinase K (20 mg/ml). Total DNA was recovered by sequential extractions with phenol and chloroform. Initially, 575 µl phenol/chloroform (1:1) was added, tubes were inverted and centrifuged at 14.000 rpm for 10 min. The aqueous upper layer (500 µl) was carefully transferred into a fresh tube and again treated with 500 µl phenol/chloroform (1:1). Tubes were inverted a few times and incubated on ice for a few minutes, then centrifuged at 14.000 rpm for 10 min. After centrifugation, upper layer (500 µl volume) was transferred into a new eppendorf tube and treated with 50 µl sodium acetate (3M, pH: 5.2) and 330 µl isopropanol (100%). Tubes were gently inverted and DNA was precipitated by centrifugation at 14.000 rpm for 10 min followed by washing with 70% (v/v) ethanol, dried, resuspended with 100 µl TE buffer containing 2 µl RNase (Sambrook *et al*., 1989). DNA samples were stored at -20^{o}C until further used for the PCR analyses.

RAPD and ERIC PCR analyses were performed using M13-10: 5'CCGCAGCCAA3', OPL-3: 5'CCAGCAGCTT3', ERIC-1: 5'CATTTAGGGGTCCTCGAATGTA3' and ERIC-2: 5'AAGTAAGTGACTGGGGTGAGCG3' oligonucleotide primers**.** PCR was carried out in a reaction volume of 25 µl containing 10X PCR buffer, 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphates (dNTPs), primer (100 pmol for RAPD, 50 pmol for ERIC), 1.25 U of Taq DNA polymerase (Thermo Scientific, USA) and genomic DNA (3 µl for RAPD and 2 µl for ERIC). Techne TC-5000 thermal cycler (California, USA) was used for all amplification reactions. Amplification conditions for RAPD included an initial denaturation step at 94^{o}C for 3 min, 34^{o}C for 1 min and 72^{o}C for 1 min during one cycle. This initial denaturation step was followed by denaturation at 94^{o}C for 30 sec, primer annealing at 34^{o}C (34^{o}C for OPL-3 and 54^{o}C for M13-10) for 30 sec, extension at 72^{o}C for 30 sec during 30 cycles and final extension at 72^{o}C for 5 min. For ERIC-PCR analysis (Versalovic *et al*. 1991), PCR conditions were as follows: 94^{o}C for 1 min, followed by 35 cycles at 94^{o}C for 45 second, 52^{o}C for 45 sec 72^{o}C for 2 min, and final extension at 72^{o}C for 10 min. PCR completion reaction mixtures were stored at 4^{o}C until electrophoresis was performed. PCR products were analyzed by agarose gel (1% for RAPD and 2% for ERIC) (Sigma-Aldrich, St. Louis, MO, USA) electrophoresis with a molecular size marker (O' Gene Ruler, 1 kb DNA Ladder, ready-to-use, Thermo Scientific, USA). The gels were stained with ethidium bromide and DNA bands were visualized under UV light.

16S rRNA-SSCP analysis

SSCP analysis was carried out essentially according to Widjojoatmodja *et al*. (1994). Primers P11P (5'-GAG GAA GGT GGG GAT GAC GT-3') and P13P (5'-AGG CCC GGG AAC GTA TTC AC-3') were used at concentrations of 0.2 µM to amplify a 216 bp fragment of the V6 region of the 16S rRNA. Amplification conditions for SSCP included an initial denaturation step at 95^{o}C for 4 min. This initial denaturation step was followed by denaturation at 95^{o}C for 30 sec, primer annealing at 55^{o}C for 30 sec, extension at 72^{o}C for 30 sec during 30 cycles and final extension at 72^{o}C for 5 min. Two microliter of PCR products were added to 10 µl of denaturation solution (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 10 mM NaOH). Mixture was heated at 95^{o}C for 5 minutes and immediately cooled on ice. The mixture was analyzed by non-denaturing polyacrylamide gel-electrophoresis (39:1 acrylamide:bisacrylamide) (16 x 18 cm) in 0.5 x TBE in a Hoefer electrophoresis system (Hoefer Inc. Holliston, MA, USA) at constant power of 5 mA, 18^{o}C for 27 h (Hongyo *et al*., 1993). The gels were silver stained according to the procedure of Byun *et al*. (2009) and dried at room temperature.

Statistical Analysis

The gels were visualized under UV transilluminator (Cleaver-MicroDOC, UK) and the photographs were documented. The molecular weight of each band was calculated with Total Lab 1D Manual R11.1, UK programme. Following installation of the gel images into the programme, the bands were determined with their pixel positions and their molecular weights were scored according to the molecular size marker. The data collected were further subjected to cluster analysis by using unweighted pair group method using arithmetic averages (UPGMA) based on Jaccard's similarity coefficient with the Phoretix 1D-Pro (Total lab, UK) programme and dendrograms were generated.

3. Results and Discussion

A total of 39 bacterial isolates were obtained from soil samples collected from Sinop, Turkey. The bacterial isolates were screened for their protease, amylase and cellulase activities. Also, the isolates were characterized by morphological, biochemical tests and genetic analyses. Moreover, the isolates were performed antibiotic susceptibility test by disc diffusion method. The present findings show that the isolates were Gram-positive, rod-shaped, motile and catalase-positive. The results of other biochemical and physiological tests were found to be variable. According to these tests, it was confirmed that 39 isolates belonged to the genus *Bacillus* (Tablo 2 and 3).

In the present study, altogether 39 isolates were tested for their ability to produce protease, amylase and cellulase. Twenty-five (64.1%), thirty-five (89.7%) and thirty-nine (100%) isolates were found positive for protease, amylase and cellulase production, respectively. The isolates produced at least two of the three enzymes, and 53.8% of strains were able to produce all three enzymes. Enzyme activities produced by the isolates ranged from 0 to 8 mm for protease, 0 to 8 mm for amylase and 2 to 10 mm for cellulase. Isolate 30 for protease, isolate 25 for amylase and isolates 36 and 37 for cellulase were form the highest clear zone than other isolates as indicate in Figure 1 and Table 2.

Tablo 2: Fermentation, biochemical and enzyme activities of *Bacillus* isolates

Strains	Galactose	Lactose	Mannose	Fructose	Arabinose	Sucrose	Trehalose	Maltose	Xylose	Catalase	Voges-P.	Nitrate	Gelatinas	Citrate	Protease	Amylase	Cellulase
1	-	-	-	-	-	-	+	+	-	+	+	+	+	-	7 mm	2 mm	2 mm
2	-	-	-	-	-	+	+	+	-	+	+	+	+	-	7 mm	2 mm	2 mm
3	+	-	+	-	+	+	+	+	+	+	+	+	-	-	-	2 mm	2 mm
4	-	-	-	-	-	+	+	+	-	+	+	+	+	-	3 mm	2 mm	5 mm
5	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	2 mm	2 mm
6	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	2 mm	2 mm
7	-	-	-	+	-	-	+	+	-	+	+	+	+	-	5 mm	3 mm	3 mm
8	-	-	-	-	-	-	+	+	-	+	+	+	+	-	3 mm	2 mm	2 mm
9	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	2 mm	3 mm
10	-	-	-	-	-	-	+	+	-	+	+	+	+	-	-	3 mm	2 mm
11	-	-	-	-	-	+	+	+	-	+	+	+	+	-	4 mm	2 mm	2 mm
12	-	-	-	+	-	+	+	+	-	+	+	+	+	-	3 mm	2 mm	2 mm
13	+	-	+	-	+	+	+	+	+	+	+	+	+	-	-	3 mm	2 mm
14	-	-	-	-	-	+	+	+	-	+	+	+	+	-	-	3 mm	2 mm
15	+	-	+	-	+	+	+	+	+	+	+	+	+	-	7 mm	2 mm	3 mm
16	-	-	-	-	-	-	+	+	-	+	+	+	+	-	-	3 mm	2 mm
17	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	2 mm	2 mm
18	-	-	-	-	-	-	+	+	-	+	+	+	+	-	-	2 mm	3 mm
19	-	-	-	-	-	+	+	+	-	+	+	+	-	-	-	2 mm	2 mm
20	-	-	-	-	-	-	+	+	-	+	+	+	+	-	-	2 mm	4 mm
21	-	-	-	-	-	-	+	-	-	+	+	+	+	-	7 mm	3 mm	3 mm
22	+	-	+	-	-	+	+	+	-	+	+	+	+	-	2 mm	-	2 mm
23	+	-	-	+	-	-	-	+	-	+	+	+	+	-	2 mm	2 mm	8 mm
24	-	-	-	-	-	+	+	-	-	+	+	+	+	-	2 mm	-	2 mm
25	-	-	-	-	-	+	+	+	-	+	+	+	+	-	3 mm	8 mm	2 mm
26	-	-	-	-	-	+	+	+	-	+	+	+	+	+	3 mm	5 mm	3 mm
27	-	-	-	-	-	+	+	+	-	+	+	+	+	-	6 mm	2 mm	2 mm
28	-	-	-	+	-	+	+	+	-	+	+	+	+	-	3 mm	2 mm	9 mm
29	-	-	-	-	-	+	+	+	-	+	+	+	-	-	3 mm	2 mm	9 mm
30	-	-	-	+	-	+	+	+	-	+	+	+	+	+	8 mm	-	2 mm
31	-	-	-	-	-	-	-	+	-	+	-	+	+	-	3 mm	-	2 mm
32	-	-	-	-	-	-	+	+	-	+	+	+	+	-	6 mm	2 mm	2 mm
33	-	-	-	-	-	+	+	+	-	+	+	+	+	-	5 mm	2 mm	2 mm
34	-	-	-	+	-	+	+	+	-	+	+	+	+	-	-	2 mm	2 mm
35	-	-	-	-	-	+	+	+	-	+	+	+	+	-	2 mm	4 mm	3 mm
36	-	-	-	-	-	-	+	+	-	+	+	+	+	-	2 mm	3 mm	10 mm
37	-	-	+	+	-	+	+	-	-	+	+	+	+	-	3 mm	2 mm	10 mm
38	-	-	-	+	-	+	+	+	-	+	+	+	+	-	-	2 mm	7 mm
39	-	-	-	-	-	+	+		-	+	+	+	+	-	5 mm	3 mm	2 mm

A study done by Mohapatra *et al*. (2003) demonstrated that the enzymes producing isolates were widely distributed in almost all bacterial genera. Especially, 10 to 35% strains of *Bacillus* were capable of producing large amounts of protease, amylase and carboxymethylcellulase. Kumar *et al*. (2014) reported that out of twelve *Bacillus* strains isolated from apple rhizosphere 9 and 7 strains were showed protease and cellulase production, respectively. Also, protease, amylase and cellulase activities from *Bacillus* species have been reported previously (Kim *et al*., 2012; Aygan *et al*., 2014; Elkhalil *et al*., 2015; Pant *et al*., 2015).

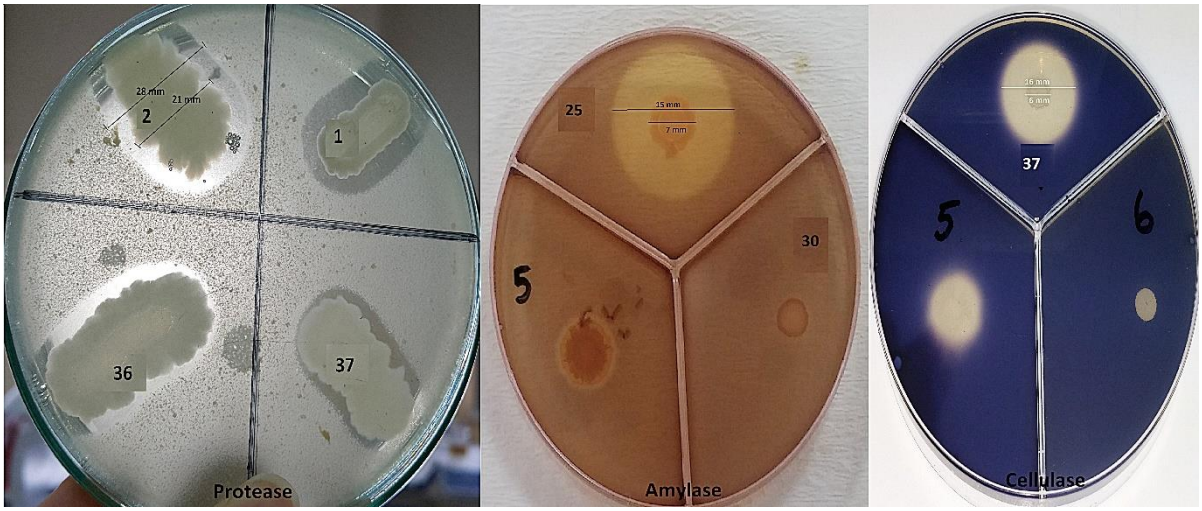

Fig. 1 Screening of protease, amylase and cellulase enzyme producing bacteria on agar medium

The 39 isolates were screened for salt tolerance and growth in nutrient broth amended with various concentrations of NaCl. Growth was measured after 24 hours until the fifteenth day. The 6, 32, 22 and 8 isolates grew in 2%, 5%, 7% and 10% NaCl, respectively. Five isolates were observed to grow at only 2% NaCl (Table 3). It was found that highest production for all three enzymes were obtained from strains capable of growth at a 5% NaCl. In addition, all isolates grew at 25°C, 37°C and 45°C, but only 10 isolates grew at +4°C (Table 3). Furthermore, all isolates grew at acid and alkaline pH. The pH range for growth of isolates were from 5.0 to 10.0 with optimum pH at 7.0-8.0, correspondingly (Table 3). Only nine isolates grew at pH 5. Beside this, isolate 29 did not growth at pH 5, 9 and 10, but produced all three enzymes.

Tablo 3: Phenotypical properties of *Bacillus* isolates

Strains	Growth in 4°C	Growth in 25°C	Growth in 37°C	Growth in 45°C	Growth in %2 NaCl	Growth in %5 NaCl	Growth in %7 NaCl	Growth in %10 NaCl	Growth in pH: 5	Growth in pH: 6	Growth in pH: 7	Growth in pH: 8	Growth in pH: 9	Growth in pH: 10
1	-	+	+	+	-	+	+	+	-	+	+	+	+	+
2	-	+	+	+	-	+	+	-	-	+	+	+	+	+
3	-	+	+	+	-	+	+	+	+	+	+	+	+	+
4	-	+	+	+	-	-	-	-	-	+	+	+	+	+
5	-	+	+	+	-	-	-	-	-	+	+	+	+	+
6	-	+	+	+	-	+	+	-	-	+	+	+	+	+
7	-	+	+	+	+	-	-	-	-	+	+	+	+	+
8	-	+	+	+	+	-	-	-	-	+	+	+	+	+
9	-	+	+	+	+	+	-	-	-	+	+	+	+	+
10	-	+	+	+	-	+	+	-	-	-	+	+	+	+
11	-	+	+	+	-	+	-	-	-	+	+	+	+	+
12	-	+	+	+	-	+	-	-	-	+	+	+	+	+
13	-	+	+	+	-	+	+	-	+	+	+	+	+	+
14	-	+	+	+	+	-	-	-	-	+	+	+	+	+
15	+	+	+	+	-	+	+	-	+	+	+	+	+	+
16	-	+	+	+	-	+	-	-	-	-	+	+	+	+
17	+	+	+	+	-	+	+	+	+	+	+	+	+	+
18	-	+	+	+	-	+	+	+	+	+	+	+	+	+

19	-	+	+	+	-	+	+	-	+	+	+	+	+	+
20	-	+	+	+	-	+	-	-	-	+	+	+	+	+
21	-	+	+	+	-	+	+	-	+	+	+	+	+	+
22	-	+	+	+	-	+	+	-	+	+	+	+	+	+
23	+	+	+	+	-	+	+	+	-	+	+	+	+	+
24	+	+	+	+	-	+	+	+	-	+	+	+	+	+
25	+	+	+	+	-	+	-	-	-	+	+	+	+	+
26	+	+	+	+	-	+	-	-	-	+	+	+	+	+
27	-	+	+	+	-	+	+	-	-	+	+	+	+	+
28	-	+	+	+	-	+	-	-	-	+	+	+	+	+
29	-	+	+	+	-	+	+	-	-	+	+	+	-	-
30	-	+	+	+	-	+	+	+	-	+	+	+	+	+
31	+	+	+	+	-	+	+	-	-	-	+	+	+	+
32	-	+	+	+	-	+	-	-	-	+	+	+	+	+
33	+	+	+	+	-	+	+	-	-	+	+	+	+	+
34	-	+	+	+	+	-	-	-	-	+	+	+	+	+
35	-	+	+	+	+	-	-	-	-	+	+	+	+	+
36	+	+	+	+	-	+	+	+	-	+	+	+	+	+
37	-	+	+	+	-	+	+	-	+	+	+	+	+	+
38	-	+	+	+	-	+	+	-	-	+	+	+	+	+
39	+	+	+	+	-	+	-	-	-	+	+	+	+	+

Antibiotic susceptibility testing results are presented in Table 4. Bacterial species of soil may have resistance to natural antibiotics or may acquire these features through genetic exchange from other bacteria (Shafiani and Malik, 2003). Antibiotic resistance pattern of the isolates showed that isolates were resistant on the percentage of 100% to penicillin; 66.6% to rifampicine; 23.7% to novobiocin; 48.7% to cefepime; 87.1% to ceftazidime; 89.7% to oxacillin; 2.5% to streptomycin; 30.7% to clindamycin; 7.6% to tetracycline; 12.8% to ampicillin/sulbactam; 10.2% to gentamicin; 56.4% to ceftriaxone; 84.6% to polymyxin B and 12.8% to amikacin were determined. In addition, all isolates were susceptible to imipenem, ciprofloxacin, meropenem and ofloxacin.

Table 4: Antibiotic susceptibility patterns of isolates

Suş No	**RD 5**	**IPM 10**	**NV30**	**SAM 20**	**FEB 30**	**CIP 5**	**CAZ 30**	**CN 10**	**MEM10**	**TE 30**	**OFX 5**	**OX 1**	**S 10**	**DA 10**	**CRO 30**	**PB 300**	**P10**	**AK 30**
1	R	S	I	R	R	S	R	S	S	I	S	R	S	S	I	R	R	S
2	R	S	I	S	R	S	R	S	S	S	S	R	S	S	R	R	R	S
3	R	S	R	S	S	S	S	R	S	S	S	R	S	R	R	R	R	R
4	I	S	S	R	R	S	R	S	S	R	S	R	S	R	R	R	R	S
5	I	S	I	S	S	S	R	S	S	I	S	R	R	S	R	I	R	S
6	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S
7	I	S	S	I	R	S	R	S	S	S	S	R	S	S	R	R	R	S
8	S	S	S	S	R	S	R	S	S	S	S	R	S	S	R	R	R	S
9	R	S	S	S	S	S	R	S	S	R	S	R	S	R	S	S	R	R
10	S	S	S	S	S	S	R	S	S	S	S	R	S	S	I	I	R	S
11	R	S	I	I	R	S	R	S	S	R	S	R	S	S	R	R	R	S
12	R	S	I	R	R	S	R	S	S	R	S	R	S	S	R	R	R	S
13	R	S	R	S	S	S	R	R	S	S	S	R	S	R	S	R	R	R
14	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	I	R	S
15	R	S	R	S	R	S	R	S	S	S	S	R	S	R	I	S	R	S
16	R	S	S	I	S	S	R	S	S	S	S	R	S	S	R	R	R	S

17	R	S	R	I	S	S	S	R	S	S	S	R	S	I	S	R	R	R
18	I	S	S	S	S	S	R	S	S	S	S	R	S	S	I	R	R	S
19	I	S	I	S	S	S	S	S	S	S	S	I	S	S	S	R	R	S
20	I	S	S	S	S	S	R	S	S	S	S	R	S	S	R	R	R	S
21	R	S	I	S	S	S	R	S	S	S	S	R	S	S	I	R	R	S
22	R	S	S	R	R	S	R	S	S	S	S	R	S	S	R	R	R	S
23	S	S	I	S	S	S	R	S	S	S	S	I	S	S	S	R	R	S
24	R	S	R	S	S	S	R	S	S	S	S	S	S	S	R	R	R	S
25	R	S	I	I	R	S	R	S	S	S	S	R	S	R	R	R	R	S
26	R	S	R	S	R	S	R	S	S	S	S	R	S	R	R	R	R	S
27	R	S	R	S	R	S	R	S	S	S	S	R	S	R	R	R	R	S
28	R	S	I	I	R	S	R	I	S	S	S	R	S	S	R	R	R	S
29	I	S	I	S	S	S	R	S	S	S	S	R	S	S	S	R	R	S
30	R	S	I	I	R	S	R	S	S	S	S	R	S	S	R	R	R	S
31	R	S	I	S	R	S	R	I	S	S	S	R	S	S	I	R	R	S
32	R	S	R	S	S	S	R	R	S	S	S	R	S	I	I	R	R	S
33	I	S	I	S	S	S	R	S	S	S	S	R	S	S	S	R	R	S
34	R	S	R	S	R	S	R	S	S	S	S	R	S	R	R	R	R	S
35	R	S	I	S	S	S	R	S	S	S	S	R	S	R	R	R	R	S
36	R	S	S	R	R	S	R	S	S	S	S	R	S	R	R	R	R	S
37	R	S	I	S	S	S	R	S	S	S	S	R	S	I	I	I	R	S
38	R	S	S	S	R	S	R	S	S	S	S	R	S	R	R	R	R	S
39	R	S	I	I	R	S	R	I	S	S	S	R	S	S	R	R	R	R

R: resistant, I: intermediate, S: sensitive

Molecular typing of *Bacillus* isolates based on 16S rRNA gene sequences are good indicators of phylogenetic relationships among bacteria at species and subspecies levels. However, identifications based on 16S rRNA gene sequences are limited and unable to discriminate among isolates (Solanki *et al*., 2012). Therefore, genotypic fingerprinting techniques (RAPD, ERIC and PCR-SSCP) were used to distinguish intra-species variability among bacterial strains. In present study, the fingerprint patterns of the 39 isolates generated by RAPD, ERIC and SSCP –PCR were complex, producing a large number of polymorphic bands of variable intensity.

A total of 39 strains generated DNA fragments with primer M13-10 and OPL-3. The RAPD analyses conducted with primers M13-10 and OPL-3 generated 15 and 14 different profiles, respectively. In the dendograms in Figure 2, the isolates were separated into two major clusters at similarity levels of 5% for M13-10 and 2% for OPL-3 or above. RAPD-PCR analysis revealed high degree of genetic diversity for all identified bacteria with primers M13-10 and OPL-3. In addition, RAPD analysis of genomic DNA using these primers revealed 100% polymorphism indicating the strength of RAPD in the differentiation of *Bacillus* isolates. The data gathered from many researches showed that RAPD-PCR was successfully applied to characterize *Bacillus* strains. For instance, Gupta *et al*. (2011) described RAPD-PCR as the best means for molecular typing of *Bacillus* species. Beric *et al*. (2009) reported that 205 *Bacillus* strains were genotyped by RAPD technique, and researchers determined 13 different groups of RAPD profiles within four (five) species: *B. subtilis, B. cereus*/*B. thuringiensis*, *B. pumilis* and *B. firmicus*.

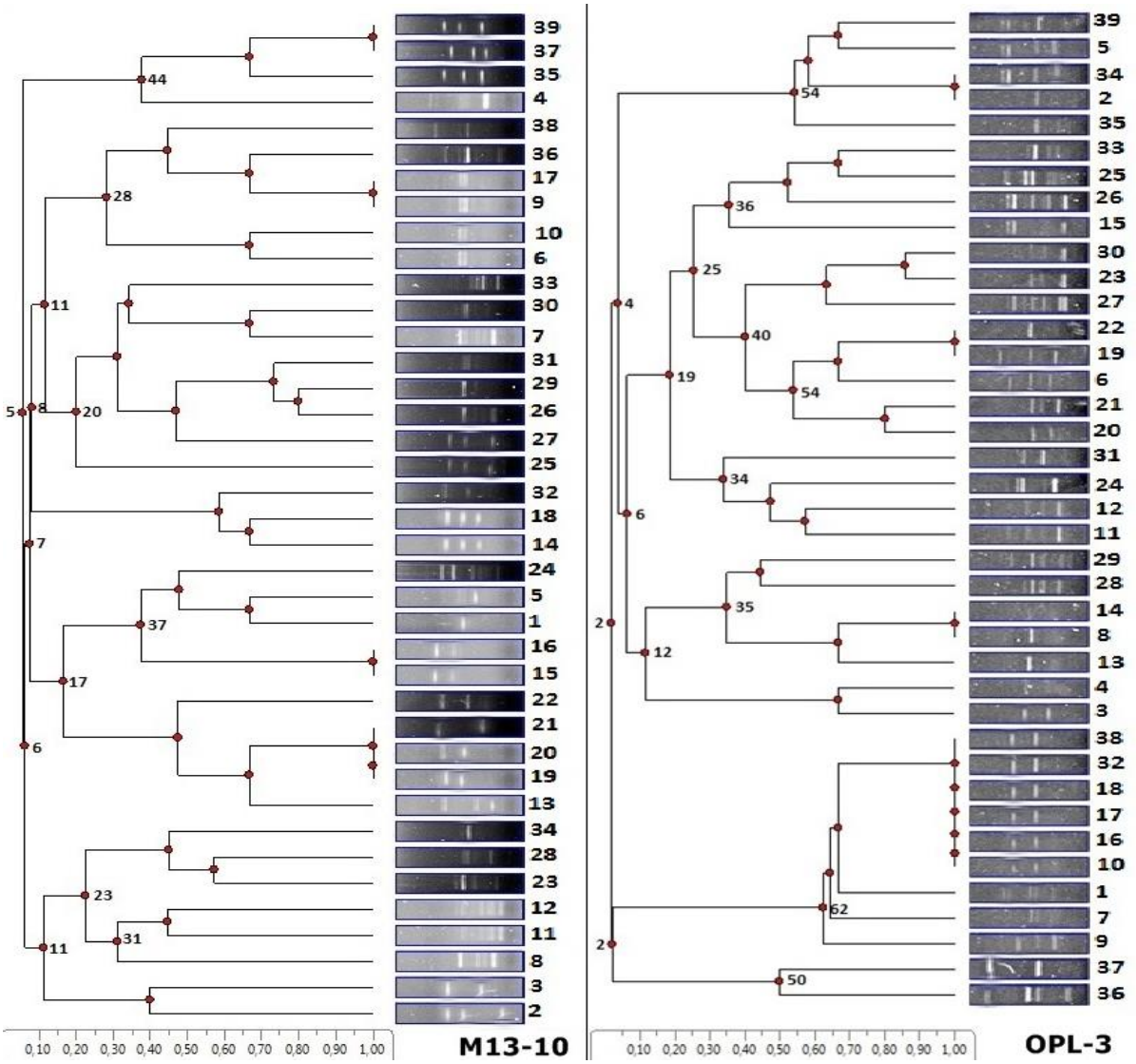

Fig. 2 Dendrograms based on RAPD-PCR analysis of M13-10 and OPL3

There were 6-14 bands with molecular weights ranging from 220 bp to 4000 bp generated by ERIC primers for a total of 39 strains. In the dendrogram, the isolates were separated into two major clusters at similarity levels between 38% and 94% (Figure 3). Our data show a high variability in ERIC patterns in agreement with Garcia *et al*. (2015). They evaluated the genetic diversity of 40 *Bacillus thuringiensis* strains recovered from environmental samples in Mexico and explained that ERIC-PCR showed 22% similarity. In addition, in the study of Freitas *et al*. (2008) 40 isolates of *Bacillus* were evaluated and identified at approx 40% genetic similarity level. In another study from a variety of soil *Bacillus* species, ERIC-PCR subgroups showed 17% similarity (Solanki *et al*., 2012).

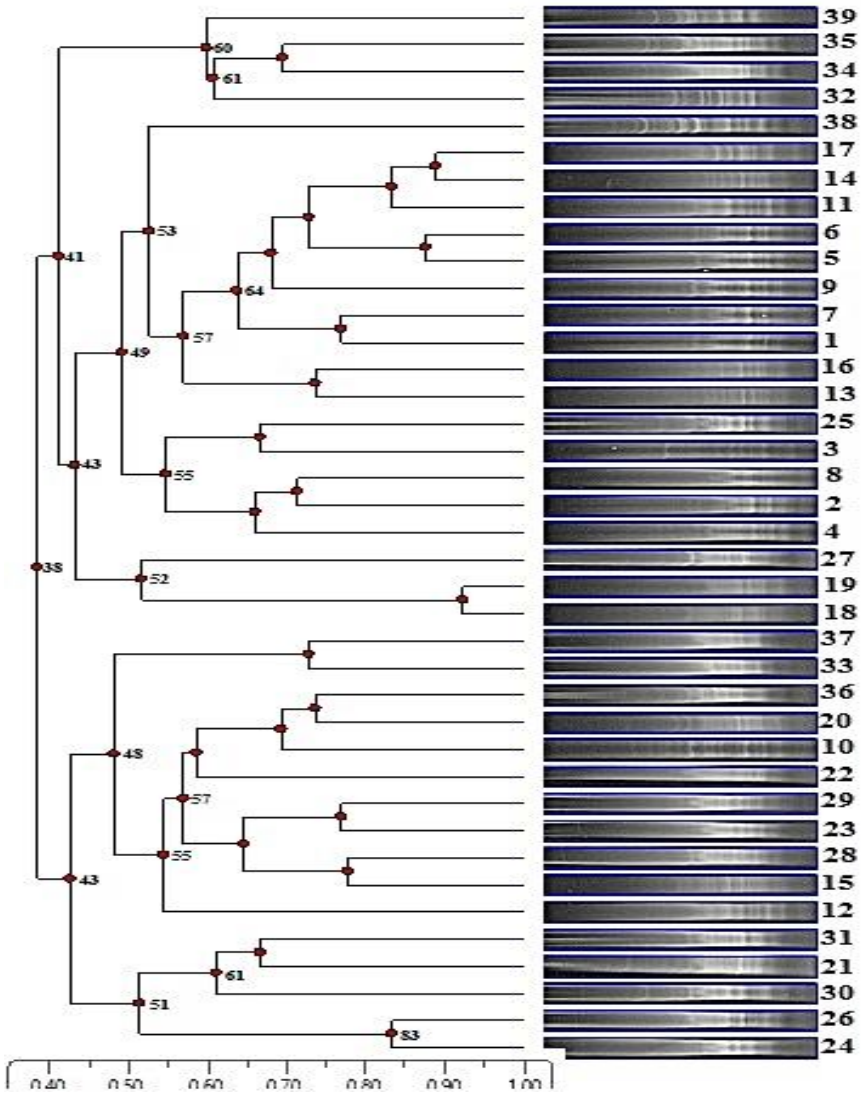

Fig. 3 Dendrogram of ERIC-PCR genotype analysis derived from *Bacillus* isolates.

The SSCP analysis conducted with primers P11P and P13P. The SSCP patterns of the isolates consisted of 2 to 7 distinguishable bands of different intensities. Most of the bands were produced by all isolates. Two or three strong bands were detected in the SSCP patterns of isolates (Figure 4). In a study done by Luis-Villasenor *et al*. (2013), diversity analyses based on PCR-SSCP showed that the bacterial community resulting from the *Bacillus* mix isolated from guts had the highest diversity.

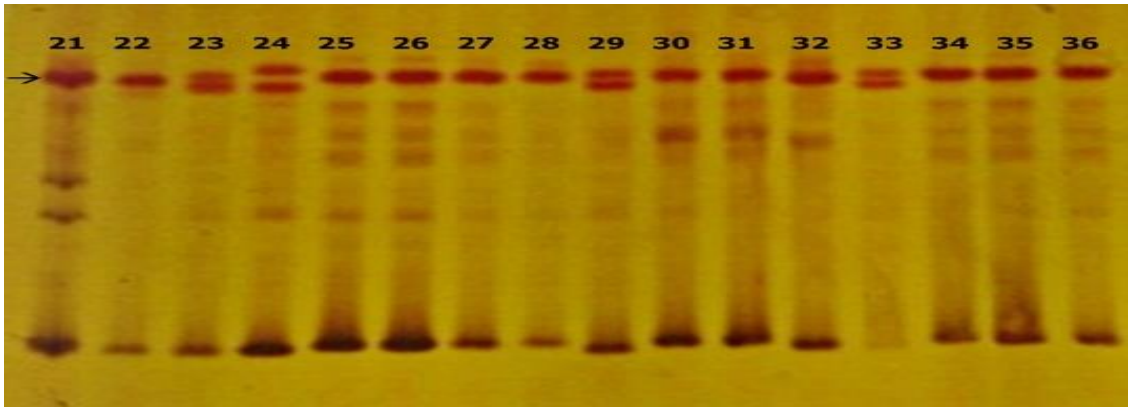

Fig. 4 SSCP profiles of PCR-amplified 16S rRNA gene fragments

Dendogram obtained by UPGMA clustering of SSCP patterns showed two main clusters at similarity levels of 15% or above (Figure 5).

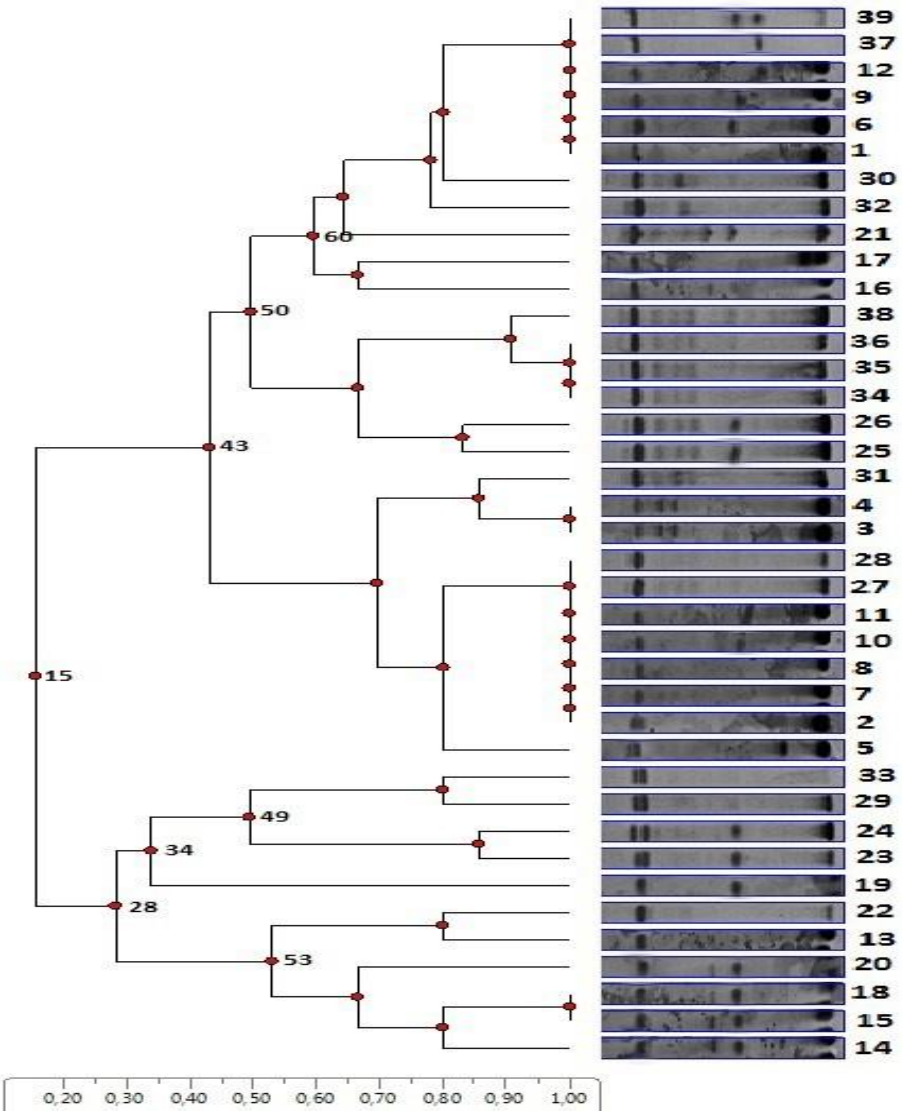

Fig. 5 Dendrograms based on PCR-SSCP analysis

4. Conclusion

As a result was revealed that it appears that *Bacillus* strains associated with soil are the source of industrial enzymes and may play an important role in enzyme catalyzed organic matter cycling in soil environments. In addition, the results of this study showed that the phylogenetic tree based on these fingerprint tools revealed close relationships among the majority of isolates. Moreover, the RAPD and SSCP techniques were selected on the basis of its higher discriminatory power at subspecies level, as compared to ERIC-PCR. Beside these, as shown with *Bacillus* strains, SSCP analysis can increase the discrimination power of rDNA-PCR fingerprinting, allowing the detection of sequence polymorphisms not detectable by conventional electrophoresis (Borin *et al*., 1997).

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