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Comparison of 16S-ITS rDNA RFLP Profiles of *Bacillus* sp. Isolated from Milk and Different Water Sources

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Abstract

The aim of this research was the isolation of thermophilic *Bacillus* sp. from different sources and grouping by 16S-ITS rDNA RFLP. Seventy-four thermophilic strains were isolated from various environmental samples. Since fifteen isolated strains were Gram (+), endospore forming rods, they were identified as *Bacillus* sp. These strains were screened for the existence of five extracellular enzyme activities. These were, lipases, amylases, proteases, xylanases, cellulases. 16S-ITS rDNA RFLP profiles were produced by using two restriction endonucleases *Taq* I and *Hae* III. The isolated strains were clustered into eigth groups by *Taq* I restriction profiles of 16S-ITS rDNA while seven groups were obtained by *Hae* III digestion profiles.

Farklı Su ve Süt Kaynaklarından İzole Edilen *Bacillus* sp.'ların 16S-ITS rDNA RFLP Profillerinin Karşılaştırılması

Anahtar kelimeler
Bacillus sp., Ekstraselüler enzim, 16S-ITS rDNA, RFLP

Özet

Bu çalışmanın amacı farklı kaynaklardan izole edilen termofilik *Bacillus* sp.'ları 16S-ITS rDNA RFLP ile gruplandırmaktır. Çeşitli çevresel örneklerden yetmiş dört suş izole edilmiştir. Gram (+), endospor oluşturan, basil şeklindeki izolatlar *Bacillus* olarak identifiye edilmiştir Bu suşlar; lipaz, amilaz, proteaz, ksilinaz ve selulazdan oluşan ekstraselülar beş enzim aktivitesi açısından incelenmiştir. 16S-ITS rDNA RFLP profilleri *Taq*I ve *Hae*III olmak üzere iki farklı restriksiyon enzimi kullanılarak çıkarılmıştır. İzolatlar *Taq*I ile sekiz, *Hae*III ile yedi farklı restriksiyon grubu oluşturmuştur.

1. Introduction

During the last decades, there was an increasing interest in development of different food safety measures to ensure safe and qualitative food products (Nada et al., 2012). Nowadays, there has been considerable increase in interest in thermophilic bacteria of the genus *Bacillus*, because of their possible contamination of heated food products (Saito, 1973; Rainey et al., 1994; Al-Awadhi et al., 1998; Touzel et al., 2000; Tyndall et al., 2002; Paes and O'Donohue, 2006; Tang et al., 2006). Raw milk provides a very suitable medium for bacterial growth, and milk quality is largely dependent on its microflora (González et al., 2013). *Bacillus* species and their spores, often present in raw milk, play an important role in the dairy industry in being responsible for spoilage of raw and pasteurized milk (González et al., 2013). Therefore, within the dairy processing context, thermophilic bacilli are used as hygiene indicators in processed product. *Geobacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus subtilis* have been isolated from raw milk and milk products (Ronimus et al., 2003). Consequently, careful microbiological monitoring of milk is required (Nada et al., 2012).

The 16S internal transcribed spacer (16S-ITS) rRNA gene RFLP method has already been used for the identification of bacteria of diverse origin; lactic acid bacteria (Bulut et al., 2005), lactobacilli (Yavuz et al., 2004a), thermophilic bacilli Yavuz et al., 2004b)and alkalophilic bacilli (Akbalik et al., 2004). Some researcher have reported that the 16S-23S ribosomal RNA intergenic transcribed spacer (ITS) has much greater variability in sequence than 16S rRNA and has been useful for differentiation of closely related bacterial species(Shaver et al., 2001; Ouoba et al., 2004). So far no reports describing the characterization of *Bacillus* sp. has been isolated from raw milk samples from Turkey have been available in the literature.

The aim of this study was to comparison of 16S-ITS rDNA RFLP profiles of *Bacillus* sp. isolated from milk and different water sources and determination of the physical growth characteristics demands.

2. Materials and Methods

2.1. Isolation and growth conditions

All chemicals were of reagent grade and were purchased from Sigma–Aldrich (USA). Five hot spring water, eleven drinking water, two waste water and forty raw milk samples from Afyonkarahisar were taken aseptically. The hot spring water samples were collected into thermos flasks in order to maintain the temperature during transportation. Enrichment method was used for isolation. 10 ml of samples were subjected to heat treatment for 10 min at 80ºC in a waterbath in order to eliminate non-spore forming bacteria (Mora et al., 1998). After pasteurization, the samples were transferred into 100 ml of Broth (yeast extract 1 g/l, glucose 1 g/l). Incubation was performed in a rotary shaker at 60°C until a turbidity obtained. Then 500 µl of the broth was plated on agar medium (yeast extract 1 g/l, glucose 1 g/l, agar agar 15 g/l) and incubated for 48-72 h at 60°C. Single colonies with different morphologies were picked.

2.2. Morphologic, biochemical and physiological characterization

Gram staining, endospore forming rods, oxidase, catalase, were determined for each isolate 15 isolated strains were determined for each isolate. *Bacillus* is a rod-shaped, gram positive, spore forming, aerobic or facultatively anaerobic, usually catalase positive, chemoorganotrophic bacterium with a fermentative or respiratory mechanism (Holt and Krieg, 1994). Therefore, if an isolate shows general characteristics of *Bacillus* genus, it is referred as *Bacillus* sp. Protease screening was performed according to the method described by

Priest et al. (1988). For amylase, xylanase and cellulase screening, the mineral medium described by Bragger et al. (1989). Lipase screening was performed according to the method described by Haba et al. (2000). Physiological parameters included growth at different ranges of temperature (37°C to 70°C), sodium chloride concentration (1% to 6%) and pH values (4,5 to 9) were determined.

2.3. Genomic DNA Isolation of Isolates and Amplification of 16S rDNA – ITS Region

DNA extraction of the bacterial isolates was carried out by using GF-1 Bacterial DNA Extraction Kit (Vivantis). The 16S rDNA – ITS Region from individual bacterial isolates was amplified by the polymerase chain reaction (PCR). PCR amplification was performed using L1 and EGE1 PCR primers. The forward primer was: L1: 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer was EGE1: 5'-CAAGGCATCCACCGT-3'(Jensen et al., 1993; Akbalik et al., 2004). The PCR reaction mixtures (50µl) contained, 2 µl of genomic DNA template, 5 µl 10x reaction bufer, 2,5 µl 25 mM $MgCl_2$, 8 µl dNTPs (each of 200 µl), 50 pmol of each of the DNA primers, 0,25 µl Taq DNA polymerase. The PCR was carried out in a thermal cycler. The reaction was subjected to 40 cycles of amplification of 16S rDNA – ITS gene region (initial cycle of 5 min at 94 °C, denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min, elongation 72 °C for 1 min and final extension step at 72°C for 10 min). Amplified DNA fragments were analyzed on 1 % (w/v) agarose gels stained with ethidium bromide and photographed with UV illumination.

2.4. Restriction Fragment Length Polymorphism (RFLP)

*Hae*III (GG.CC) and *Taq*I (T.CGA) restriction endonucleases were used for RFLP. Ten µl of the PCR products were digested with 5 units of each of the enzymes in a final volume of 50 µl. Ten µl of each PCR product was transferred into eppendorf tubes and 40 µl restriction enzyme mixtures (5 µl restriction enzyme bufer, 35 µl sterile deionized water, 10 µl DNA, 0.5 µl (5U) restriction enzyme) were added. *Taq*I digestion samples were overlaid with mineral oil and the restriction reactions were carried out in a water bath at 65 °C. *Hae*III digestions were performed at 37 °C. Both reactions were incubated overnight. After the digestion, DNA was extracted as described and dissolved in 15 µl 1xTE (Yavuz et al., 2004b). For the seperation of restriction fragments 1% (w/v) agarose gels were prepared. Electrophoresis was carried out for 20 min at 145-150 V. At the end oft the electrophoresis, the gel was removed and DNA was visualised on a UV illuminator.

3. Results

In this study, 74 thermophilic microorganisms were isolated from different samples (twenty isolates from hot spring water, eleven isolates from tap water, two isolates from waste water and forty isolates from milk samples) (Table 1). Colony morphologies were determined under the light microscope. Coccus shaped bacteria, gram negative and unpure isolates were eliminated. In total fifteen isolated strains were found to be spore-forming, gram-positive, catalase-positive and rod-shaped were selected for further studies. Eighty per cent of the isolates were from raw milk, 13% from hot water and 7% were from waste water samples.

Physiological and enzymatic characteristics status of the isolates were shown in Table 1. All isolates tested were able to grow 35-45 °C but only R1, R2, R34 were able to grow 60 °C. Only milk samples were able to grow ≥55 °C. None of them were able to grow 75 °C. However no growth was observed at 75 °C suggesting that these isolates could be thermophil. The isolates were tested for growth at different pH. All the isolates were able to grow at pH ≥ 6.5. Also R1, R34 were able to grow pH 4.5 and 5.0. This result could indicate that isolates were alkalophiles. Growth of the isolates were also observed at 0,3% , 1%, 3%, 6% NaCl concentrations

on agar plates. Test results showed that all of this isolates except that isolates W12 and R35 were able to grown 0,3-6% salt concentrations. The isolates were screened for lipase, amylase, protease, xylanase, cellulase activity. Only isolate W12 was able to produce amylases. None of the enzymes were detected at other isolates (Table 2).

Table 1. Samples, isolated from from different sources

Samples	Number of isolate	Name of the isolate
Hot Water		
Aydın thermal	2	H1, H2
Kadı thermal	13	H3,H4,H5, H6, H7, H8,H9, H10, H11, H12, H13, H14, H15
Hilal thermal	2	H16, H17
Gazlıgöl thermal	1	H18
Kızılcahamam	2	H19, H20
Waste water		
Tınaztepe	1	W1
Bolvadin	1	W2
Bolvadin	2	W3,W4
Kadınana	1	W5
Savran	1	W6
Cumhuriyet	1	W7
Kocaeli	2	W8, W9
Aydın Çine	1	W10
Bilecik	1	W11
Şuhut	1	W12
Antalya	1	W13
Isparta Barla	1	W14
Raw Milk		
Fetibey	10	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10
Erenler	5	R11, R12, R13, R14, R15
Gazlıgöl	1	R16
Çobanlar	17	R17, R18, R19, R20, R21, R22, R23, R24, R25, R26, R27, R28, R29, R30, R31, R32, R33
Sinanpaşa	5	R34, R35, R36, R37, R38

Genomic DNA was successfully extracted from fifteen bacterial isolates. Amplification of 16S-ITS region mostly produced one distinct DNA fragment of approximately 2000-2500 bp in length. In order to determination of 16S-ITS rDNA restriction profiles, two restriction enzymes, *Taq* I and *Hae* III were used and similar profiles are grouped. 16S-ITS rDNA RFLP profiles of *Taq* I groups of isolates were shown in Table 3. According to the results of *Taq* I enzyme digestion, strains were clustered into eigth genotypic groups (T1, T2, T3, T4, T5, T6, T7, T8). While hot spring water isolates H16 and H17 were located in T1 group (1035, 500, 328 bp) milk sample isolates were clustered into different genotypic groups (T2 T3, T4, T5, T7, T8). Waste water isolate W12 took part in a separate group in T2 (845, 535, 379, 291 bp). Isolates were divided into seven major groups depending on the results of the digestive restriction enzyme *Hae* III (HA1, HA2, HA3, HA4, HA5, HA6 and HA7) (Table 4).

H16 and H17 isolated from hot spring water were located in HA1 group (514- 383 bp) but waste water isolates W12 was located in HA2 group. Most of isolates isolated from milk samples (R1, R2, R3, R11, R12, R13, R16) located HA3 group (682, 514, 365, 327 bp). According to the two enzymes digestion dairy isolates in different groups took part from water-borne isolates.

Table 3. 16S-ITS rDNA RFLP profiles of *Taq* I genotypic groups of isolates.

Groups of 16S-ITS rDNA RFLP profiles	Isolate No	Fragment length after digestion by *Taq* I (bp)
T1	H16, H17	1035, 500, 328
T2	R37	845, 535, 379, 291
T3	R1, R2, R3, R11	568, 474, 412, 182

T4	R12, R16	1035, 535, 389, 291
T5	R13	535, 389, 291
T6	W12	293, 412, 318
T7	R17, R34	535, 379
T8	R35, R36	1018, 845, 461, 379

The results of the Table 4 generated from both of the restriction enzyme profiles were combined and ten distinct homology groups of the isolated strains were obtained. Hot spring waters isolates (H16 and H17) located in G1 (514- 383 bp). Waste water Isolates W12 was located in G1 group. Milk sample isolates were clustered into eigth different genotypic groups (G1, G2, G3, G4, G5, G6, G7, G8). Analysis of the homology groups indicated that isolates were isolated from different sources could be separated by 16S-ITS rDNA RFLP.

Table 4 16S-ITS rDNA RFLP profiles of *Hae* III genotypic groups of isolates.

Groups of 16S-ITS rDNA RFLP profiles	Isolate No	Fragment length after digestion by *Hae* III (bp)
HA1	H16, H17	514, 383
HA2	W12	285, 188
HA3	R1, R2, R3, R11, R12, R13, R16	682, 514, 365, 327
HA4	R37	300
HA5	R36, R19	682, 527, 300
HA6	R34	682, 514
HA7	R35	278, 175

Table 2. Physiological and enzimatic characteristics of rod-shaped, gram positive, spore form*i*ng isolates.

	Temperature (ºC)					pH							Salt concentrations (%)				Catalase	Oxidase	Amylases	Proteases	Cellulases	Lipases	Xylanase
H16	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
H17	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
W12	**+**	**+**	**-**	**-**	**-**	**-**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**-**	**-**	**+**	**+**	**-**	**-**	**-**	**-**
R1	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R2	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R3	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R11	**+**	**+**	**+**	**-**	**-**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**-**	**-**	**-**	**-**	**-**
R12	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R13	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R16	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R37	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R19	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R34	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
R35	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-
R36	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-

4. Discussions and Conclusions

In this study, thermophilic *Bacillus* sp. from different sources were isolated and grouped by 16S-ITS rDNA RFLP. Seventy four thermophilic microorganisms were isolated from different samples and coccus shaped bacteria, gram negative and unpure isolates were eliminated. Fifteen isolates with different colony morphology were found to be spore-forming, Gram-positive, catalase-positive and rod-shaped were selected for further studies.

Thermophilic *Bacillus* species, with growth temperature optima between 45 and 70°C, have been isolated from a wide range of environments (Peng etal., 2003). In the dairy industry, the facultative thermophiles belong to the *Bacillus* genus and tend to grow at both mesophilic and thermophilic temperatures, depending on the strain. Some examples of species include *Bacillus licheniformis, Bacillus coagulans, Bacillus pumilus, Bacillus sporothermodurans* and *Bacillus subtilis* (Burgess et al., 2010). Nielsen et al. (1995) have demonstrated that a strain of *B. clausii* has tolerated 8 % NaCl whereas other membershave tolerated about 10 % NaCl. Moderate halophiles are capable to grow over a wide range of salt concentrations (optimal growth at 3–15% NaCl) (Joo and Chang, 2005). According to Krulwich (1995) alkalophiles can be divided into two groups; alkaline-tolerants (optimal growth at the pH range 7.0-9.5) and alkalophilics (optimal growth at the pH range 10.0 and 12.0).

This result could indicate that isolates were alkaline-tolerants and moderate halophiles. Our results showed that all isolates except W12 and R35 were able to grown 0,3-6% salt concentrations and at pH ≥6,5. it is shown that our results, as well as, milk sample isolates were able to grow ≥55 °C (Table 1). Burgess et al. (2010) reported that the presence of thermophiles in dairy products is an indicator of poor hygiene during processing.

We found that only waste water isolate W12 was able to produce amylases. Extracellular enzymes from alkalophilic microorganisms can be active at alkaline pH (Ghanem etal., 2000) and most of the alkalophilic enzymes show optimal activity alkali pH (Takami and Horikoshi, 2000).

In recent study the 16S-ITS rRNA gene RFLP method has been successfully applied to the species-level identification of thermophilic bacilli (Yavuz et al., 2004b) and alkalophilic bacilli (Akbalik et al. 2004). Consequently, fifteen isolated strains were characterized according to their 16S-ITS rDNA restriction profiles. Yavuz et al. (2004b) reported that seventeen different genotypic groups have been obtained after 16S rDNA-ITS Region RFLP of 112 isolated strains by *Taq* I and *Hae* III. On the other hand Bulut et al. (2003) has used the same method for further identification; isolates which could'nt have been identified phenotypically, could have been identified by using 16S rRNA-ITS region RFLP. Akbalık et al. (2004) results indicated that 16S-ITS rDNA RFLP could be a rapid method in revealing genetic diversity of the large collections of alkalophilic bacteria at species level. They were reported that Both *Taq*I and *Hae*III were found to

be necessary for the discrimination of the strains by this method. Similar to this study, on the identification of thermophilic bacilli from marine thermal vents of Eolian Islands, seventy-four thermophilic isolates have been compared according to their restriction patterns of amplified 16S rDNA with eigth type strains of *Bacillus* (Caccamo et al., 2001). They have obtained thirteen different *Alu* I restriction patterns and %78 of isolates were recognized as representatives of different *Bacillus* species. In another work Blanc et al. (1997) the *Hae* III restriction profiles of 16S rDNAs have been compared with four different reference strains. Five different groups have been found, four of the them including the reference strains. Kuisine et al. (2002) has evaluated the thermophilic proteolytic isolates from a geothermal site in Lithuania based on 16S rDNA-RFLP and ITS- PCR analyses. Acording to PCR-RFLP 42 strains have been divided into six distinct groups. Another study indicated that 16S rDNA and ITS regions were used separately for RFLP analysis (Caccamo et al., 2001).

In the present study, most isolates showed common into same physiological characteristics and showed common enzyme production On the other hand, some of them differed from each other in terms of genotypic group. Isolates were clustered into different genotypic groups by both *Taq* I and *Hae* III. According to the results of *Taq* I enzyme digestion, alkaline-tolerants and moderate halophiles bacillus strains were clustered into eigth genotypic groups. Those isolates were divided into seven major groups depending on the results of the digestive restriction enzyme *Hae* III. The results of the Table 4 generated from both of the restriction enzyme profiles were combined and 10 distinct homology groups of the isolates. According to the two enzymes digestion dairy isolates in different groups took part from water-borne isolates. Analysis of the homology groups indicated that isolates were isolated from different sources could be separated by 16S-ITS rDNA RFLP and This metot is suitable for distinguishing among *Bacillus* sp. where were isolated.

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