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RAPD ANALYSIS OF GENETIC DIVERSITY AND QUALITATIVE ASSESSMENT OF HYDROLYTIC ACTIVITIES IN A COLLECTION OF *BACILLUS* SP. ISOLATE

TANJA BERIĆ[1], MARIA C. URDACI[2], S. STANKOVIĆ[1], and JELENA KNEŽEVIĆ-VUKČEVIĆ[1]

[1]*Chair of Microbiology, Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia
[2]*Laboratoire de Microbiologie et Biochimie Appliquee,* 33175 Gradignan, France

Abstract — Genetic diversity and production of hydrolytic enzymes of 205 *Bacillus* isolates from different geographical and ecological niches in Serbia were studied. Combining RAPD analysis and 16S DNA sequencing, we determined 13 different groups of RAPD profiles within four (five) species: *B. subtilis, B. cereus*/*B. thuringiensis, B. pumilus*, and *B. firmus*. Screening for production of hydrolytic enzymes showed that there was no correlation of enzyme production with species. Most of the isolates from all habitats produced amylase, protease, lipase, mannanase, and xylanase to some extent at 25°C and 37°C. The number of isolates that retained enzyme production ability at 55°C is considerably lower and they predominantly came from manure.

Key words: *Bacillus* isolates, RAPD, enzyme production

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INTRODUCTION

Bacillus species are known as producers of different exoenzymes, a trait that is linked with their way of life and places where they thrive. In addition, these bacilli often grow at temperatures that are higher than mesophilic temperatures. Extracellular hydrolytic enzymes that retain their activity at higher temperatures have a great potential for commercial application. Amylase degrades starch components of different waste material into simple sugars that find many uses in the feed and fermentation industries (Hyun and Zeikus, 1985). Proteases are highly exploited enzymes in the food, leather, detergent, and pharmaceutical industries, as well as for diagnostic purposes and waste management. The protease enzymes constitute two thirds of all enzymes used in various industries (Gupta et al., 2002). Lipase catalyzes a variety of biotechnologically relevant reactions such as production of free fatty acids, interesterification of oils and fats, and synthesis of esters (Macrae and Hammond, 1985; Harwood, 1989). Mannanases have potential use in the paper industry for complete hydrolysis of softwood sulfite liquors to fermentable sugar (Ratto and Poutanen, 1988). Recently, interest in the xylanases has markedly increased due to potential application in pulping and bleaching processing using cellulose free preparations, in the food and feed industries, in textile processes, in the enzymatic saccharification of lignocellulosic materials, and in waste treatment (Van der Broeck et al., 1990; Gilbert et al., 1992; Mechaly et al., 1997).

It was reported that high metabolic activities of *Bacillus* strains often correspond to their specific genotypes (Pinchuk et al., 2002). This genetic heterogeneity is interesting in view of the production of extracellular enzymes. Use of the random amplified polymorphic DNA (RAPD) method has extensive application for rapid typing of microorganisms at the species and subspecies levels (Stephan et al., 1994; Ronimus et al., 1997; Pinchuk et al., 2002). In this study, the RAPD method was used to investigate the genetic heterogeneity of *Bacillus* strains isolated from different habitats in relation to production of extracellular enzymes.

From various habitats (straw and hay, soil, and manure) in Serbia, we isolated 205 different Gram-positive, spore-forming bacilli on the basis of colony formation (Stanković and Lazarević, 2001). Our goals were to determine the genetic diversity of this large collection of *Bacillus* sp. isolates and screen it for production of amylase, protease, lipase, mannanase, and xylanase at 25, 37, and 55°C on solid media.

MATERIAL AND METHODS

Isolation and preliminary characterization of Bacillus sp. strains

The method of isolating various *Bacillus* strains was based solely on the resistance of their endospores to elevated temperatures. Test tubes containing a mixture of app. 1 g of sample (straw, soil, or manure) and 1 ml of nutrient broth were placed in a water bath at 80°C for 10 min so that endospores would be separated from vegetative cells (Walker et al., 1998). Concentrated samples, as well as two following decimal dilution, were spread on the surface of LA plates and incubated at 30°C for 48 h. Distinct single colonies were subcultured onto fresh LA plates. The subcultured bacterial isolates were preliminarily characterized on the basis of microscopic appearance and the results of Gram staining and the catalase test.

DNA extraction

Genomic DNA from *Bacillus* strains was prepared as described earlier (Le Marrec et al., 2000). In short, after centrifugation and two washes in TE buffer (10 mmol/l Tris-HCl, pH 8.0; 1 mmol/l EDTA), cells were resuspended in 1 ml of a lysis buffer (50 mmol/l Tris, pH 8.0; 1 mmol/l EDTA; 25% saccharose) containing 20 μgm/l of lysozyme (Merck) and incubated for 45 min at 37°C. The reaction was stopped using 1ml of EDTA (250 mmol/l, pH 8.0) for 5 min. The samples were then treated with 400 μl of 20% (w/v) SDS and 20 μl of a 20 mg/ml proteinase K (Sigma) solution. The mixture was incubated at 65°C until it became clear and less viscous, after which phenol-chloroform extraction was performed. The DNA was precipitated in ethanol and resuspended in 100 μl of TE buffer with 10 μl of RNase (10 mg/ml).

RAPD analysis

Arbitrary 10-mer primers for the series OPA and OPL (Bioprobe, Montrevils-sous-Bois, France) were used for random amplification of DNA. Briefly, 25 μl of reaction mixture contained 25 to 50 ng of genomic DNA, 10 pmol of primer, 50 μM of each dNTP, 0.6 U of Taq DNA polymerase, and 2.5 μl of 10x PCR buffer with $MgCl_2$. Control reaction mixtures lacking template DNA were included in each experiment. The reaction involved initial denaturation at 95°C for 5 min; 45 subsequent cycles, each consisting of 94°C for 30 s, 36°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 5 min. Measured batches (25 μl) of the PCR fingerprinting samples were electrophoresed in agarose gel (1.5%) in TBE buffer at 10 V/cm, stained with ethidium bromide, and photographed under UV light. The lambda *Eco*RI/*Hind*III ladder was used as a size standard.

Amplification of 16S rDNA by PCR and sequence determination

Genomic DNA from *Bacillus* strains was prepared. The 16S rDNA gene fragments were amplified by PCR using the universal primers pU1 (5'-TGTTCCCATCCCAGATTCC-3') and pU3 (5'-GCGTGGCTGCGGGTCCCT-3'), which amplify the maximum number of nucleotides in 16S rDNA from a wide variety of bacterial taxa. The following thermocycling program was used: 5-min denaturation at 95°C; 35 subsequent cycles of 1-min denaturation at 95°C, 40-s annealing at 46°C; 1-min extension at 72°; and a final extension step of 3 min at 72°C.

Testing for hydrolase activities

Hydrolase activities were screened on plates containing the substrate for each particular enzyme. Plates containing 30 ml of growth medium were used throughout the work. *Bacillus* isolates were applied to plates as 5-μl drops of overnight culture.

Amylases

Plates for detecting amylase activity contained: nutrient broth (0.8% w/v), NaCl (0.4% w/v), agar

(1% w/v), and soluble starch (0.5% w/v) (Sigma, CAS No. 9005-84-9). After overnight incubation at appropriate temperatures, the plates were flooded with Lugol solution. The absence of a dark blue pellet around amylase-producing colonies was noted and measured.

Proteases

Plates for detecting protease activity contained: nutrient broth (0.8% w/v), NaCl (0.4% w/v), agar (1% w/v), and gelatin (0.4% w/v). The plates were flooded with 1% (w/v) tannic acid for 10 min (Harrigan and McCance, 1966). After overnight incubation at appropriate temperatures, the absence of a white pellet around protease-producing colonies was observed.

Lipase

Plates for detecting lipase activity contained: nutrient broth (0.8% w/v), NaCl (0.4% w/v), agar (1% w/v), olive oil (2.5% w/v), and Rhodamine B (0.1% w/v). After overnight incubation at appropriate temperatures, the plates were observed under UV light and pale pink fluorescence around lipase-producing colonies was noted (Kouker and Jaeger, 1987).

Mannanase

Plates for detecting mannanase activity were prepared with yeast extract (0.2% w/v), peptone (0.5% w/v), $MgSO_4$ (0.5% w/v), NaCl (0.05% w/v), $CaCl_2$ (0.015% w/v), agar (2% w/v), and locust bean gum (1% w/v) (Fluka, CAS No. 9000-40-2). After overnight incubation at appropriate temperatures, the plates were flooded with 0.2% (w/v) aqueous Congo red for 15 min, followed by repeated washing with 1 M NaCl (Gessesse and Gashe, 1997). Appearance of a clearing zone around colonies that produce the enzyme was observed.

Xylanase

Plates for detecting xylanase activity were prepared with yeast extract (0.2% w/v), peptone (0.5% w/v), $MgSO_4$ (0.5% w/v), NaCl (0.05% w/v), $CaCl_2$ (0.015% w/v), agar (2% w/v), and xylan from beechwood (0.1% w/v) (Sigma, CAS No. 9014-63-5). After overnight incubation at appropriate temperatures, the plates were flooded with 0.1% (w/v) aqueous Congo red for 15 min, followed by repeated washing with 1 M NaCl (Gessesse and Gashe, 1997). Appearance of a clearing zone around colonies that produce the enzyme was noted.

For each isolate, three replicates were prepared. Plates were incubated at 25, 37, and 55°C for 24 h. For each of the three replicates, the diameter of each colony and activity zone was measured in two dimensions at 90° to each other and the values averaged. The index of relative enzyme activity (RA) for each isolate, substrate, and temperature combination was calculated by dividing the total area of activity (area of the clearing zone minus area of the colony) by area of the colony (Bradner et al., 1999).

RESULTS

Isolation and preliminary characterization of Bacillus sp. isolates

Using the feature of members of the genus *Bacillus* to form endospores resistant to elevated temperatures, 205 strains were isolated from 33 localities and from three ecological niches in Serbia: from straw and hay, 22; from soil, 126; and from manure, 57. Colonies with different morphology were subcultured on LA agar plates. All 205 isolates proved to be Gram-positive, endospore forming, catalase-positive rods.

RAPD and 16S rDNA analysis

After initial testing with several primers, we selected OPL3 and OPL12, which produced clear and reproducible polymorphic banding patterns, to test the whole collection of *Bacillus* strains. The RAPD analyses conducted with primer OPL3 generated 13 different profiles. Using primer OPL12, we were able to distinguish several subgroups within the 13 profiles, as shown in Table 1 and Fig. 1. The DNA from one representative strain from each group/subgroup was extracted and 16S rDNA gene fragments were amplified and sequenced. The GenBank database was used to compare 16S rDNA sequences and nucleotide identity was between 99.8 and 99.9% with the corresponding species, four in total. *Bacillus pumilus* was represented with three groups of differ-

Table 1. Characteristics of *Bacillus* strains isolated from different ecological niches.

Strain number	Isolation source	RAPD group	16S rDNA identification
2.1	Straw	I	*B. pumilus*
2.13		IIa	
4.1		IIb	
5.1		III	
37.7	Soil	IV	*B. subtilis*
6.1		Va	
14.4		Vb	
2.11	Straw	Vc	
10.3	Soil	VI	
1.1	Straw	VIIa	
21.6	Soil	VIIb	
26.5	Manure	VIII	
26.2	Manure	IX	*B. cereus/B. thuringiensis*
32.1	Manure	X	
20.1	Soil	Xia	
22.5	Soil	Xib	
37.2	Soil	XII	
17.3	Soil	XIII	
28.2	Manure	XIV	*B. firmus*

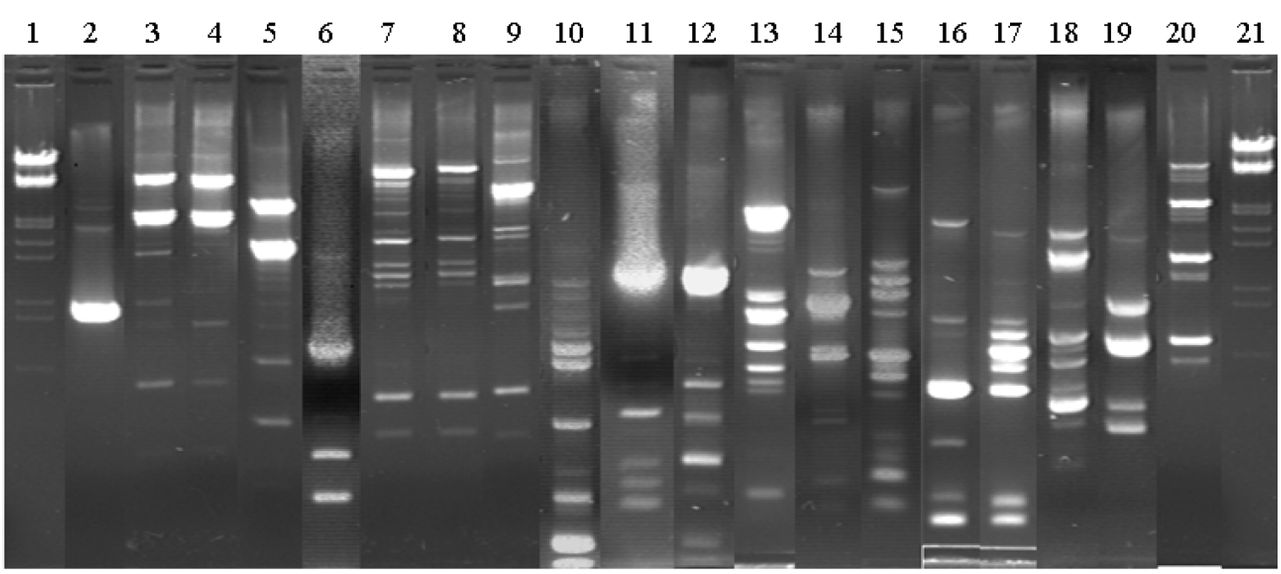

Fig. 1. Fingerprints obtained by RAPD analysis with OPL12 primer of *Bacillus* strains represented in Table 1. One strain of each group is represented. Lanes: 2, 3, 4, 5, *B. pumilus*; 6, 7, 8, 9, 10, 11, 12, 13, *B. subtilis*; 14, 15, 16, 17, 18, 19, *B. cereus*/*B. thuringiensis*; 20, *B. firmus*; 1 and 21, λEcoR1/HindIII ladder.

ent RAPD profiles (I, II, and III) with subgroups (IIa and IIb), 34 strains overall. *Bacillus subtilis* was represented with five groups of distinctive profiles (IV, V, VI, VII, and VIII) with subgroups (Va, Vb, Vc, VIIa, and VIIb), 110 strains in total. Sixty strains of *B. cereus*/*B. thuringiensis* isolates fell into five groups (IX, X, XI, XII, and XIII) with subgroups (XIa and XIb). *Bacillus firmus* had only one representative, strain 28.2. Distribution of the determined species of *Bacillus* in different habitats that were the source of isolation is represented in Fig. 2. From straw, 64% of isolates were *B. pumilus*, 27% *B. subtilis*, and 9% *B. cereus*/*B. thuringiensis*. From soil, the predominant species was *B. subtilis* (65%), followed by *B. cereus*/*B. thuringiensis* with 23% and *B. pumilus* with 12%. Among strains from manure, *B. subtilis* was

also dominant with 49%, followed by *B. cereus/B. thuringiensis* with 39%, *B. pumilus* with 11%, and only one strain of *B. firmus*.

Analysis of hydrolytic activity

The results of screening for hydrolytic activities of *Bacillus* isolates are shown in Table 2. As expected, most strains, produced amylase at 25 and 37°C. The percent of producing strains in each particular habitat ranged from 73 (hay and straw at 25°C) to 100 (manure at 25°C, and soil and manure at 37°C). At 55°C, production of amylase was retained in four strains isolated from straw and hay, 22 from soil, and 10 from manure.

Similar results were obtained for production of protease. However, fewer strains isolated from manure were positive for protease (68% at 25°C and 65% at 37°C), but as much as 37% of these strains kept their activity at 55°C. All strains that thrived in straw produced protease at 25 and 37°C, but none of them retained activity at 55°C. As much as 90% (at 37°C) and 93% (at 25°C) of strains isolated from soil produced protease to some extent, but only 14% of these strains still produced the enzyme when incubated at 55°C.

Hydrolysis of oil was observed for considerably fewer strains and only at 25 and 37°C: at 25°C, 23, 18, and 5%, from straw and hay, soil, and manure; respectively. Slightly more producers were found at 37°C: 27% from straw, 25% from soil, and 13% from manure.

The number of mannanase-producing strains isolated from straw and hay ranged from 95% at 25°C to 91% at 37°C. None of the strains produced the enzyme at 55°C. Eighty-five percent of strains isolated from soil produced mannanase at 25°C and 81% retained that ability at 37°C. Only two strains produced the enzyme at 55°C. From manure, 79% of strains produced mannanase at 25°C, 84% at 37°C, and as much as 33% at 55°C.

Xylanase at 25°C was produced by 81% of strains from straw, 65% from soil, and 63% from manure. At 37°C, there were 91% of producers from straw, 73% from soil, and 59% from manure. Only one strain, isolated from manure, produced xylanase at 55°C.

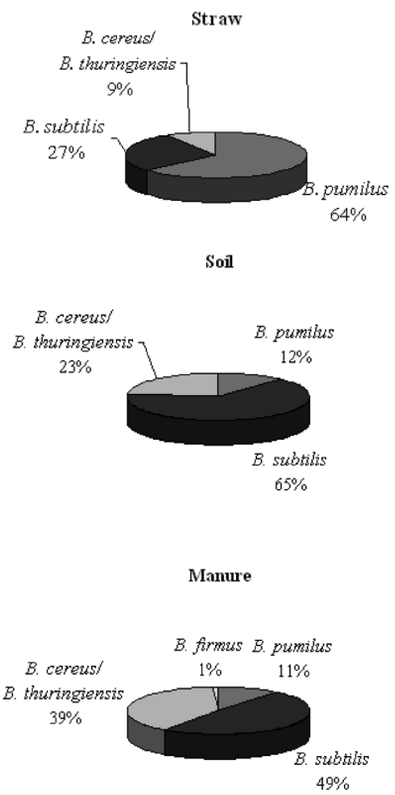

Fig. 2. Distribution of *Bacillus* species in different habitats.

The results of measuring relative activity of enzyme-producing isolates at different temperatures are shown in Fig. 3. For all enzymes tested, most producing isolates had low to medium hydrolytic activities (RA of 0-5) and only a small portion (or for some enzyme none) of isolates showed a high production rate (RA of 5-10).

DISCUSSION

Bacteria of the genus *Bacillus* are among the most widespread microorganisms in nature. In this work, as a source for isolation of strains we used straw and hay, soil, and manure from different locations in Serbia. Two hundred and five strains were preliminarily identified as members of the genus *Bacillus* (Stanković et al., 2007). Analysis by RAPD revealed considerable genetic diversity, represented by 13 distinctive groups (and six subgroups) of band profiles. Using sequencing of 16S rDNA, we established that all the observed diversity was from only four (five)

Table 2. Screening for hydrolytic activity in a *Bacillus* collection.

Temp. of incubation		25°C			37°C			55°C	
Habitat	S & h	Soil	Mn	S & h	Soil	Mn	S & h	Soil	Mn
Amylase producers	16/22 73%	118/120 98%	57/57 100%	21/22 95%	120/120 100%	57/57 100%	4/22 18%	22/120 18%	10/57 17%
Protease producers	22/22 100%	111/120 93%	39/57 68%	22/22 100%	107/120 90%	37/57 65%	0	17/120 14%	21/57 37%
Lipase producers	5/22 23%	22/120 18%	3/57 5%	6/22 27%	30/120 25%	7/57 13%	0	0	0
Mannanase producers	21/22 95%	102/120 85%	45/57 79%	20/22 91%	98/120 81%	48/57 84%	0	2/120 1%	19/57 33%
Xylanase producers	18/22 81%	79/120 65%	36/57 63%	20/22 91%	88/120 73%	34/57 59%	0	0	1/57 2%

species of *Bacillus*. More than half of the strains isolated belonged to *B. subtilis* (110) and, as statistically expected, this cluster of groups had considerable diversity. Nevertheless, *B. cereus/B. thuringiensis*, with 60 corresponding isolates has also considerable genetic heterogeneity, with five distinctive groups of profiles (but only one subgroup). *Bacillus cereus* and *B. thuringiensis* are essentially identical (Carlson et al., 1994) and can not be separated by 16S rDNA analysis. *Bacillus thuringiensis* can be distinguished only by the presence of intracellular protein crystals during sporulation (Hegason et al., 1998). The fact that some *B. thuringiensis* and *B. cereus* strains have a very efficient conjugation system (Gonzales et al., 1982; Jensen et al., 1996) facilitates the exchange of genetic material between strains (Hegason et al., 1998), making separation of DNA on the molecular level impossible. High local genotypic diversity in the cases of *B. subtilis* and *B. cereus/B. thuringiensis* can be attributed to recombination, which appeared to be frequent (Istock et al., 1992). *Bacillus pumilus* was represented by 34 isolates with three groups and one subgroup and less pronounced heterogeneity than in the other species. Finally, *B. firmus* was rep-

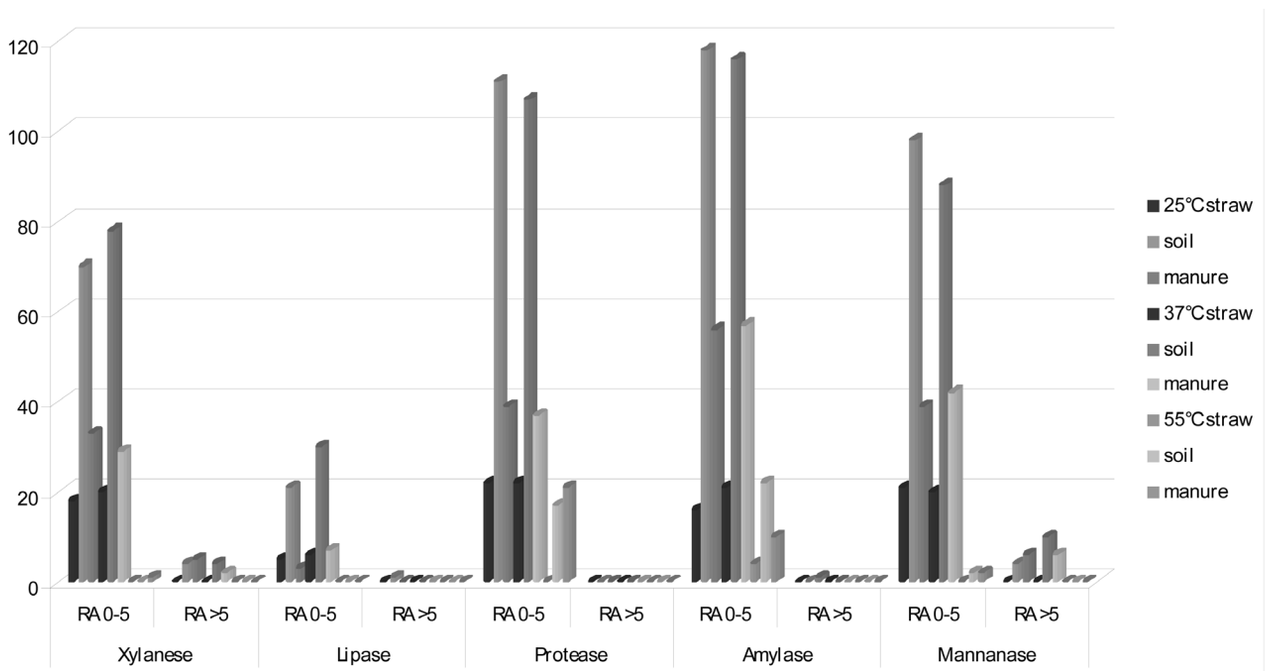

Fig. 3. Relative activity of enzyme-producing isolates at different temperatures.

resented with only one strain. As can be seen from Fig. 2, the dominant species in straw was *B. pumilus*, while *B. subtilis* was dominant in soil and manure. Manure was the habitat harboring the greatest diversity, with representatives of all four species. This is in accordance with previous findings that bacteria are the predominant microorganisms cultured during the entire composting process. They accounted for 80-90% of the microorganisms typically found in a gram of compost (Trautmann and Olynciw, 2000).

Examination of hydrolytic activities at temperatures of 25 and 37°C revealed no correlation with the specific habitat, i.e., straw and hay, soil, and manure were equally good sources for isolating producing strains. However, when monitoring hydrolytic activity at 55°C, manure stood out as the best source of enzyme-producing isolates (51) and soil as the second best (41). No correlation was perceived between enzyme production at 55°C and the particular species of *Bacillus*.

Regarding the efficacy of enzyme production (Fig. 3), for all enzymes and for all temperatures tested, most producing isolates had low to medium hydrolytic activities (RA of 0-5) and only a small portion (or for some enzymes none) of isolates showed a high production rate (RA of 5-10). In addition, no correlation was detected between the efficacy of enzyme production and the particular *Bacillus* species.

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RAPD АНАЛИЗА ГЕНСКОГ ДИВЕРЗИТЕТА И КВАЛИТАТИВНА ПРОЦЕНА ХИДРОЛИТИЧКЕ АКТИВНОСТИ ИЗОЛАТА ИЗ ИЗОЛАТА *BACILLUS* SP.

ТАЊА БЕРИЋ[1], MARIA C. URDACI[2] , С. СТАНКОВИЋ[1] и ЈЕЛЕНА КНЕЖЕВИЋ-ВУКЧЕВИЋ[1]

[1]*Катедра за микробиологију, Биолошки факултет, Универзитет у Београду,* 11000 Београд, Србија
[2]*Лабораторија за микробиологију и примењену биохемију,* 33175 Градињан, Француска

У овом раду проучавани су генетски диверзитет и производња хидролитичких ензима у колекцији од 205 изолата рода *Bacillus* који воде порекло из различитих еколошких ниша у Србији. Комбиновањем метода анализе RAPD и секвенцирања 16s rDNK издвојено је тринаест различитих група RAPD профила у оквиру четири (пет) врста: *B. subtilis*, *B. cereus/B. thuringiensis*, *B. pumilus* и *B. firmus*. Испитивање на производњу хидролитичких ензима је показало да већина изолата, из свих станишта, производи амилазу, протеазу, липазу, мананазу и ксиланазу у некој количини, на 25 и 37°C. Број изолата који задржава могућност производње ензима на 55°C је значајно мањи. Они су претежно изоловани из стајског ђубрива и није уочена корелација између производње ензима и припадности одређеној врсти.