Characterization of Indigenous *Bacillus* Isolates from Stabilized Sludge in Petrochemical Industry

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ABSTRACT

Bacillus species are rod-shaped, endospore-forming aerobic or facultatively anaerobic, Gram-positive bacteria; in some species cultures may turn Gram-negative with age. The many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment. The spores are resistant to heat, cold, radiation, desiccation, and disinfectants. *Bacillus* species are used in many medical, pharmaceutical, agricultural, and industrial processes that take advantage of their wide range of physiologic characteristics and their ability to produce a host of enzymes, antibiotics, and other metabolites. Certain *Bacillus* species are important in the natural or artificial degradation of waste products.

We isolated 15 indigenous *Bacillus* isolates from stabilized sludge in petrochemical plant in Serbia (FOV – HIP "Petrohemija", Pancevo) and investigated their morphological and biochemical characteristics, emulsification activity and sensitivity to antibiotics and heavy metals. In addition, we estimated the genetic diversity of isolates by RAPD and rep-PCR.

Three of 15 isolates showed very strong emulsification ability of xylol (E_{24} from 95 to 100). Six isolates showed strong emulsification of mineral oil (E_{24} from 78 to 100). All isolates were tolerant to 100µg/ml of Zn and Co, 10µg/ml of Hg and Mo, while eleven isolates showed tolerance to 10µg/ml of Cd and six isolates to 100µg/ml Hg. Only one isolate was sensitive to trimethoprim (5 µg). All isolates were sensitive to bacitracin (40U), cephalexin (30 µg), clindamycin (2 µg) and neomycin (120µg), while five isolates were resistant to novobiocin (5µg) and two to bacitracin (40U). Based on PCR analysis, we assessed genetic similarity of investigated *Bacillus* isolates.

Key words: *Bacillus* sp., hydrocarbon utilization, heavy metal tolerance, emulsification activity, BOX PCR, RAPD

INTRODUCTION

Safe disposal of oil sludge generated during the processing of crude oil is big problem in petroleum industry and oil refineries. The petroleum hydrocarbons from oil sludge have adverse effects on ecosystem and lead to environmental pollution. Oil sludge is a complex mixture of specific compounds such as alkane, aromatic, asfaltene fraction and heavy metals, and soil contamination may be caused by improper disposal. More than seventy microbial genera contain species able to degrade almost all fractions of hydrocarbons present in oil sludge, but degradation capability depends on many factors (Ryu et al., 2006). Isolation of indigenous microbial population from contaminated site may

help the bioremediation process because indigenous microorganisms can degrade specific constituents and have a higher tolerance to toxicity. Soil microorganisms are typically associated with organic fractions of the soil microenvironment and they participate in the metal dynamics typically ascribed to these fractions. Bacteria have a high surface-to-volume ratio and should have a high capacity for sorbing metals from solution (Beveridge, 1988). Metal binding by gram-positive and gram-negative bacterial cell walls has been evaluated (Marquis 1976, Doyle et al., 1980, Beveridge et al., 1985). Soil bacteria utilize and solubilize a variety of hydrocarbons by producing a variety of surface active agents named biosurfactants. Wide spectra of microbial compounds (glycolipids, lipopeptides, fatty acids, polymeric biosurfactants) have been found to have surface activity (Morikawa et al., 2000).

The heavy metals and hydrocarbons from oil sludge act as stress or selection agents and give rise to resistant and even hyper-resistant bacterial populations in the polluted areas. Considering that hydrophobic pollutants present in petroleum hydrocarbons require solubilization before being degraded by microbial cells, it is necessary to select appropriate microorganisms with environmental compatibility. In this work, we assessed the natural abiotic selection leading to resistant microbial population in the contaminated sites and investigated indigenous *Bacillus* (as the most abundant) population in stabilized sludge of HIP "Petrohemija", Pancevo, Serbia. The present study applies commonly used molecular techniques as rep-PCR and RAPD to estimate diversity of this population.

METHODS

Sensitivity of bacterial isolates to six antibiotics was assessed by agar diffusion method with followed antibiotics: novobiocin (5µg), trimethoprim (5 µg), bacitracin (40U), cephalexin (30 µg), clindamycin (2 µg) and neomycin (120µg). The investigated heavy metals were added in nutrient agar (NA) in following concentrations (µg/ml): 10, 20 cadmium sulphate (Cd); 100 cobalt sulphate (Co); 2, 4, 10, 15, 20 mercuric chloride (Hg); 100 zinc sulphate (Zn) and 50 sodium molybdate dihydrate (Mo). Substrate utilization of hydrocarbons was tested as described by Toledo et al. (2006). Biopolymer production and emulsification ability of xylole, toluene and mineral oil was carried out as described by Cooper and Paddock (1983). rep-PCR analysis using BOX type $(GTG)_5$ primer was performed as recommended by de Bruijn (1992), while RAPD analysis was done according to Dooley et al. (1993). Similarity was estimated by means of the simple matching coefficient (*S*SM) and clustering was based on the unweighted pair group arithmetic average-linkage algorithm (STATISTICA 7 software).

RESULTS and DISCUSION

In situ bioremediation using indigenous microorganisms is the most widely used technique for decontamination of affected sites, and reintroduction of indigenous isolates after enrichment is frequently used for this process (Mishra et al., 2001). We have isolated indigenous population of bacteria from stabilized sludge and polluted soil, for characterization and selection of isolates for possible use in bioremediation process.

Bacillus population was the most abundant, with 15 of 25 isolates, and it was compared with two strains previously isolated from oil polluted soil (BZi1 and BZi2). All isolates showed high sensitivity to applied concentrations of antibiotics, except to trimethoprim. All isolates were sensitive to cephalexin (30 µg), clindamycin (2 µg) and neomycin (120µg), while five isolates were resistant to 5µg novobiocin (BZi1, BZi2, 5B, 6B and 8B). Only one isolate was sensitive (BZi2) to 5 µg trimethoprim. Isolates BZi1 and 6B showed intermediate sensitivity to 40U bacitracin and 5 µg trimethoprim.

Tested *Bacillus* isolates were tolerant to 50 µg/ml Mo, 100 µg/ml Zn and Co, except isolate 10B. Isolate 17B was sensitive to 10 µg/ml Cd, while other isolates grew well on this concentration, but showed differences on 20 µg/ml Cd (Table 1.). All isolates showed tolerance to 2 µg/ml Hg. Nine isolates were tolerant to 4 and 10 µg/ml Hg, while 7 showed tolerance to 15 and 20 µg/ml Hg. Results showing significant differences in tolerance of isolates to heavy metals are presented in Table 1.

Isolate	heavy metals (µg/ml)			substrate utilization				emulsification activity (E_{24})		
	Hg 10	Hg 20	Cd 20	toluene 1%	xylole 1%	mineral oil 0,5%	crude oil 0,5%	toluene	xylole	mineral oil
BZi1	-	-	-	-	+	+	+	74.42	62.50	78.05
BZi2	-	-	-	-	-	+	-	67.44	65.00	89.74
5B	+	+	+	±	-	+	±	90.30	75.61	80.03
6B	+	+	+	+	±	+	±	97.62	95.12	80.49
7B	+	+	+	±	±	+	±	97.67	82.50	78.05
8B	+	-	+	±	±	+	±	95.35	97.56	75.61
9B	-	-	-	±	±	-	+	95.45	98.12	78.05
10B	-	-	-	±	-	-	-	88.10	49.98	78.51
11B	-	-	±	±	±	-	-	nd	71.00	99.80
12B	+	+	-	+	+	+	+	97.67	73.17	87.50
15B	+	-	+	-	-	+	+	78.05	58.54	85.71
16B	-	-	-	-	-	-	±	64.29	58.62	85.37
17B	-	-	-	±	-	-	±	71.43	78.05	88.10
18B	±	±	+	+	+	+	+	45.45	85.37	78.05
19B	±	-	+	+	+	+	+	90.70	85.00	99.12
23B	-	-	-	+	+	+	+	90.48	65.00	92.68
25B	+	-	+	-	±	-	-	57.14	65.00	73.14

(-) no growth; (±) poor growth; (+) good growth; (nd) not detected

Table 1. Substrate utilization, emulsification activity and heavy metal tolerance of *Bacillus sp.* isolates

Fourteen tested *Bacillus* isolates from stabilized sludge and two from oil-polluted soil showed multiple tolerances to heavy metals. Obtained results were in agreement with several previous reports (Silver and Phung, 1996). Bacteria have developed a variety of resistance mechanisms to counteract heavy metal stress. These mechanisms include the formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and direct efflux of a metal out of the cell. In bacteria, efflux systems are a more common resistance mechanism for dealing with heavy metals. One type of efflux systems found in gram-positive bacteria pumps out Cd^{2+} and Zn^{2+} by using a phospho-aspartate intermediate (Nies, 1999, Outten et al., 2000) and enables multiple tolerances.

Utilization of toluene, xylole, mineral and crude oil as a sole carboin source added to Bushnell-Hass (BH) medium is shown in Table 1. Four isolates exhibited abundant growth on all substrates, three isolates grew on all substrates but with different efficiency, and two isolates could utilize only three substrates. Four isolates grew only on one substrate. Growth of different *Bacillus* strains in the presence of different PAHs was previously reported by Hubert et al., (1999), Baldwin et al (2000), Barathi et al., (2001) and Toledo et al. (2006). *B. macroides* strain exhibited a degradation ability of mixed hydrocarbon consisting of benzene, toluene and xylole, while *B. pseudomegaterium* degraded mixture of toluene and naphthalene (Green et al., 2000).

Hydrophobic pollutants present in petroleum hydrocarbon require solubilization before being degraded by microbial cells. Many bacteria produce extracellular compounds that can emulsify or disperse this phase and make it available for utilization. Some *Bacillus subtilis* strains produced high yields of active surfactant when the growth medium contained a hydrocarbon as only carbon sources, while *Corynebacterium fascians* was able to stabilize emulsions of water and hydrocarbons even if grown on medium which did not contain hydrocarbon (Cooper and Paddock, 1983). In our investigation, we tested emulsification activity of soluble extracellular agents from cell-free broth as recommended by Cooper and Paddock (1983). E_{24} index (percent of emulsified volume after 24h) ranged from 45.45 to 97.67 for toluene, 49.98- 98.12 for xylole and 73.14 to 99.80 for mineral oil. Isolates from polluted soil had significantly lower index then many isolates from stabilized sludge. Many isolates showed very high emulsification activity on all investigated substrates (5B, 6B, 7B, 8B, 9B, 12B, 19B, 23B) and were also able to utilize them. Similar results for emulsification activity of *B.subtilis* strains were described by Toledo et al., (2006): 65.6 on xylole, 70 on toluene, 42.6 on mineral oil and 75.9 on crude oil. Our isolate 11B showed almost 100% emulsification of mineral oil, but wasn't able to degrade it. Contrary to conventional arguments that microbial emulsifiers are produced solely to facilitate the uptake of water-insoluble substrates, Cooper and Paddock (1983) described the yeast strain *Torulopsis petrophylum* effective at generating surface-active agents, but without possibility to uptake hydrocarbons. The complex physiological roles of biosurfactants (bacterial motility, signaling, differentiation, biofilm formation, toxicity) and roles in bioremediation were reviewed by Van Hame et al. (2003).

Repetitive sequence-based PCR (rep-PCR) genomic fingerprinting is used for species and strain specific fingerprinting of different bacteria (Versalovic et al., 1994; Laguerre et al. 1996; Jussila et al., 2006). DNA primers corresponding to BOX elements sequences and repetitive trinucleotide $(GTG)_5$ as one of them were the most useful methods for monitoring the diversity of culturable bacteria during in situ bioremediation (Jussila et al., 2006). In our investigation, $(GTG)_5$-PCR produced clear and well-separated fingerprints consisting of short and long PCR products in most of the isolates (Figure 1). Results derived from $(GTG)_5$ -genotyping are presented as dendrogram in Figure 2. One group of isolates (9B, 10B, 12B and 18B) showed only small-size bands and clustered separately with 75% dissimilarity from the other cluster. The similarity level in second cluster varied from 42 (isolate 25B) to 100% (isolates 5B and 8B; 16B and 17B). Isolates from polluted soil were distinguished into two subclusters: BZi1 isolates was in one cluster with 67% similarity to 19B and 23B, while BZi2 isolate was in the second cluster and showed lower similarity (58%) to subgroup of 8 (71% similar) isolates.

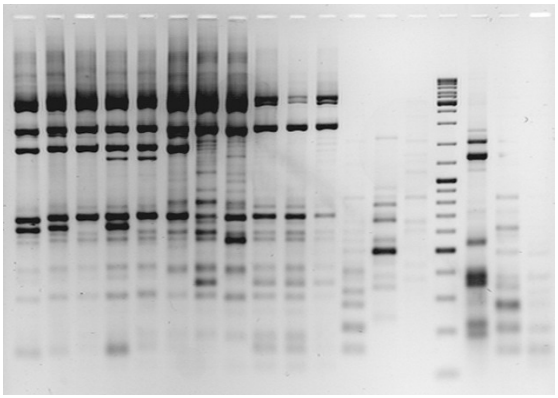

Figure 1. $(GTG)_5$ -PCR fingerprinting of some *Bacillus* isolates from stabilized sludge in petrochemical plant in Serbia (lane 1-5: **5B; 8B; 7B; 6B; 15B;** lane 9-11: **16B; 17B; 11B;** lane 16-18: **25B; 19B; 23B**, respectively) and from polluted soil (lane 12: **BZi1**; lane 14: **BZi2**). Lane 15: Molecular marker GeneRuler DNA Ladder mix SM0331 (Fermentas)

To confirm that isolates 5B and 8B, and 16B and 17B are genetically identical as obtained by $(GTG)_5$ -genotyping, we used additional PCR methods- Random Amplified Polymorphic DNA (RAPD) fingerprinting. This method has already been used to differentiate several related species and strains of bacteria (Nakamura, 2000) and closely related *Bacillus sphaericus* strains (Woodburn et al., 1995). Results of RAPD analysis of fifteen sludge isolates and two isolates from polluted soil using SPH1 primer are shown on Figure 3.

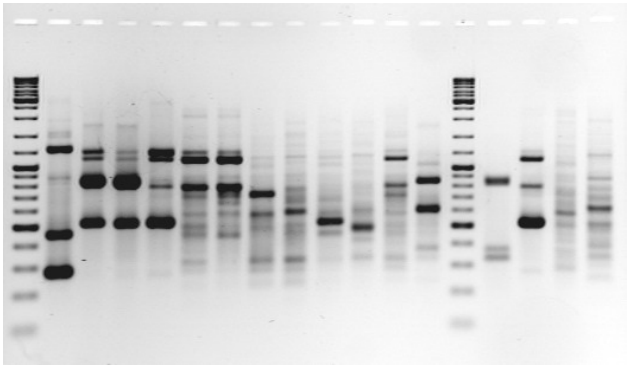

Figure 3. RAPD fingerprinting of some *Bacillus* isolates from stabilized sludge in petrochemical plant in Serbia (lane 3-8: **5B; 8B; 6B; 16B; 17B; 9B;** lane 10-13: **7B; 10B; 11B; 12B;** lane 15-18: **14B; 15B; 18B; 19B** , respectively) and from polluted soil (lane 2: **BZi2**; lane 9: **BZi1**). Lane 1 and 14: Molecular marker GeneRuler DNA Ladder mix SM0331 (Fermentas)

Isolates were grouped in tree clusters with 55 and 67% similarity between clusters (Figure 4.). Isolates 23B and 25B formed one cluster (45% differences) that was distant from all other isolates (72%). The level of similarity for isolates in the other two clusters ranged from 67 to 94%.

Genetic identity between isolates 5B and 8B was confirmed by RAPD analysis. Isolates 16B and 17B, identical by $(GTG)_5$ -genotyping, showed very high similarity level (94%) by RAPD, but were not identical. These isolates also exhibited differences in toluene utilization and very small differences in emulsification activity. In contrary, isolates 5B and 8B, genotypically identical, showed different tolerances on 20 µg/ml Hg and utilization and emulsification of xylole. It might be necessary to apply more then one RAPD primer to confirm genetic identity of these isolates.

The grouping results derived from two PCR methods were generally in good agreement. Group of isolates (5B, 8B, 6B and 11B) showed 67% similarity by SPH1 and 71% by $(GTG)_5$ primer. Isolates 9B, 10B and 18B also clustered together with both primers, while isolates 25B, 12B, 23B, BZi1 and BZi2 grouped differently. These results are comparable to data reported by Jussila et al. (2005) which grouped 5 *B. macroides* strains in 3 groups by $(GTG)_5$ genotype, and Woodburn et al. (1995) with 15 to 84% similarity levels of *B. sphaericus* isolates assessed by RAPD method.

Characterization of *Bacillus* isolates obtained by using two different primers, different substrate utilization and emulsification ability, and sensitivity to heavy metals, represents the first step towards selection of the most tolerant and active isolates for future bioremediation applications.

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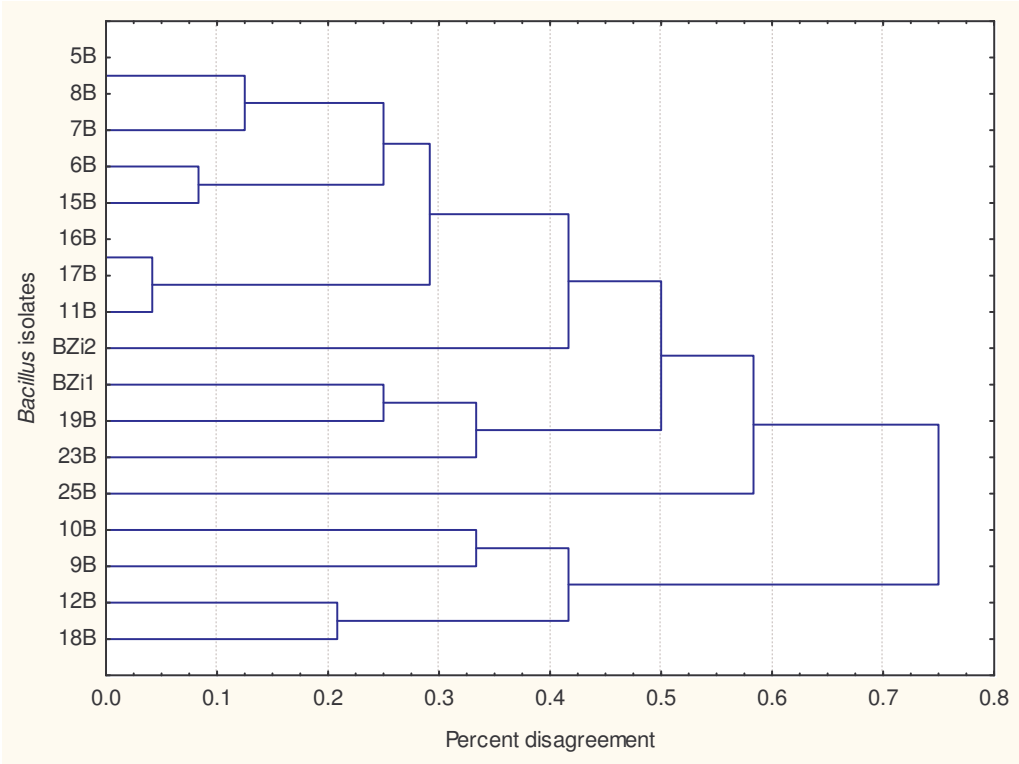

Figure 2. Dendrogram of $(GTG)_5$ -PCR fingerprinting of *Bacillus* isolates from stabilized sludge in petrochemical plant in Serbia and from polluted soil (BZi1 and BZi2)

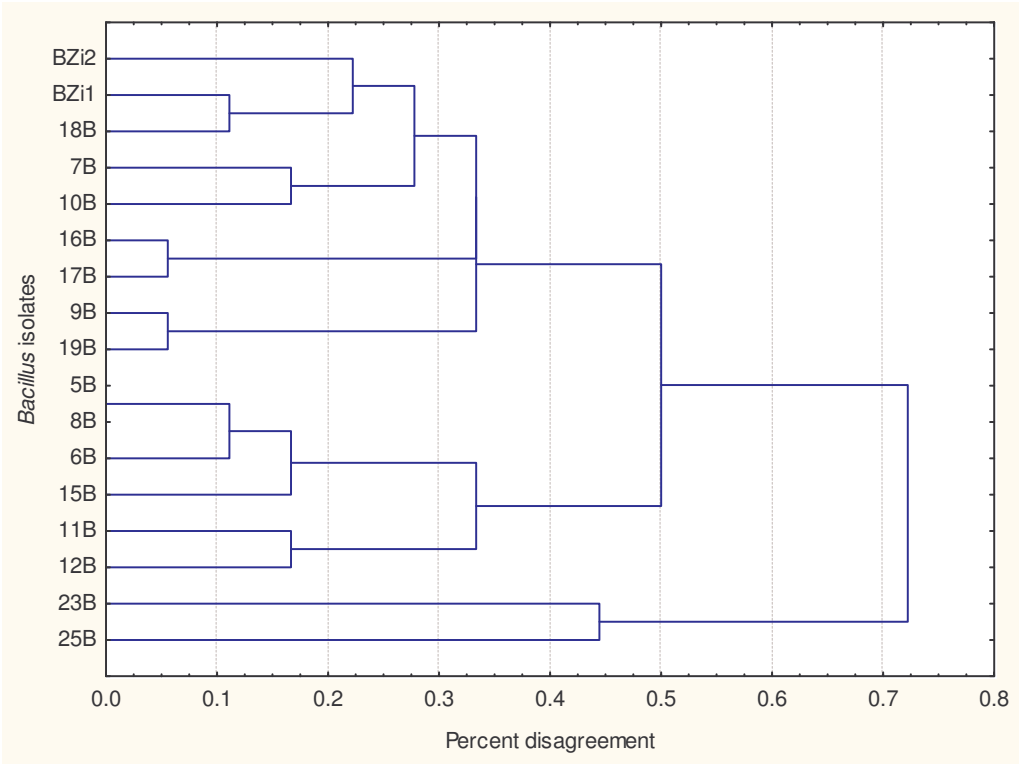

Figure 4. Dendrogram of RAPD fingerprinting of *Bacillus* isolates from stabilized sludge in petrochemical plant in Serbia and from polluted soil (BZi1 and BZi2)