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ISOLATION AND CHARACTERIZATION OF BACTERIA AND YEASTS FROM CONTAMINATED SOIL

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Abstract: Plant growth promoting (PGP) bacteria and yeasts play an important role in bioremediation processes. Thirty bacterial and ten yeast isolates were obtained from PAH and PCB contaminated soil with an aim of determining the presence of PGP mechanisms (production of ammonia, indoleacetic acid, siderophores and solubilization of inorganic phosphate). As a result, three bacterial (*Serratia liquefaciens*, *Micrococcus* sp. and *Serratia* sp.) and two yeast isolates (*Candida utilis* and *Candida tropicalis*) were recognized as PGP strains. Among them, *Serratia* sp. showed the highest indole production (25.5 μg/ml). Analyses of metal tolerance (Cu^{+2}, Cr^{+6} and Ni^{+2}) revealed that *Serratia liquefaciens*, *Micrococcus* sp., *Serratia* sp. and *Candida tropicalis* were capable to tolerate significant concentration of metals. As a result of this study several bacterial and yeast strains were attributed as potential plant growth promoters which can be applied in future remediation activities and environmental quality improvements.

Key words: plant growth promotion, microorganisms, heavy metals.

Introduction

The constant application of chemicals and mineral fertilizers in agriculture exerted a negative impact on soil microorganism fund and environmental conditions in general (Rodrigez et al., 2006; Smith and Read, 2008). Concern for the environmental health has developed in parallel with the interest for plant growth promoting (PGP) microorganisms. Plant growth promoting (PGP) microorganisms as inhabitants of rhizosphere, root surface and root inner tissues

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(Hayat et al., 2010) are well known for their beneficial effects on plant growth. This group consists of plant growth promoting bacteria (PGPB) and plant growth promoting yeasts (PGPY). Plant growth promotion and stabilization of plant physiological state and health are a result of wide range of mechanisms. Some of them are well studied while others are not fully described. The positive effect on plant growth is a result of plant mineral nutrition improvements (N_2 fixation, solubilization of mineral phosphate and other nutrients), melioration of phytohormone status, mechanisms that raise the resistance to biotic and abiotic stresses and soil-borne diseases (Glick, 2012).

The products that contain live formulations of beneficial microorganisms are referred to as biofertilizers or biocontrol agents and those products are capable to completely or partly supplant mineral fertilizers and pesticides (Compant et al., 2005; Berg, 2009). The application of such products leads towards soil health improvements, re-establishing of biological balance (Christry and Ramaligam, 2005), increase of soil fertility and soil contamination reduction (Tilak et al., 2005).

Literature data emphasize the role of PGPB and PGPY in bioremediation processes (Huang et al., 2005; Kavamura and Esposito, 2010). Those microorganisms are capable to change metal bioavailability, availability of the pollutants, alter plant cell metabolism, and raise plant tolerance towards heavy metals (Welbaum et al., 2004) with simultaneous growth promotion (Ma et al., 2011). In these combinations, some microbes are directly involved in the degradation of the organic soil contaminants while others can positively affect plant growth and health. All this leads to higher phytoremediation effectiveness. The role of yeasts in plant growth promotion has received less attention even though literature data show that a diverse range of soil yeasts use similar mechanisms as soil bacteria (Mirabal et al., 2008).

Over the time, microorganisms originated from contaminated soil or waste waters have been proven as powerful bioremediation agents (Trama et al., 2014). Keeping this in view, a field survey was conducted to collect samples from PAH and PCB contaminated soil followed by isolation of microorganisms and *in vitro* characterization. The objective was to find out the promising native PGP strains of bacteria and yeasts which can be used in further bioremediation practice.

Material and Methods

The site of interest was City Park in Tivat, Montenegro. Previous researches of this site emphasize the problem with heavy metals (nickel, chromium), PAHs, PCBs and organotin compounds (Jovičić Petrović et al., 2014; Karličić et al., 2014). Chemical characteristics of a composite sample are presented in Table 1.

Isolation and purification of bacteria and yeasts. The isolation of bacteria was conducted by the serial dilution method in combination with plating on nutrient

agar (Torlak, Serbia), citrimide (Merck, USA), Fjodorov agar (Anderson, 1958). Yeasts were isolated by the enrichment technique followed by plating on yeast maltose (YM) agar (Bajić et al., 2015). Randomly picked colonies were purified and maintained on the respective slants until further testing.

Table 1. Chemical characteristics of the soil.

pH		Humus %	Organic C %	Nitrogen			C/N	P_2O_5 mg/100g	K_2O mg/100g
H_2O	KCl			Total %	NH_4^+ mg/kg	NO_3^- mg/kg			
7.25	7.08	3.62	2.101	0.197	14	47.6	10.7:1	26.8	19.7

In vitro screening for plant growth promoting (PGP) activities.

NH_3 production. Isolates were tested for the production of ammonia in peptone water. Tubes with peptone water (10 ml) were inoculated with freshly grown cultures and incubated for 48 to 72 h at 28±2ºC. Nessler's reagent (0.5 ml) was added in each tube according to the previously described method (Cappuccino and Sherman, 1992).

Assay for indoleacetic acid (IAA) production. IAA was quantified by using the colorimetric assay based on the Salkowsky reagent (Patten and Glick, 2002). Absorption was read at 535 nm (T70 UV/VIS Spectrometer, PG Instruments LTD, UK).

Phosphate solubilizing activity. Bacterial strains were evaluated for their ability to solubilize inorganic phosphate by screening on National Botanical Research Institute's phosphate growth medium (NBRIP) containing l^{-1}: glucose, 10 g: $Ca_3(PO4)_2$, 5 g; $MgCl_2x6H_2O$, 5 g; $MgSO_4x7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g; agar, 1.5%. Tri-calcium phosphate represented an insoluble inorganic form of phosphate. The experiment was performed three times with three replicates for each bacterial and yeast strain. After 14 days of incubation at 30°C, the halo zones and colony diameters were measured. Solubilisation Index (SI) = halo diameter (mm)/colony diameter (mm) was calculated (Berraquero et al., 1976).

Siderophore production. Assaying for siderophores production was performed on the Chrome azurol S agar medium (Sigma Aldrich, USA) described by Schwyn and Neilands (1987). Chrome azurol S agar plates were prepared and divided into three equal sectors and spot inoculated with the test organism (10 µl of 10^6CFU/ml) and incubated at 28±2°C for 72 h.

Identification of isolates and enzyme profiling. The isolates that were characterized as PGP were identified by the API20NE, API Staph kits for bacterial isolates and API AUX gallery for yeasts (bioMérieux, France). The API ZYM kits (bioMérieux, France) were used for detecting enzyme profiles of selected isolates.

Analyses of metal tolerance. The Minimal Inhibitory Concentration (MIC) was determined using Mueller–Hinton (Merck, USA) medium (bacteria) and YPD medium (yeasts) containing Cu^{+2} added in a form of $CuSO_4 \cdot 5H_2O$, Cr^{+6} ($K_2Cr_2O_7$) and Ni^{+2} ($NiCl_2 \cdot 6H_2O$) at concentrations ranging from 0.15 mM to 20 mM.

Results and Discussion

Soil samples used as a source of PGP isolates showed the satisfying content of humus which is very important for growth of diverse microbial communities. Thirty bacterial and ten yeast isolates were obtained from the samples. The bacterial isolates were determined as members of fam. *Enterobacteriaceae, Micrococcaceae* and *Bacillaceae* and yeasts as members of *Candida* sp., *Kloeckera sp*. genera based on cultural, morphological and biochemical characteristics (data not shown).

The PGP screening of those isolates revealed the presence of several promising strains. After PGP characterization, the most promising isolates were identified by the appropriate API kit as: *Serratia liquefaciens* (BKC ZI), *Micrococcus* sp. (BKC ZI1), *Serratia sp.* (BKC 333), and in case of yeasts *Candida tropicalis* (YST 3T), *Candida utilis* (YST 2K). Table 2 shows the isolates that expessed two or more PGP attributes.

Table 2. PGP features of isolates.

Isolates	NH_3 production	IAA production µg/ml	P solubilisation index (S.I.)	Siderophore production
Bacteria				
BKC ZI	+	8.4 ± 0.21	1.3	+
BKC ZI1	+	9.6 ± 0.25	1.6	+
BKC G1SP	+	2 ± 0.13	GNS	/
BKC 2T	+	10.5 ± 0.25	GNS	/
BKC GSP	+	5.75 ± 0.18	GNS	/
BKC 3O	+	1.1 ± 0.11	GNS	/
BKC 333	+	25.5 ± 0.42	1.11	+
BKC F	+	3.7 ± 0.18	/	/
Yeasts				
YST 3T	/	2.6 ± 0.15	1.09	/
YST 2K	+	1.1 ± 0.10	GNS	/

GNS = Grew and did not solubilise.

Many of tested isolates were capable to produce ammonia and the rarest feature was the production of siderophores. The production of siderophores can affect iron availability of heavy metal-bearing Fe (Braud et al., 2009) and *Serratia liquefaciens, Micrococcus* sp. and *Serratia* sp. tested in our study showed an ability to bind Fe^{3+} ions and facilitate their absorption which was confirmed in previous

studies (Koo and Cho, 2009). One of the most interesting mechanisms is the production of indoleacetic acid and *Serratia liquefaciens, Micrococcus* sp., *Serratia* sp., *Candida tropicalis* and *Candida utilis* were positive for IAA production. In this research, the isolate identified as *Serratia* sp. showed the highest IAA production (25.5μg ml^{-1}). Root development can be promoted by much smaller amounts (0.7 μg ml^{-1}) (Teixeira et al., 2007). Both *Serratia* strains, *Micrococcus* sp. and *Candida tropicalis* showed the ability to solubilize the inorganic form of phosphorus and all of them were positive for the presence of acid phosphatase, the enzyme responsible for inorganic phosphate solubilization (Behera et al., 2013). Enzymatic profiles suggest that some of our isolates have the potential antagonistic activity towards phytopathogenes. Lytic enzymes (*β*-glucosidase, N-acetyl-*β*-glucosaminidase, lipases) included in the suppresion of soil-borne pathogens (Glick, 2012) were produced by several isolates that we marked as the most promising ones.

Qualitatively, enzimatic profiles of promising stains are presented in Table 3. *Serratia liquefaciens*, *Serratia* sp. and *Candida tropicalis* were positive for the presence of *β*- glucosidase and N-acetyl-*β*-glucosaminidase, enzymes involved in biocontrol mechanisms.

Table 3. Enzymatic profiles of PGP stains.

Enzymes	Bacteria			Yeasts	
	S. liquefaciens	*Micrococcus sp.*	*Serratia sp.*	*C. tropicalis*	*C. utilis*
Alkaline phosphatase	+	-	+	+	-
Esterase (C4)	+	+	+	-	+
Esterase Lipase (C8)	+	-	+	+	+
Lipase (C14)	+	-	+	-	-
Leucinearylamidase	+	-	+	+	+
Valinearylamidase	+	-	+	-	+
Cysteine arylamidase	+	-	+	-	-
Trypsin	-	-	+	+	-
α-chymotripsin	-	-	+	-	-
Acid phosphatase	+	+	+	+	-
Naphthol-AS-BI-hosphohydrolase osphohydrolase	+	-	+	+	+
α-galactosidase	-	-	-	-	-
β-galactosidase	+	-	+	-	-
β-glucuronidase	-	-	-	-	-
α-glucosidase	+	-	+	+	-
β-glucosidase	+	-	+	+	+
N-acetyl-β-glucosaminidase	+	-	+	+	-
α-mannosidase	-	+	-	-	-
α-fucosidase	+	-	-	+	-

Isolates that were set aside due to their PGP characteristics were further tested for heavy metal tolerance (Table 4).

Table 4. Heavy metal tolerance of PGP isolates.

Heavy metal concentrations (mM)	Bacteria			Yeasts	
	S. liquefaciens	*Micrococcus spp.*	*Serratia sp.*	*C. tropicalis*	*C. utilis*
Copper					
1.2	+	+	+	+	+
2.5	+	-	+	+	-
5	+	-	+	-	-
10	+	-	+	-	-
20	-	-	-	-	-
Chromium					
0.6	+	+	+	+	+
1.2	+	+	-	+	-
2.5	+	+	-	-	-
5	-	-	-	-	-
Nickel					
1.2	+	+	+	+	+
2.5	-	-	-	+	-
5	-	-	-	+	-
10	-	-	-	-	-

The MICs of the isolates ranged from 0.15 mM to 20 mM. *Serratia* stains were capable to tolerate Cu^{+2} concentrations of 10mM. Those stains showed a higher tolerance compared to results obtained by Sarma et al. (2013) where MICs were at 5 mM Cu^{2+}. Among yeasts, only *Candida tropicalis* was able to grow at 2.5 mM Cu^{2+} concentration. *Serratia liquefaciens* was able to tolerate higher Cr^{+6} concentrations (2.5 mM) which is a good result since heavy metal resistance is not a common property of fam. *Enterobacteriaceae* (Campos et al., 2013). The 5 mM concentration of Cr^{+6} caused inhibitions of all tested isolates while Ni^{+2} concentration of 2.5 mM caused inhibition of all isolates except *Candida tropicalis.* A similar level of tolerance was shown by *Micrococcus* sp. where Ni^{2+} caused microbial growth supression at 1.2 mM. The only isolate that can tolerate a higher amount was *Candida tropicalis* (5 mM Ni^{2+}) which showed higher resistance compared to *Candida maltosa* which was able to tolerate 3 mM of Ni^{2+} (Breierova et al., 2008).

Apart from being capable to promote plant growth, selected isolates were also capable to propagate at high concentrations of some heavy metals. These results suggest that our strains can promote plant growth even in soils contaminated with heavy metals. The fact that they were isolated from PAH and PCB contaminated

soil confirms their fitness to higher concentrations of such contaminants. The plant growth promoting bacteria and yeasts are capable to accelerate germination, seedling emergence, plant growth and biomass production (Lucy et al., 2004), to increase resistance to environmental stresses (Glick, 2012) and Huang et al. (2005) consider those effects responsible for the pollutant removal.

Conclusion

The main pool of PGP bacteria and yeasts suitable for application in bioremediation is a particular contaminated site. The presented study showed that indigenous bacteria and yeasts had plant growth promoting features and also an ability for the successful propagation in presence of heavy metals. The potential carried by those microorganisms needs to be incorporated in all future actions directed towards ecosystem recovery.

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R e z i m e

Zemljište predstavlja dinamičan ekosistem naseljen brojnim mikroorganizmima među kojima su bakterije najbrojnije. Najveći broj je skoncentrisan u uskoj zoni zemljišta koja okružuje koren i koja se naziva rizosfera. Procenjuje se da 1–2% bakterija koje naseljavaju ovu zonu imaju sposobnost da promovišu rast biljaka (engl. *plant growth promoting bacteria – PGPB*). Pored bakterija, sve više je podataka da i zemljišni kvasci poseduju ove sposobnosti. Mikroorganizmi koji stimulišu rast biljaka koriste različite mehanizme kojima povećavaju dostupnost nutrijenata biljkama, regulišu njihov hormonski status kao i odnos prema biljnim patogenima. Bakterije i kvasci koji stimulišu rast biljaka igraju važnu ulogu i u procesima bioremedijacije. Trideset bakterijskih i deset izolata kvasaca je izolovano iz kontaminiranog zemljišta (PAH i PCB) i testirano na prisustvo mehanizama kojima se pospešuje rast biljaka. Cilj je bio doći do sojeva koji su prilagođeni na život u zagađenom zemljištu, a istovremeno imaju i potencijalno stimulativno dejstvo. Nakon izvršenih biohemijskih analiza (produkcija amonijaka, indol-sirćetne kiseline, siderofora, rastvaranje neorganskog fosfora) u stimulatore biljnog rasta svrstana su tri bakterijska (*Serratia liquefaciens*, *Micrococcus* sp. i *Serratia* sp.) i dva izolata kvasaca (*Candida utilis* i *Candida tropicalis*). Među njima najveću sposobnost produkcije indol-sirćetne kiseline je pokazao izolat *Serratia* sp. (25,5 μg/ml). Analize tolerancije na prisustvo teških metala (Cu^{+2}, Cr^{+6} i Ni^{+2}) pokazale su da su izolati *Serratia liquefaciens*, *Micrococcus* sp., *Serratia* sp. i *Candida tropicalis* sposobni da podnesu više koncentracije. Rezultati ovih istraživanja mogu imati praktičnu primenu u budućim remedijacionim aktivnostima i unapređenju kvaliteta životne sredine.

Ključne reči: stimulacija rasta biljaka, mikroorganizmi, teški metali.

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