Stimulation of diesel degradation and biosurfactant production by aminoglycosides in a novel oil-degrading bacterium *Pseudomonas luteola* PRO23

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Abstract

Bioremediation is promising technology for dealing with oil hydrocarbons contamination. In this research growth kinetics and oil biodegradation efficiency of *Pseudomonas luteola* PRO23, isolated from crude oil-contaminated soil samples, were investigated under different concentrations (5, 10 and 20 g/L) of light and heavy crude oil. More efficient biodegradation and more rapid adaptation and cell growth were obtained in conditions with light oil. The 5 to 10 g/L upgrade of light oil concentration stimulated the microbial growth and the biodegradation efficiency. Further upgrade of light oil concentration and the upgrade of heavy oil concentration both inhibited the microbial growth, as well as biodegradation process. Aminoglycosides stimulated biosurfactant production in *P. luteola* in the range of sub-inhibitory concentrations (0.3125, 0.625 μg/mL). Aminoglycosides also induced biofilm formation. The production of biosurfactants was the most intense during lag phase and continues until stationary phase. Aminoglycosides also induced changes in *P. luteola* growth kinetics. In the presence of aminoglycosides this strain degraded 82% of diesel for 96 h. These results indicated that *P. luteola* PRO23 potentially can be used in bioremediation of crude oil-contaminated environments and that aminoglycosides could stimulate this process.

Keywords: biodegradation, crude oil, aminoglycosides, *Pseudomonas luteola*.

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Oil pollution accidents have become a common phenomenon and have caused serious environmental problems, such as introduction of toxic compounds in food chains and changes in physical and chemical properties of the soil [1]. One of the highly efficient methods in remediation of oil polluted soil is bioremediation, where contaminants are degraded or transformed to less hazardous compounds through biological processes [2].

Bacteria of the genus *Pseudomonas* are highly capable to adapt on conditions in oil contaminated sites and can use different hydrocarbons as energy sources [3,4]. Therefore, *Pseudomonas* strains are commonly applied in *ex situ* bioremediation methods [5]. In these methods the first step is to isolate and characterize microorganisms that can use the oil contaminant as an energy source. Another step is to define conditions in applied bioreactors which are optimal for microbial growth and biodegradation [6]. These conditions include the type of oil and its concentration [7]. Some oil types stimulate microbial activity with an upgrade of its concentration. This is happening until reaching a specific concentration threshold, afterwards toxic compounds in oil can inhibit microbial growth and the biodegradation process [8]. This raises the importance of defining optimal concentrations for specific oil types.

Furthermore, microbial activity could be stimulated by adding chemical inducers in the bioreactors. For defining such inducers it is crucial to know how the strain of interest adapts to oil as its only carbon source. In genus *Pseudomonas* adaptation is achieved by biosurfactant production and biofilm formation [9]. Biosurfactants allow more efficient oil emulsification, which increases its accessibility for degradation. Biosurfactant production and oil degradation could be stimulated by aminoglycosides [10]. In subinhibitory concentrations aminoglycosides start acting as alternative signaling molecules, modulating gene expression, biosurfactant production and biofilm formation in *Pseudomonas* [11].

The aim of this research was isolation and identification of new oil and diesel degrading bacterial strain, and stimulation of biosurfactant production and diesel degradation using the aminoglycosides in sub-inhibitory concentrations.

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MATERIAL AND METHODS

The soil samples were aseptically collected from the zone of used oil re-refining facility in the Belgrade Oil Refinery (Serbia). Isolation of microorganisms resistant on light and heavy crude oil was achieved by diluting 1 g of soil in 100 mL of liquid mineral medium [12], containing 1g/L NH_4NO_3, 0.25g/L K_2HPO_4, 50mL/L soil extract, with addition of 10 g/L light or heavy crude oil (oil drill "Rusija", Serbia). Those enriched suspensions were then incubated on orbital shaker (ES-20, Biosan, Latvia) at 30 °C and 200 rpm for 30 days. Isolation and enumeration of *Pseudomonas* sp. from enrichment suspensions were proceeded on *Pseudomonas* Cetrimide agar with addition of *Pseudomonas* C-N SR0102E supplement (Oxoid). After 48 h of incubation (30 °C) all developed colonies were morphologically identical and further purification and identification was continued on nutrient agar. Ability of the isolate to use crude oil as the only carbon source was investigated by cultivating the strain on solid mineral medium with addition of 10 g/L of heavy or light crude oil. Biosurfactant production was investigated by drop-collapse test [13].

Identification of isolate was performed by API and APIWEB technique (Biomerieux, France).

Effect of different oil type concentrations was studied by cultivating the strain in shaking flask bioreactors [14]. Gas phase–liquid phase ratio in the shaking flasks was 1:1, while the total bioreactor volume was 500 mL. The liquid phase was composed of liquid mineral medium (described above) with 10% of isolated strain suspension. The tested concentrations of oil were: 5, 10 and 20 g/L for each oil type. An un-inoculated shaking flask bioreactor represents control for each oil concentration. For each oil concentration an abiotic control group was created, where no bacterial culture was added to the liquid phase. All bioreactors were incubated (30 °C, 200 rpm) for 6 days. Cell number in liquid phase was measured every 24 h by quantitative plate method. The absorbance (OD_{600}) of the liquid phase was measured using a T70 UV/Vis spectrometer (PG Instruments, UK).

The efficiency of crude oil biodegradation was determined by measuring the residual amount of crude oil present in the experimental and abiotic groups. The remaining oil was extracted from bioreactors after 6 days of incubation by *n*-hexane as previously described [15], where the efficiency of biodegradation was expressed as a percentage of degraded oil and calculated according to previous research [16], as follows:

1) Weight of residual crude oil = weight of beaker containing extracted crude oil – weight of empty beaker;

2) Amount of crude oil degraded = weight of crude oil added in the media – weight of residual crude oil;

3) % degradation = amount of crude oil degraded/amount of crude oil added in the media×100

Different tobramycin and gentamicin concentrations (0.3125–1.25 μg/mL) were used for testing the effect of aminoglycosides on diesel degradation. The bioreactors contained 1g/L of diesel D-2, while the basic liquid mineral medium and incubation conditions were as described previously. Quantitative plate method was used for measuring the microbial growth. Biosurfactant production was simultaneously measured by the oil-spreading test [17]. Diesel degradation was determined after 96 h by *n*-hexane extraction, as described above [15].

RESULTS AND DISCUSSION

Strain characterization

The selected isolate, named PRO23, could grow on solid medium where heavy or light oil was the only carbon source. By drop-collapse test the strains ability to produce biosurfactants was observed (data not shown).

The results of biochemical test showed positive reactions for arginine, citrate, urea, glucose, sucrose, melibiose and arabinose, and negative for oxydase, lysine, ornithine, mannitol, inositol, sorbitol, rhamnose and amygdalin. Based on APIWEB database, this strain shows maximal similarity with species *Pseudomonas luteola*.

Light and heavy crude oil degradation

The strain had adapted to all concentrations of light oil and lower concentrations of heavy oil in period shorter than 24 h (Figs. 1 and 2). The longest lag phase was expressed for the highest concentration of heavy oil. In all conditions log phase of growth is noticeable during the first 48 h of incubation. The highest cell number occurred in conditions with 10 g/L of light oil, where the upgrade of oil concentration from 5 to 10 g/L stimulated the microbial growth. Further upgrade of light oil concentration to 20 g/L, as well as all the upgrades in heavy oil concentration, inhibited the microbial growth. Cell number growth in the first 48 h of incubation was followed by absorbance growth in all experimental groups, but cell number decrease was not followed by absorbance decrease.

The most efficient biodegradation and highest cell number in the lag phase was obtained in 10 g/L of light oil (Fig. 3). In a case of heavy oil as a carbon source, the most efficient biodegradation (29.7 %) was obtained in the lowest oil concentration.

So far, there is no literature data referring to the ability of *P. luteola* to degrade crude oil, but some studies show that it was capable to degrade a selective

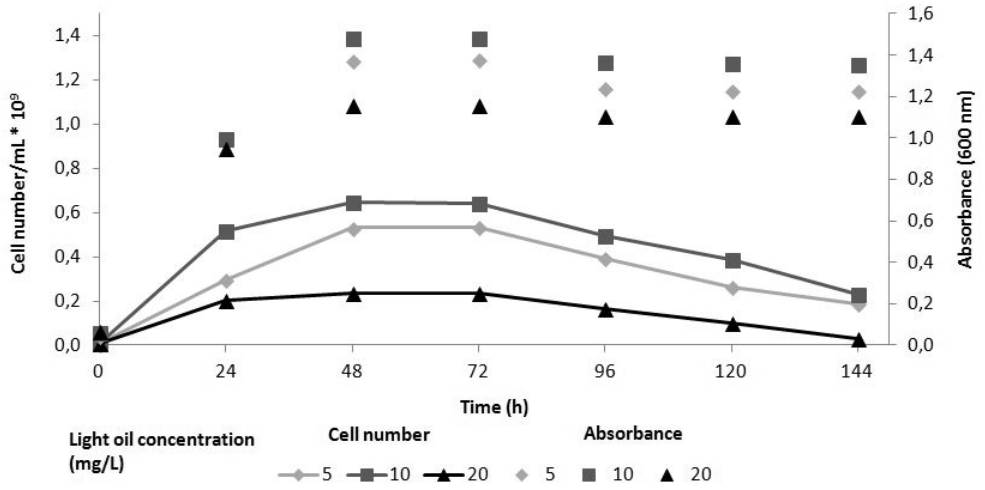

Figure 1. Growth of Pseudomonas luteola PRO23 in the presence of light crude oil.

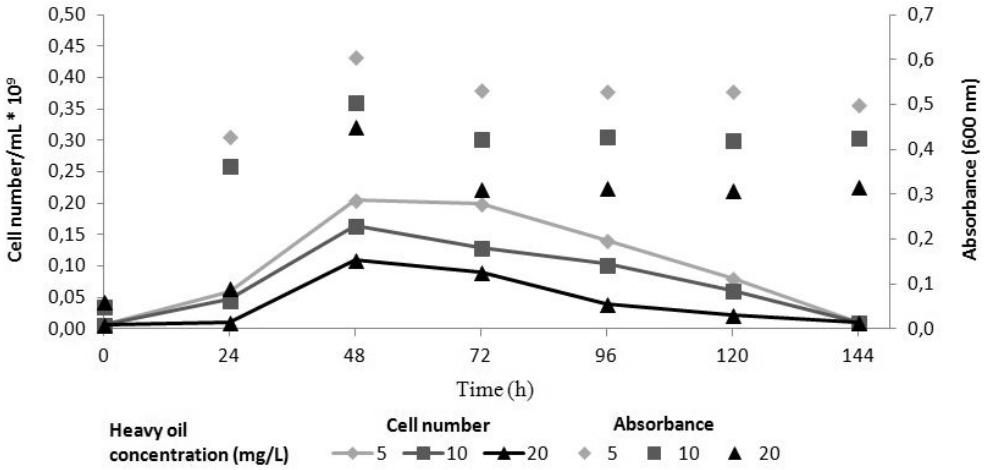

Figure 2. Growth of Pseudomonas luteola PRO23 in the presence of heavy crude oil.

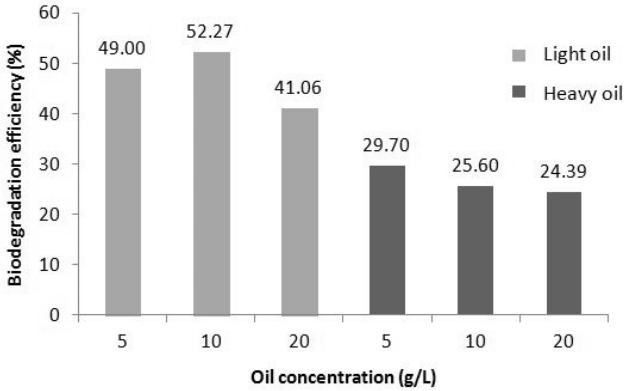

Figure 3. Biodegradation of light and heavy crude oil by Pseudomonas luteola PRO23.

herbicide diclofop-methyl [18], phenolic compounds [19], and decolorize several groups of azo dyes [20].

In genus *Pseudomonas* the main way of adaptation on conditions where the nutrients are hydrophobic is formation of oil in water emulsion with biosurfactants [13]. Higher hydrophobic phase shares [21] and higher viscosity of oil [22] can inhibit the emulsification process. This explains why lag phase was shorter in the light oil and why the highest concentration of heavy oil prolonged it. The highest light and heavy oil concentrations inhibited the microbial growth. In this higher concentration range, some toxic compounds could

express their bacteriostatic effect, which lead to the described growth inhibition. A previous studies show that many crude oil compounds can express bacteriostatic effect for genus *Pseudomonas* after reaching a specific concentration threshold [23]. The bacterial growth was more intensive in conditions with light than in conditions with heavy oil. This can be explained with higher content of small chain alkanes in this type. It is assumed that the isolated strain showed highest affinity for these alkanes, which led to more intensive growth in light oil. This is in accordance with results of previous studies of *Pseudomonas* bacteria [24]. Cell number decrease was not followed by absorbance decrease, which indicates that the changes of the absorbance are the consequence of bacterial growth, as well as oil emulsification and biosurfactant production [25].

Microbial growth stimulation with upgrade in light oil concentration (5 to 10 g/L) was followed by more efficient biodegradation. Heavy oil concentration upgrade induced a decrease in biodegradation efficiency, as well as bacterial growth inhibition.

The isolated strain degraded 41–52% of light and 24–30% of heavy oil in period of 6 days, so it could find a great use in bioremediation technologies.

Stimulation of diesel degradation by aminoglycosides

Tobramycin and gentamicin were added to the bioreactors in three different concentrations. The lowest and the middle concentrations have stimulated biosurfactant production (Fig. 4), biofilm formation (Fig. 5), microbial growth (Fig. 6) and diesel degradation (Fig. 7). Biofilm formation was observed after 48 h of incubation, when a plateau in biosurfactant production was reached (Fig. 5). This stimulatory effect was dose dependent, where 0.625 μg/mL had a higher stimulatory effect than 0.3125 μg/mL. The highest concentration had an inhibitory effect on all of the aforementioned processes. Biofilm induction by aminoglycosides in genus *Pseudomonas* was observed in previous studies [26]. It was also shown that tobramycin could stimulate transcription of biosurfactant production genes in a dose dependent manner [27]. Therefore we suppose that sub-MICs (minimal inhibitory concentrations) of tobramycin and gentamicin have induced biosurfactant production and biofilm formation probably by acting as regulators on a transcriptional level.

Aminoglycosides have also modulated the microbial growth kinetics. The lag phase was shorter and the

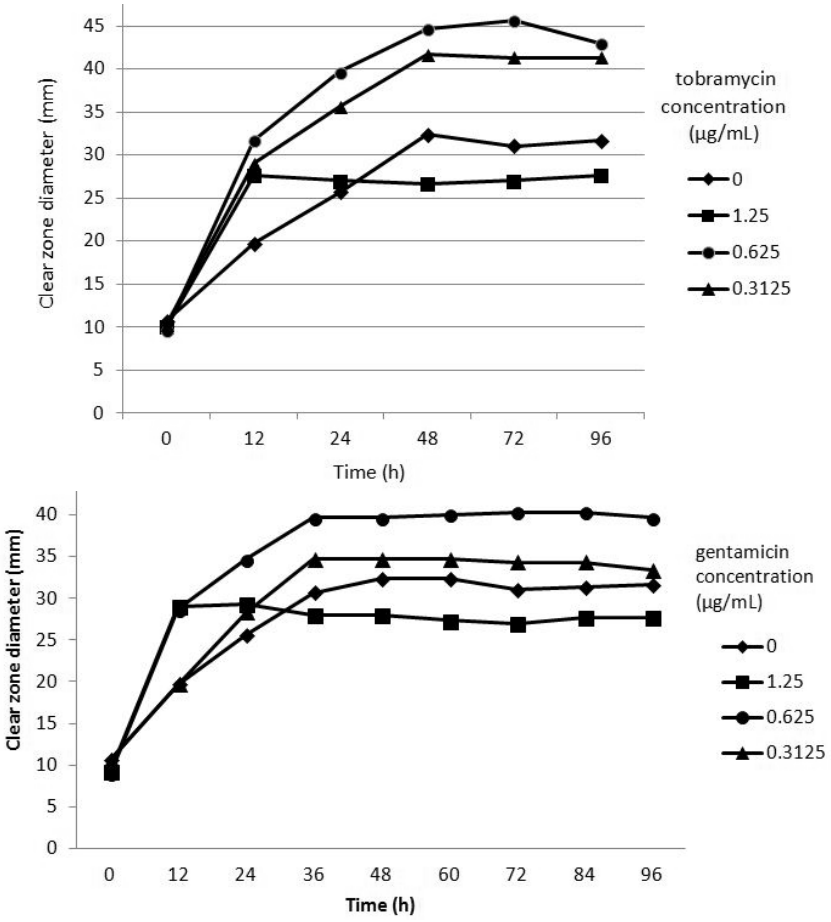

Figure 4. Effect of tobramycin and gentamicin subinhibitory concentrations on biosurfactant production in Pseudomonas luteola PRO23.

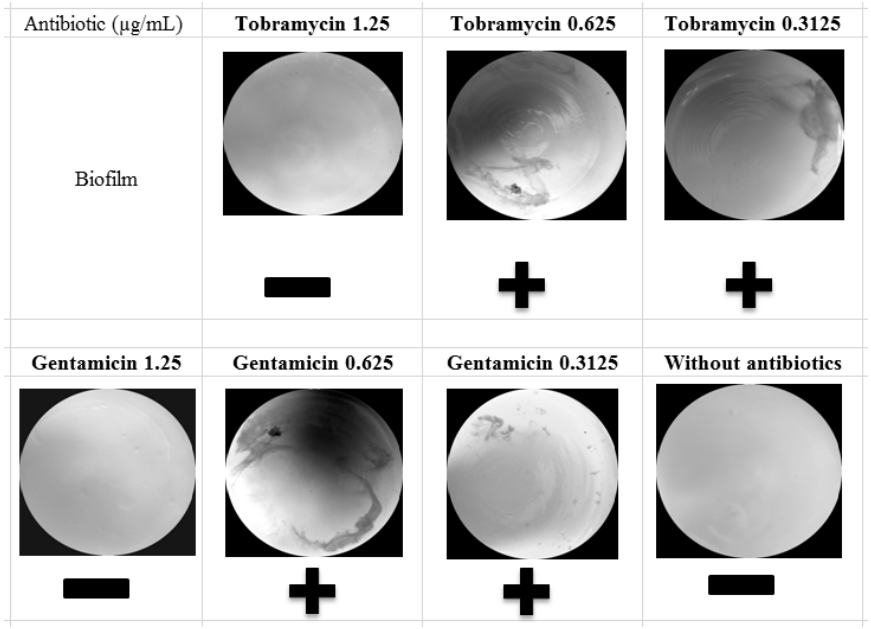

Figure 5. Biofilm appearance in bioreactors after 48h incubation (+); no biofilm appearance (–).

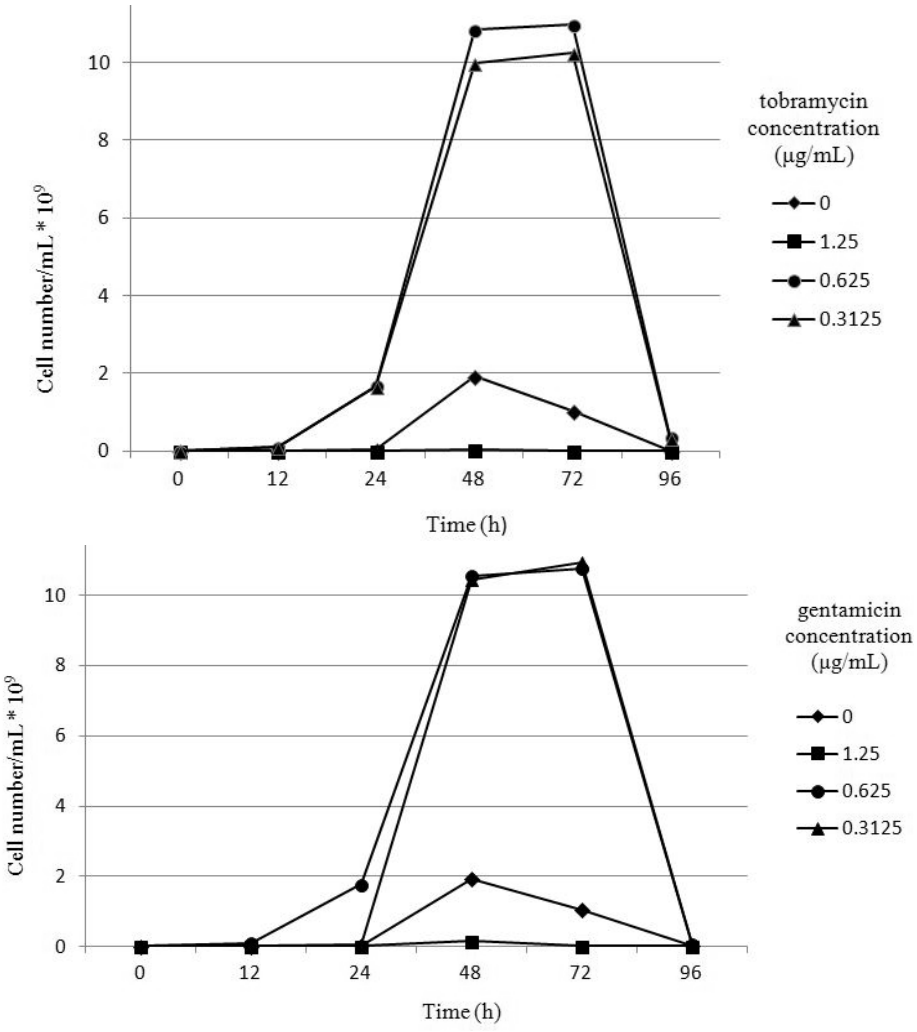

Figure 6. Effect of tobramycin and gentamycin subinhibitory concentrations on growth kinetics in Pseudomonas luteola PRO23.

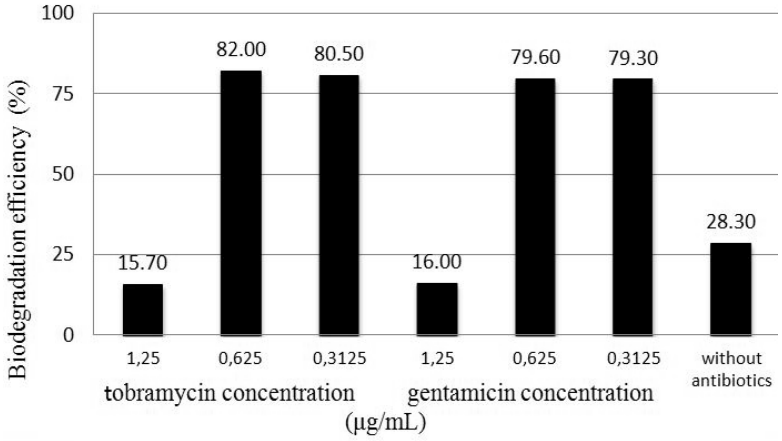

Figure 7. Effect of aminoglycosides on diesel biodegradation efficiency in Pseudomonas luteola PRO23.

higher cell number was reached in the presence of tobramycin and gentamicin sub-MICs (Fig. 6). In these conditions stationary phase was longer then in the control group, probably because biosurfactant production increased the nutrients availability. The optical density of the cultures was growing even in the lag phase, when no growth of bacterial cell number was observed. This *OD* increase is probably caused by biosurfactant production, which starts before the ending of the lag phase. *OD* changes could also be induced by the orange pigmentation of the strain, or other metabolite production. It didn't drop during the decrease of CFUs, which was only observed in biofilm containing bioreactors. *OD* consistency in the liquid phase of biofilm containing cultures was previously described [28].

We also compared the kinetics of biosurfactant production with the growth kinetics. The highest rate of biosurfactant production was observed in the lag phase, when the strain was adapting to diesel as its only nutrient source. The production level reaches a plateau in the stationary phase, although viable cells are still present in the culture. This result is in accordance with previous studies, which show that ramnolipid production stops after reaching a certain threshold [29].

Subinhibitory concentrations of tobramycin and gentamicin have also stimulated diesel biodegradation. The highest rate of biodegradation was observed for 0.625 μg/mL of tobramycin, where 82 % of diesel was degraded in 96 h. This degradation rate was three times higher than in the control group, where no aminoglycoside was present. In previous studies it was shown that biosurfactant production and biofilm formation led to more efficient oil degradation [14]. We suppose that tobramycin and gentamicin stimulate these processes in *P. luteola*, which resulted in better diesel degradation and higher cell growth. Both chemicals had a very similar effect on strains' adaptation to diesel. They exhibit the similar effect probably because they both belong to the same class of antibiotics. Therefore, we propose the structure of aminoglycosides as a reference for new biodegradation inducing agents.

CONCLUSION

Pseudomonas luteola PRO23 strain, isolated from oil contaminated soil, is capable of using light and heavy crude oil as the only carbon and energy source. The strain showed different growth kinetics depending on oil type and concentration. The growth was more intensive in conditions with light oil as a sole carbon source. The most intensive growth was obtained in 10 g/L light oil concentration. The increase of heavy oil concentration and the highest light oil concentration inhibited the microbial growth. According to these results, optimal conditions for *ex situ* oil biodegradation and growth of *P. luteola* PRO23 are 5–10 g/L of light oil type. Aminoglycosides tobramicin and gentamicin stimulated microbial growth in the bioreactors. Growth stimulation was followed by more intense biosurfactant production, biofilm formation and more efficient oil degradation. Therefore aminoglycosides could serve as reference molecules for designing new inducers of bioremediation.

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IZVOD

STIMULACIJA DEGRADACIJE DIZELA I PRODUKCIJA BIOSURFAKTANATA POMOĆU AMINOGLIKOZIDA I NOVE BAKTERIJE *Pseudomonas luteola* PRO23 KOJA RAZGRAĐUJE NAFTU

Iva M. Atanasković[1], Jelena P. Jovičić Petrović[2], Marjan B. Bioč anin[1], Vera M. Karličić[2], Vera B. Raičević[2], Blažo T. Lalević[2]

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(Naučni rad)

Jedna od naprednih tehnologija za rešavanje problema kontaminacije naftom je bioremedijacija. U ovim istraživanjima ispitana je kinetika rasta i efikasnost biodegradacije nafte bakterije *Pseudomonas luteola* PRO23, izolovane iz uzoraka zemljišta kontaminiranih sirovom naftom, pri različitim koncentracijama (5, 10 and 20 g/L) lake i teške sirove nafte. U uslovima prisustva lake nafte konstatovana je znatno efikasnija biodegradacija, brža adaptacija i brži rast ćelija. Povećanje koncentracije lake nafte od 5 do 10 g/L stimulisala je mikrobni rast i efikasnost biodegradacije. Dalje povećanje koncentracije lake nafte, kao i povećanje koncentracije teške nafte, inhibiralo je mikrobni rast, kao i biodegradaciju. Aminoglikozidi su stimulisali produkciju biosurfaktanata kod *P. luteola* pri subinhibitornim koncentracijama (0,3125, 0,625 μg/mL). Aminoglikozidi su takođe indukovali stvaranje biofilma. Produkcija biosurfaktanata bila je najintenzivnija tokom lag faze i nastavila se do stacionarne faze. Aminoglikozidi su izazvali promene u kinetici rasta *P. luteola*. U prisustvu aminoglikozida, stepen degradacije dizela u prisustvu ovog soja iznosio je 82% za 96 h. Ovi rezultati ukazuju da *Pseudomonas luteola* PRO23 se potencijalno može koristiti u bioremedijaciji ekosistema kontaminiranog sirovom naftom i da aminoglikozidi mogu da stimulišu ovaj proces.

Ključne reči: Biodegradacija • Sirova nafta • Aminoglikozidi • *Pseudomonas luteola*