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PRODUCTION OF BIOSURFACTANTS BY *ARTHROBACTER SP. N3*, A HYDROCARBON DEGRADING BACTERIUM

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Abstract. *Different screening methods, such as emulsification capacity and oil spreading assays, hydrocarbon overlay agar and modified drop collapse methods were used to detect biosurfactant production by hydrocarbon degrading Arthrobacter sp N3 strain. It was indicated that oil spreading assay was the most reliable method to detect biosurfactant production. To investigate biosurfactant production, batch cultivation of Arthrobacter sp N3 was carried out in a fermenter with complex nutrient medium supplemented by sunflower oil as a carbon source. The highest oil displacement activity was achieved when Arthrobacter sp N3 strain was cultivated in two stages (with aeration for cell production and without aeration for biosurfactant synthesis). Then, two forms of the biosurfactant (crude preparation and partially purified biosurfactant) were recovered from the culture liquid. Furthermore, the biosurfactant produced by Arthrobacter sp N3 strain was analyzed by thin layer chromatography and it was estimated that even a few compounds have surface activity. The effect of temperature and pH on biosurfactant activity was also studied. It was observed that no appreciable changes in biosurfactant activity occurred at temperature and pH values ranges of 4–125 ºC and 5–10, respectively.*

Keywords: *biosurfactant, Arthrobacter, fermentation, hydrocarbon degrading bacterium.*

Introduction

Pollution caused by crude oil and its products is the most prevalent problem in the environment [1, 2]. Various physical, chemical and biological techniques are used to reduce its negative impact on human health, flora and fauna. Bioaugmentation, one of the most efficient biological techniques, is based on remediation of the contaminated environment by adding of hydrocarbon degrading microorganisms [3]. Unfortunately, biodegradation of hydrocarbons often is limited by bioavailability which is associated with their poor solubility in water and sorption to soil particles. Thus, to enhance hydrocarbon bioavailability, synthetic surfactants and biosurfactants are used [4, 5]. Biosurfactants have several advantages over synthetic surfactants: higher biodegradability, lower toxicity, higher specificity and effectiveness at the extreme temperature and pH values [3, 6].

The biosurfactant producing microbes are distributed among a wide variety of genera. For instance, bacteria that belong to such genera as *Bacillus, Pseudomonas, Sphingomonas*, *Rhodococcus* and *Arthrobacter* [7-12] and yeasts belonging to genera *Candida* and *Yarrowia* [13] have been reported to produce surface active compounds. Microorganisms produce the surface active compounds either extracellularly or these compounds are attached to microbial cells [6, 14]. In general, their structure includes hydrophilic and hydrophobic moieties. The major classes of biosurfactants include glycolipids (rhamnolipids, trehalolipids, sophrolipids), lipopeptides and lipoproteins, fatty acids, phospholipids, neutral lipids and polymeric biosurfactants [3, 6].

Biosurfactants often are used in processes of oil removal from contaminated sites and for enhancement of hydrocarbon biodegradation [2, 4, 5]. In some cases, not only biosurfactant itself but also biosurfactant producing microorganisms are applied for cleaning up of the hydrocarbon polluted environment [15].

Recently, increasing attention has been focused on the hydrocarbon degrading microorganisms with biosurfactant-producing capability [9]. Since these microorganisms are able to produce biosurfactants when grow on hydrocarbon compounds presented in polluted environment, they are highly promising for bioremediation purposes.

JSC "Biocentras" owns extensive microbial cultures collection with more than 200 strains of hydrocarbon degrading microorganisms. The strains were isolated from hydrocarbon polluted environment around the world. Furthermore, in our previous work [16], we have discovered that some of these microorganisms have emulsification activity. So, these findings lead to the presumption that our microorganisms might be applicable for remediation of hydrocarbon polluted environment not only as hydrocarbon degraders but also as biosurfactant producers.
The aims of the present work were to determine capability of *Arthrobacter* sp N3 strain of producing biosurfactant, and to investigate biosurfactant production in batch cultivation. The biosurfactant was recovered from the culture liquid and its partial characteristic was determined.

Materials and methods

Microorganism. Hydrocarbon degrading and biosurfactant producing *Arthrobacter* sp N3 strain obtained from the culture collection of JSC "Biocentras: was used.
Media and cultivation conditions. Nutrient agar (*Oxoid*, UK) was used for plating, and nutrient broth (*Oxoid*, UK) was used for the subculture and preculture of *Arthrobacter* sp N3 strain. For flask cultures, 750 ml Erlenmeyer flasks containing 100 ml of nutrient broth and 4 % (v/v) inoculums' culture were incubated with shaking at 200 rpm and 30 °C for 16-48 h. For biosurfactant production, batch cultivation was carried out in a 14 l benchtop fermenter BioFlo 110 (New Brunswick Scientific, USA) with 7 l of optimized complex medium. The medium was inoculated with 10 % (v/v) of 16 h-old seed culture. The fermenter was operated at 30 ºC and 200 rpm agitation, with or without aeration at 0.5-1 vvm and uncontrolled pH.
During the cultivation experiments, samples of culture liquid were removed periodically and analyzed for bacterial growth and biosurfactant production.
Biosurfactant recovery. Culture liquid of *Arthrobacter* sp N3 strain was centrifuged at 18500 x g for 20 min to obtain culture supernatant (named crude preparation). Then, two volumes of chilled acetone were added and, after keeping at 4 °C overnight, precipitate was collected by centrifugation at 3250 x g for 20 min. The biosurfactant from precipitate material was extracted with isopropanol, which was evaporated away leaving behind biosurfactant heaving an oil-like appearance. This product was named partially purified preparation of biosurfactant.
Thin layer chromatography (TLC). To determine chemical nature of biosurfactant, the TLC was conducted on Silica gel 60 glass plates (Fluka, Germany). The developing solvents systems were as follows: ***1***, n-hexane – diethyl ether – acetic acid (70:30:2, v/v/v); or ***2***, chloroform – methanol - water (65:25:4, v/v/v). After developing, the spots were revealed by: a) saturated iodine steam, for detection of lipids; b) spraying with 0.2 % w/v ninhydrin (in ethanol) and heating at 100 °C for 5 min for detection of compounds with free amino groups. To detect biosurfactant compounds, all spots were scraped off the plates, dissolved in n-hexane – 2-propanol (3:2, v/v) mixture and tested for surface activity by the oil spreading assay.
Biosurfactant stability. Stability studies were carried out using either crude preparation or partially purified biosurfactant. To determine thermostability, crude preparation of the biosurfactant was autoclaved at 121 ºC for 30 min, while partially purified biosurfactant was exposed at 4, 20, 30, 50, 75, 100 and 125 ºC for 1 h, after which the oil displacement area was measured. To study the pH stability, the pH of crude preparation of the biosurfactant was adjusted to different pH values (2 to 12) and oil displacement area was measured after 24 h exposure.
Emulsification capacity measurement. Emulsification activity was determined by the procedure described in our previous work [16]. The emulsification activity was given as a percentage of emulsified layer height divided by total height of the liquid column.

Oil spreading assay. The oil spreading assay was adapted from the method described by authors [11]. The 20 ml of distilled water was added to a Petri dish (10cm diameter) and followed by the addition of 10 µl of crude oil to the surface of water. 10 µl of biosurfactant solution were then added to the oil surface. The diameter of clear zone formed on the oil surface was measured. Biosurfactant oil spreading (displacement) activity was defined as oil displacement area in cm^2.

Drop collapse method. A modified drop collapse method was carried out using microscope slides coated with crude oil. 10 µl of the sample tested were placed on the slides. Biosurfactant production was considered positive when the drop diameter was larger than those produced by distilled water and also by culture medium as negative controls.

Blue agar plate method. Pure bacterial culture was plated onto blue agar [17]. Anionic biosurfactant-producing colonies on blue agar plate are identified following the formation of dark blue halos around the colonies on a light blue plate background.

Hydrocarbon overlay agar method. Hydrocarbon overlay agar method [18] was performed with some modifications. Mineral agar plates [19] were coated individually with 100 µl of diesel fuel or fuel oil. Plates were inoculated with *Arthrobacter* sp N3 strain and incubated at 30 ºC for 48–72 h. Colony surrounded by an emulsified halo was considered being positive for biosurfactant production.

Bacterial growth was monitored by viable cell count which was determined by plating of serial dilutions of samples on nutrient agar plates and incubating at 30 °C for 24 h.

Statistics. All experiments were carried out in duplicate or triplicate. Calculations were performed with Origin software.

Results and discussion

For investigation of *Arthrobacter* sp N3 strain ability to produce biosurfactants, various screening methods were performed. According to Satpute et al [18], combination of various methods is required for effective screening of biosurfactants since a single method is not suitable to identify all types of biosurfactants. As *Arthrobacter* sp N3 strain is being used as bioremediation agent for cleaning of hydrocarbon polluted environment, methods indicating biosurfactant activity toward hydrocarbons were preferred in this investigation.

Firstly, *Arthrobacter* sp N3 strain was examined for emulsification capacity. The experiment revealed that *Arthrobacter* sp N3 strain was positive in the emulsification test and showed 49.5 % activity.

Blue agar method is known as fast semi-quantitative method for detection of microbial cultures producing extracellular glycolipids or other anionic surfactants [18]. *Arthrobacter* sp N3 strain grew weakly on blue agar plates and produced no halos. This indicates that *Arthrobacter* sp N3 produces not glycolipids.

On the contrary, *Arthrobacter* sp N3 strain was positive for biosurfactant production in the hydrocarbon overlay agar method. The results (Fig. 1) indicated that *Arthrobacter sp N3* strain produced biosurfactant growing on both, diesel fuel and fuel oil.

Surface activity of the compounds produced by *Arthrobacter sp N3* strain was detected by modified drop collapse method, too. However, the method is more qualitative than quantitative [20].

Oil spreading assay was shown to be rapid and more sensitive for detection of surface active compounds [18, 20]. *Arthrobacter* sp N3 strain demonstrated oil displacement activity toward crude oil. Furthermore, it was observed that the oil displacement activity, as measured by the area of the clear zone on the oil-water surface, decreased proportionally with a decrease in concentration of the biosurfactant. Thus, oil spreading assay was found to be the most suitable for quantitative measurement of biosurfactant activity. The oil spreading assay was used to detect, quantify, and compare biosurfactant activities throughout all experiments in this work.

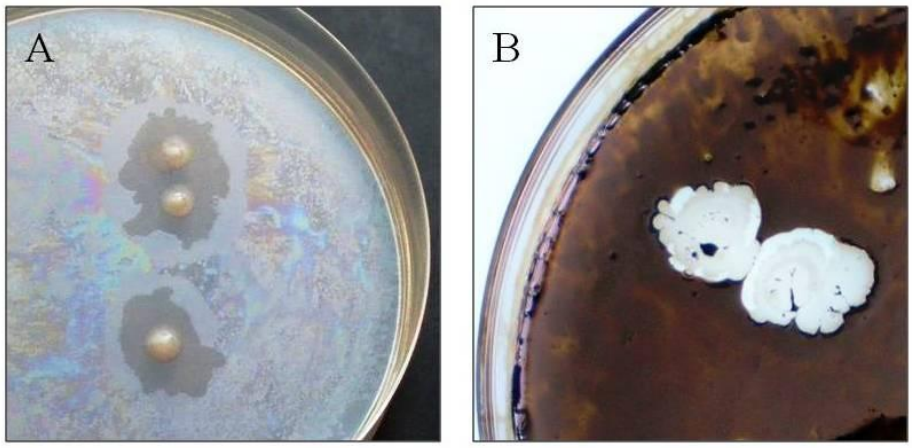

Fig.1. **Biosurfactant production by *Arthrobacter sp N3* strain grown on mineral agar with diesel fuel (A) and fuel oil (B)**

When *Arthrobacter* sp N3 strain was grown in shake-flask culture, it was observed that biosurfactant production depended on nutrient medium composition (data not shown). The highest oil displacement activity (88.2 cm^2) was determined in optimized nutrient medium with sunflower oil as carbon source. The medium was used for further experiments on biosurfactant production in a fermenter.

The batch cultivation of *Arthrobacter sp N3* strain was carried out in a fermenter with 7 l of medium containing sunflower oil by two different aeration modes: a) constant aeration; b) with and without aeration. The profiles of cell growth, pH changes and production of biosurfactant are shown in Fig 2.

With *Arthrobacter* sp N3 strain cultivated at constant aeration of 0.5-1.0 vvm and agitation of 200 rpm (Fig. 2A), the most intensive increase in bacterial cell count was detected during the first hours of cultivation. At 8 h of the cultivation, when stationary phase of cell growth was reached, cell count of $7.9 \cdot 10^9$ CFU/ml was obtained. Further microbial population increased slightly. The change in profile of pH was practically inconsiderable. However, surface activity of the culture liquid was much lower than expected. At the end of cultivation, only 19.6 cm^2 oil displacement activity was registered. It is likely that too intensive aeration and agitation inhibited synthesis of biosurfactant by *Arthrobacter sp N3* strain.

At the next stage of the investigation, *Arthrobacter sp N3* strain was cultivated in the fermenter under varied aeration and agitation conditions: the first 8 hours agitation of 200 rpm and aeration of 0.5-1.0 vvm were maintained; next, the culture was cultivated without aeration and agitation (Fig. 2B).

As shown in Fig. 2B, profiles of cell growth and pH changes were similar to these measured in batch fermentation with constant aeration (Fig. 2A.), on the contrary, biosurfactant production had different character. The first 8 hours, while fermenter was operated with aeration and agitation, *Arthrobacter sp N3* strain intensively grew but oil displacement activity was low. The oil displacement activity was gradually increased after 12 h. The highest oil displacement activity of 174.6 cm^2 was achieved at 24 hour of the cultivation. If to compare to shake flask culture, it is obvious that activity in the batch fermentation was about two times higher. Furthermore, the results confirmed that *Arthrobacter sp N3* strain growth and biosurfactant synthesis are separated processes: oxygen is needed for bacterial cells growth, while intensive aeration inhibited biosurfactant production.

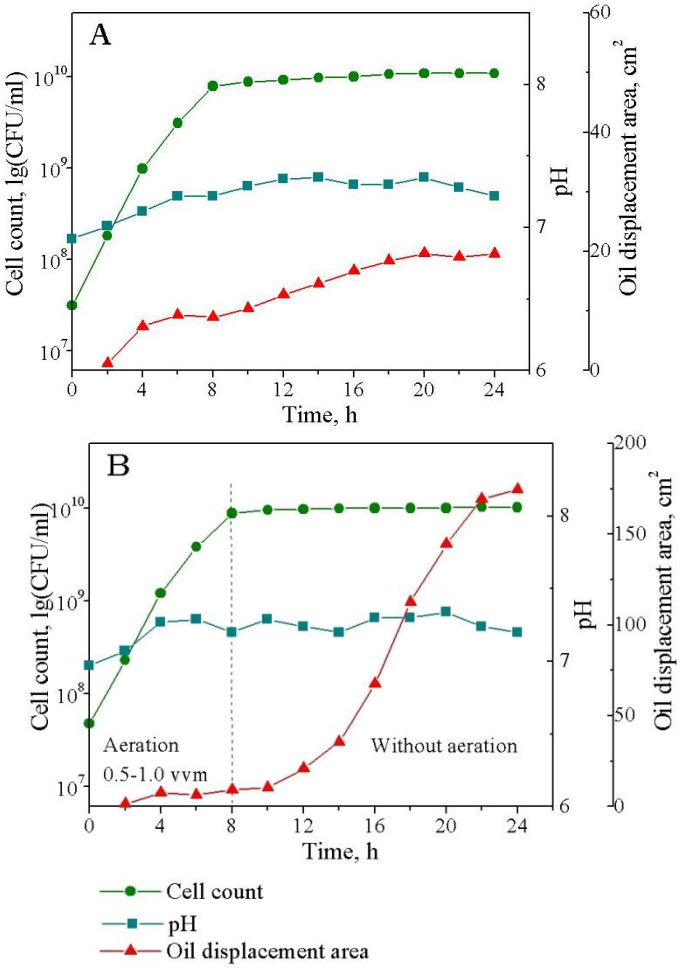

Fig.2. **Profiles of cell growth, biosurfactant production and pH changes during *Arthrobacter sp N3* strain batch cultivation carried out with constant aeration (A) and with and without aeration (B)**

The importance of aeration and agitation in producing of biosurfactant has been shown and by researchers [7, 13, 21]. For example, authors [13, 21] have reported that microbial growth and biosurfactant production increases when aeration and agitation rates are increased. On the contrary, Suwansukho et al. [7] have reported that aeration stimulated growth of *Bacillus subtilis* MUV4 strain but inhibited the biosurfactant production.
To recover biosurfactant from culture liquid, the effect of extraction and precipitation was investigated (data not shown). Based on the results obtained the procedure for biosurfactant recovery was developed. Following the procedure, the steps for the biosurfactant recovery are: centrifugation, acetone precipitation, 2-propanol extraction and evaporation. As a result, two types of the biosurfactants were obtained: crude preparation and partially purified biosurfactant.

The biosurfactants produced by *Arthrobacter* sp N3 strain, as shown by TLC analysis (Fig. 3), appear to be composed of a mixture of a few neutral lipids (Fig. 8A) and polar lipids (Fig. 8C). Moreover, purple colour of spots developed after visualization with ninhydrin (Fig. 8D) indicates that these surface active compounds have amino functional group, which is characteristic for lipopeptides and lipoproteins.

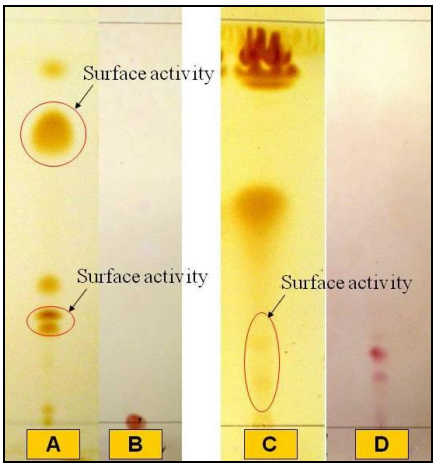

Fig. 3. **TLC of partially purified biosurfactant from *Arthrobacter* sp N3 strain, using solvent *system 1* (A and B) and solvent *system 2* (C and D).The plates were visualized by iodine steam (A and C) and ninhydrin (B and D)**

To evaluate the effect of environmental conditions on performance of biosurfactants produced by *Arthrobacter* sp N3 strain, the thermal and pH stability were studied.
The thermal stability of the biosurfactant was tested over a range of temperature (4-125 °C). The partially purified biosurfactant was shown to be thermostable (Fig. 4). Heating of the biosurfactant to 125 °C caused no significant effect on oil displacement activity. Furthermore, oil displacement activity of crude biosurfactant preparation was not lost after autoclaving at 121 °C for 30 min. Besides, the biosurfactant activity was 20 % higher than that was before the thermal treatment. The increase in oil displacement activity can be attributed to the fact that remaining in crude preparation bacterial cells were destroyed under thermal action and cell bound biosurfactant was released.

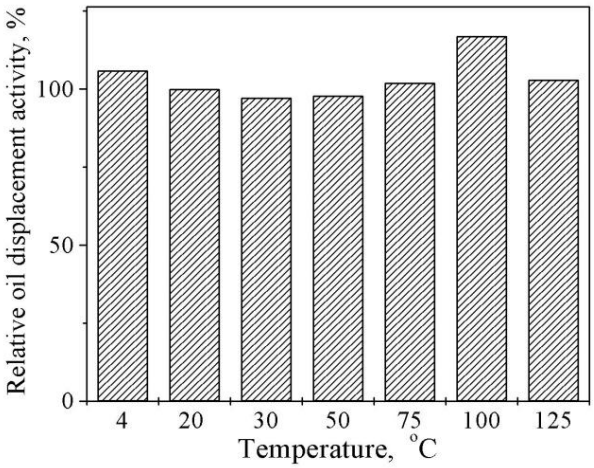

Fig.4. **Effect of temperature on the stability of the partially purified biosurfactant**

For pH stability, the pH value of crude preparation of biosurfactant was varied from 2 to 12. As seen in Fig. 5, no appreciable change in biosurfactant activity was observed in the pH ranges of 5–10. Precipitation of crude biosurfactant preparation occurred at pH below 5, which led to a decrease of biosurfactant activity about 43 %. Loss of biosurfactant activity at pH below 4 due to precipitation has been observed and by authors [8].

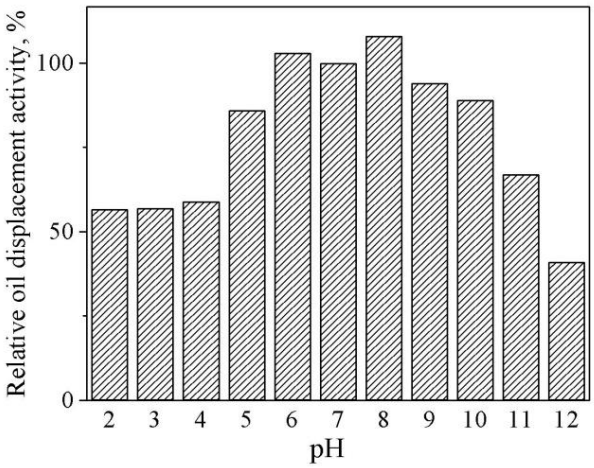

Fig. 5. **Effect of pH on the stability of crude biosurfactant preparation**

So, biosurfactants produced by *Arthrobacter* sp N3 strain has properties necessary for performance in oil polluted environment.

Acknowledgment

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Conclusions

1. The ability of *Arthrobacter* sp N3 strain to produce biosurfactants was confirmed by hydrocarbon overlay agar and modified drop collapse methods, emulsification capacity and oil spreading assays. In addition, it was indicated that oil spreading assay is a reliable method to detect biosurfactant production.
2. Batch cultivation in two stages (with aeration for cell production and without aeration for biosurfactant synthesis) resulted in about 2-fold oil displacement activity, if compared to that in shake flasks culture.
3. Two forms of the biosurfactant (crude preparation and partially purified biosurfactant) were recovered from the culture liquid. Thin layer chromatography of the biosurfactants revealed that even a few compounds have surface activity.
4. Biosurfactants from *Arthrobacter* sp N3 strain were found to be stable at temperature and pH ranges of 4-125 ºC and 5-10, respectively.
5. Hydrocarbon degrading and biosurfactant producing *Arthrobacter sp N3* strain, as well as its biosurfactant, might be applicable in bioremediation of the sites contaminated with petroleum hydrocarbons.

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