CHARACTERISTICS OF THE STRAIN RHODOCOCCUS RUBER -3/4/3 - BIOSURFACTANT PRODUCER

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ABSTRACT. **Aims:** The study is to characterize the local strain of *Rhodococcus sp. – 3/4/3*, isolated from environmentally polluted wastewater, to study the ability of the strain to synthesize BS. **Methodology and results:** The natural strain of *Rhodococcus sp. - 3/4/3*, a biosurfactant (BS) producer, was isolated from the polluted wastewater of Navoiyazot JSC. Based on morphological, cultural, physiological, and biochemical properties, sequencing the gene of the 16S rRNA strain was identified as *Rhodococcus ruber - 3/4/3*. The study used qualitative and quantitative methods for the biosynthesis of biosurfactants by *Rhodococcus sp. – 3/4/3*. **Conclusion, significance, and impact of study:** It has been established that the strain *Rhodococcus ruber - 3/4/3* is a BS producer. For BS biosynthesis, the strain was grown on a medium with the simultaneous use of a hydrophilic 2% hexadecane (HD) and a hydrophobic carbon source of 1% glucose, and 0.1% urea was used as a nitrogen source. It was shown that the strain *Rhodococcus ruber - 3/4/3* synthesizes cell-bound BS, and BS biosynthesis is associated with the amount of cell biomass. BS was extracted with methyl tert-butyl ether (MTBE), and thin layer chromatography (TLC) revealed only one fraction of glycolipids with Rf=0.64, the yield of partially purified biosurfactant was 4.8 g/l.

Keywords*:* Rhodococcus, biosurfactant production, biosynthesis, hexadecane.

INTRODUCTION

Recently, there is a growing interest in the study and practical application of surfactants of biological origin of BS as environmentally safe and biologically active biomolecules. Microbial BS are secondary metabolites produced by microorganisms such as bacteria, fungi and yeast (Cappelletti M. et al. 2018; 2020; Kim D. et al. 2018; Liu K. et al. 2020 Abaturov A.E. et al. 2021). They are widely used to clean the environment from pollutants through biodegradation and bioremediation, as emulsifiers and solubilizers of hydrophobic substances. Demonstrating pronounced biological activity (antibacterial, antiviral,

immunomodulatory, and antitumor) not found in synthetic surfactants, they have a high potential for use in medicine (Kuyukina S. et al. 2015; 2019).

Bacteria of the genus *Rhodococcus* are BS producers, the biosynthesis of which is currently being actively studied. In large quantities, they produce BS in response to the presence of liquid hydrocarbons (LH) in the nutrient medium. The physiological role of BS is associated with participation in the solubilization of water-insoluble substrates and enhancement of cell adhesion to a hydrophobic surface. In terms of their physicochemical characteristics, such as surface and interfacial tension, critical micelle concentration, and emulsifying activity, *Rhodococcus* BSs compete with other microbial and synthetic surfactants (Thi Mo L. et al. 2022). *Rhodococci* are characterized by a minimum number of pathogenic species and are of great interest as BS producers. The strains belonging to the *Rhodococcus erythropolis* species are the most studied in terms of BS characterization and biosynthesis; at the same time, it has been shown that other *Rhodococcus* species are also active BS producers (Xin Hu et al. 2019; Lang S et al. 1998). In Uzbekistan, a detailed study of bacteria of the genus *Rhodococcus* and their biological properties has not been carried out.

In this regard, the aim of this study is to characterize the local strain of *Rhodococcus sp*. – 3/4/3, isolated from environmentally polluted wastewater, to study the ability of the strain to synthesize BS.

MATERIALS AND METHODS

Characteristics of the strain. The strain *Rhodococcus sp.-3/4/3* was isolated from the polluted wastewater of Navoiyazot JSC (Alimova B. et al. 2022; Sharifov Sh.M. et al. 2021). The isolated strain was preliminary identified by phenotypic features. Cell morphology and development cycle were studied on live preparations after 6, 24, 48, 72 and 96 hours of cultivation on nutrient agar medium (Himedia) using a light microscope (Model N300M (NDCE - X5). Gram stain and determination of the acid resistance of the strain studied according to generally accepted methods (Egorov N.S. 1976).

Genetic identification was carried out according to the Sanger method. A standard phenol-chloroform method was used to isolate genomic DNA and it was analyzed on a 0.8% agarose gel using a GelDoc^{TM} XR+Imager with software from Bio-Rad Laboratories, USA. To determine the size of the isolated DNA and PCR products on agarose gel, DNA markers with sizes of 0.5–12.0 Kbp were used. PCR amplification of the 16S rRNA gene of the strain was performed using master-mix PLATINUM HS PCR 2X (Invitrogen, USA) and universal primers:

27F_5'-AGA GTT TGA TCM TGG CTC AG-3' and U1492R_5'-GGT TAC CTT GTT ACG ACT T- 3' on C1000 Touch Thermal Cycler (Bio-Rad, USA). The PCR product was analyzed in 1.0% agarose gel and isolated from the gel using the QIAquick Gel Extraction Kit (QiaGen, USA). Sequencing of the gene of the strain 16S rRNA was performed on a SeqStudio Genetic Analyzer using a BigDye Terminator v.3.1 cycle sequencing kit (Thermo Fischer Scientific, USA).

Nutrient medium for BS biosynthesis. To obtain the inoculum, the strain was grown in a liquid nutrient medium trypton-soy broth (TSB) (HiMedia) for 48 h. The inoculum in an amount of 4% was introduced into the nutrient medium PSBS of the following composition (g/L): glucose - 10.0; peptone -2.0; KH_2PO_4 -0.5; K_2HPO_4 -0.6; $MgSO_4$ x $7H_2O$ - 0.5; $CaCl_2$ x $2H_2O$ - 0,02; NaCl - 0.5; $FeCl_3$ x $6H_2O$ - 0.1; $CoCl_2$ - 0.03; urea at a concentration of 1 g/L was used as a source of nitrogen. Additionally, microelements were added to the medium in a volume of 1.0 ml, with the following composition (g/l): H_3BO_3 – 0.1; $MnCl_2$ x $4H_2O$ – 0.1; $ZnSO_4$ x H_2O x 0.1; $FeCl_3$ x $6H_2O$ – 0.1; $CaCl_2$ x $2H_2O$ – 1; $CuCl_2$ x $2H_2O$ – 0.05 (Drews G. 1974), 2.0% HD was used as a carbon source. The pH value of the nutrient medium is 7.0 - 7.5. The strain was cultivated in a 250 ml Erlenmeyer flask containing 100 ml of nutrient medium on an orbital shaker (orbital shaker incubator BioSan ES-20/60) at 150 rpm, temperature 28°C for 10 days. When cells grow on PSBS medium, the formation of two phases is observed during the entire cultivation period. To study the ability of the strain to synthesize BS, the culture liquid was centrifuged or separated from the cells in a separating funnel, two phases were obtained separately - hydrophilic (supernatant 1) and hydrophobic. The hydrophobic part was destroyed by ultrasound (US) for 5 minutes, centrifuged at 6000 rpm for 10 minutes, and supernatant 2 was obtained, in which the BS content was subsequently determined.

Determination of surface activeness. The surface tension was measured using an automatic tensiometer (Model GD8541A, China) in supernatant 1 after centrifugation of the culture liquid, as well as in supernatant 2. The critical micelle concentration (CMC) and the critical dilution factor obtained by BS were determined by the dilution method with distilled water until the minimum surface tension was reached.

Emulsifying activity. The emulsifying ability was evaluated by analyzing the emulsifying index (EI_{24}), based on the property of the surfactant to form a stable emulsion when vigorously mixed with a hydrocarbon. Emulsifying activity (EA) was expressed as the optical density (OD) of the emulsion at 540 nm. EI_{24} was determined and calculated as the percentage ratio of the volume of the formed

dense emulsion layer to the total liquid volume according to the method of Cooper and Goldenberg (Cooper D. G. et al. 1987). To do this, an equal volume of diesel fuel was added to 2 ml of the culture liquid. The mixture was vigorously stirred for 2 min using a vortexer (MX-S Biosan). The results were taken into account after 24 h of incubation at 20°C.

The emulsification index was calculated by the formula:

$$EI\ 24 = \frac{Thickness\ of\ emulsification\ layer}{Total\ height\ of\ the\ liquid\ column} \times 100$$

Here, EI_{24}– emulsification index

When determining the hydrophobicity of the cell surface by the Rosenberg method (Rosenberg, M. 1984), we used cells grown on PSBS medium without HD and on PSBS medium with 2% HD in the strain growth dynamics. When cultivating on the medium without HD, the cells were precipitated by centrifugation at 10,000 rpm, while when cultivating on the medium with HD, the cells were separated from the culture liquid on a separating funnel. Next, the cells were washed with 10 mM phosphate buffer, the washed cells were resuspended in PB until the optical density of the cell suspension was 0.5-0.6 units, measured on a spectrophotometer (UV / VIS Scanning Spectrophotometer Model TP-X8) at λ = 540 nm and the thickness of the optical layer 10 mm. In test tubes containing 5 ml of cell suspension, 1 ml of chloroform was added. The mixture was vortexed for 2 minutes. After that, the upper aqueous phase was carefully taken and transferred to an optical cuvette for measurements. The hydrophobicity index (HI) of the cells was calculated as a percentage using the formula: PG=100-((E*100)/E0), where E0 is the initial optical density of the microbial suspension, E is the optical density of the suspension after the interaction of cells with chloroform.

BS extraction. Extraction of BS was performed using methyl tert-butyl ether (1:2) from supernatant 2. To supernatant 2 after destruction and centrifugation in a GT1-3A centrifuge at 6000 rpm for 10 min, methyl tert-butyl ether was added in a ratio (1:2), left for rocking chair 200 rpm for 3 hours at 28°C. The solvent layer was separated from the aqueous phase by centrifugation at 4°C and 3000 rpm for 10 min or settled on a separating funnel for 20 min, then the upper volatile fraction was collected and evaporated on a rotary evaporator to obtain crude BS (Thi Mo L. 2022).

Thin layer chromatography (TLC) of isolated BS. Partially purified BS was loaded onto silica gel coated thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany) along with the standard. The solvent system used to

separate the compounds (Wittgens et al. 2011) consisted of chloroform, methanol and acetic acid in a volume ratio of 65:15:1. Detection of glycolipids was carried out using saturated iodine vapor.

Determination of the concentration of carbohydrates. The content of GL was determined from the concentration of sugars by the colorimetric method after interaction with the phenol-sulfuric acid reagent in supernatant 2 (Dubois M. 1956). Cell-free supernatant 2 in a volume (0.5 ml) was placed in a test tube, 500 µl of a 5% phenol solution in water was added. With vigorous stirring, 2.5 ml of concentrated sulfuric acid (p = 1.84 g/ml) was added, left for 10 min. The optical density (OD) of the obtained solution was measured at a wavelength of 480 nm. The reference solution was prepared similarly, replacing the sample with distilled water.

The mass fraction of sugars was calculated by the formula:

$$X = \frac{D \times n \times K \times V}{m} \times 100$$

X – mass fraction of sugar, %; D - the optical density of the solution at a wavelength of 490 nm; n - dilution; K - the coefficient of the calibration curve (0.35); V - the volume of the hydrolyzate, ml; m - the weight of the sample, mg.

Determination of protein concentration. The content and concentration of protein in the samples were determined by the Lowry method spectrophotometrically (Lowry, O.H et al. 1951).

RESULTS AND DISCUSSION

The production of microbial BS is an expensive process due to the low yield of the product, the laboriousness and complexity of methods for their isolation. Improvement in production efficiency can be achieved through the use of appropriate cheap raw materials, optimization of cultivation conditions, selection of optimal methods for isolating BS, and selection of highly active producer strains (Adu S. et al. 2020; Eras-Muñoz, E. et al. 2022).

Previously, 15 strains of bacteria belonging to the genus *Rhodococcus* were isolated from polluted wastewater and soils of the Republic of Uzbekistan (Sharifov Sh.M. et al. 2021). The highest decrease in surface tension and the highest emulsification index was observed in *Rhodococcus sp. - 3/4/3*, in this regard, it was selected for further research. It should be noted that the strain was isolated from the activated sludge airlift of Navoiyazot JSC.

Morphological-cultural and physiological-biochemical features of the strain. The cultural characteristics of the strain were studied on nutrient agar medium (Himedia). It is shown that the strain forms orange-red, round, matte, convex colonies, medium in size, has tree-like dry shoots along the edges. A distinctive feature of the strain is the most pronounced mycelial stage of cell development. In the initial stage of growth, germination and branching of the original coccoid and short rod-shaped cells begins with the formation of one to three growth tubes. The coccus germinates into short sticks, and then into the mycelium. In the morpho-cycle, three forms of cells are found: germinating, branching, rod-shaped. By 72 hours of cultivation, abundant mycelium is observed, which is fragmented into shortened uneven rod-shaped and coccal elements. At the age of 4–5 days, a noticeable predominance of coccoid (0.5–0.8 μm) cells is observed. It has been noted that after removal of colonies from the agar plate and subsequent examination of their location, ingrowth of cells into the agar is detected. Spore does not form.

The strain does not form acids on arabinose, galactose, rhamnose, raffinose, lactose, inositol. Acid formation was observed on glucose, mannitol, sucrose, maltose, glycerol. No gas formation was observed in any of the polyhydric alcohols and carbohydrates. The strain is able to grow in a medium containing 3-5% NaCl and is resistant to NaCl concentrations of 7-8% in the medium. The strain decomposes tyrosine, but does not show urease activity.

Genetic identification of the strain. As a result of isolation of genomic DNA of *Rhodococcus sp.* - 3/4/3 and subsequent amplification of the 16S rRNA gene, a PCR amplicon of 1450 bp was obtained, which corresponds to the size of this gene (Fig. 1). Next, the PCR product was isolated and purified from a 1% agarose gel according to the QiaGen kit manufacturer's instructions. The gene sequence, consisting of 496 base pairs, was registered in the GeneBank database under registration number OQ676950 (*Rhodococcus sp.-3/4/3* GenBank accession number: SUB12977596 Seq1 OQ676950). Analysis of the homologous sequence of this sequence in the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that *Rhodococcus sp. - 3/4/3* has 99.80% homology with known strains of *Rhodococcus ruber*.

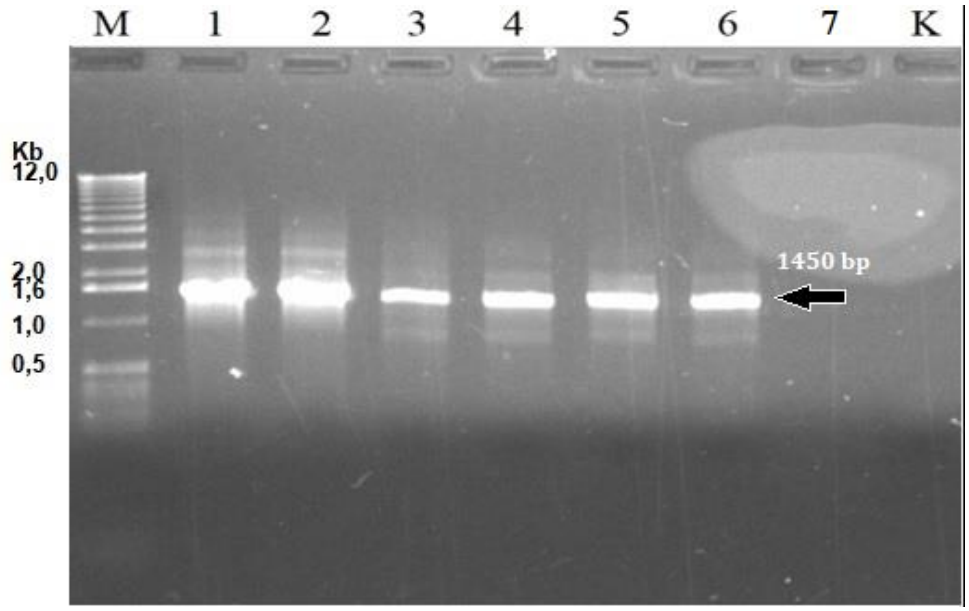

Figure 1. PCR amplification of the 16S rRNA gene: M, DNA markers, 1-5, other bacterial samples, 6, *Rhodococcus sp. - 3/4/3*, 7 and K - control without primer and DNA.

Based on the data obtained and the homology of the partial nucleotide sequence, a phylogenetic tree of the strain was constructed using the Neighbor Joining method based on BLAST, which showed a very close distance to the species *Rhodococcus ruber*, genus *Rhodococcus*, family *Actinomycetes* (Fig. 2).

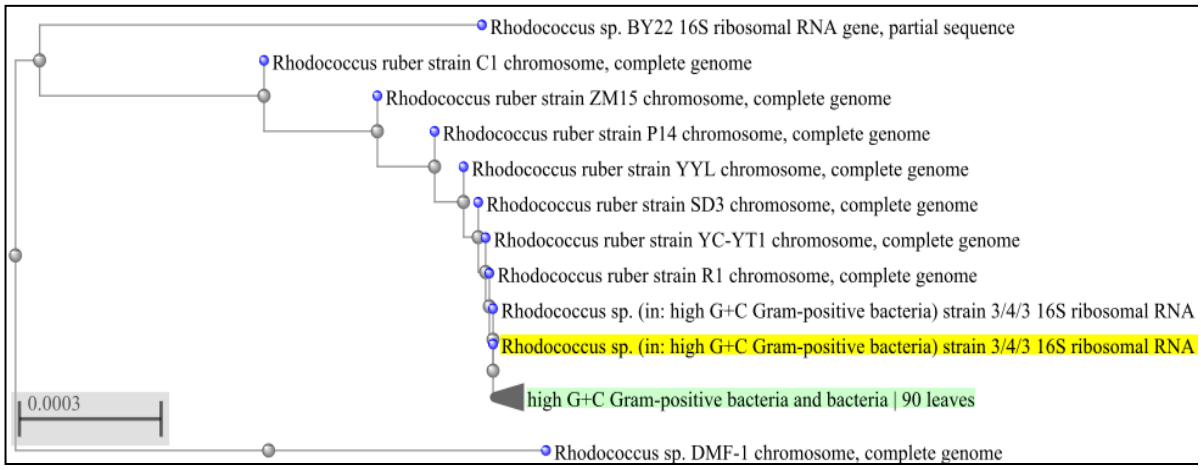

***Figure 2**. Phylogenetic tree of Rhodococcus sp. - 3/4/3 within the genus Rhodococcus.*

Biosynthesis of BS. It is known that for the maximum yield of metabolites, BS are considered secondary metabolites, the nutrient medium used for cultivation is one of the most important conditions. For BS biosynthesis, the most important components of the nutrient medium that regulate metabolism and affect their synthesis are carbon and nitrogen. As a carbon source, both hydrophilic carbon

sources (glucose, sucrose, mannitol), which are mainly used by microorganisms for cellular metabolism and synthesis of the polar part of BS, and hydrophobic carbon sources - hydrocarbons, which are used for the biosynthesis of the hydrophobic part of BS, are usually used (Nurfarahin A. H. et al. 2018; Cooper D. G. 1986). According to the literature data, the simultaneous use of both hydrophilic and hydrophobic substrates is possible for the synthesis of two BS fractions. In addition, most published studies emphasize that the addition of hydrophobic substrates enhances and simultaneously accelerates the process of BS biosynthesis (Cappelletti M. et al. 2020; Kuyukina S. et al. 2019).

In this regard, to study the ability of the strain *Rhodococcus ruber - 3/4/3* to synthesize BS, the strain was grown on PSBS medium for 10 days, where 1% glucose in combination with 2% HD was used as a carbon source, and nitrogen - urea in the amount of 1 g/l. Samples were taken every 24 hours. Since one of the main characteristics of BS biosynthesis is their emulsifying activity, which shows the ability of strains to emulsify various hydrocarbons and oils, as well as surface tension, the content of BS was estimated by EI, as well as by changes in surface tension in supernatant 1 and supernatant 2.

The dependence of the process of BS biosynthesis by *Rhodococcus* cells on the stage of the growth cycle was revealed. As the results of the studies showed, the formation of a film was observed on the surface of the culture liquid after 24 h of cultivation. In the dynamics of growth, the layer of cells (films) adsorbed on HD increased, the yield of BS and IE in supernatant 2 also increased, while in supernatant 1 only an insignificant amount of BS released from the cell surface into the fermentation medium was observed (Fig. 3). A sharp decrease in surface tension from 72 mN/m to 42 mN/m is observed after 48 hours of cultivation in supernatant 2, while in supernatant 1 the surface tension remained practically unchanged compared to the control medium, the EI_{24} value in supernatant 1 was significantly low and amounted to 8 %.

Further studies were carried out with supernatant 2. When studying the biosynthesis of BS in the dynamics of strain growth, it was found that over the next day in supernatant 2, a gradual decrease in surface tension was observed at 144 hours of cultivation, it amounted to 36 mN/m, and after 240 hours the surface tension showed 33 mN/m. According to Cooper's definition, if a strain during growth reduces the surface tension of the liquid to 40 mN/m and below, this strain is considered an active BS producer (Pavlova O. N. et al. 2019). According to the results of our studies, the *Rhodococcus ruber - 3/4/3* strain during growth reduced the surface tension in the supernatant from 2 to 33 mN/m.

At the same time, in the dynamics of growth with a decrease in surface tension, an increase in EI_{24} and the quantitative content of BS are observed. The maximum EI_{24} was observed at 120 hours of cultivation and amounted to 68%, with a quantitative content of BS 3.3 g/l., with further cultivation as the strain grew, the yield of BS increased and at 144 hours of cultivation amounted to 4.8 g/l. Based on the studies performed during the cultivation of the strain *Rhodococcus ruber - 3/4/3* in the dynamics of growth on PSBS medium, it was found that the strain mainly synthesizes cell-bound BS.

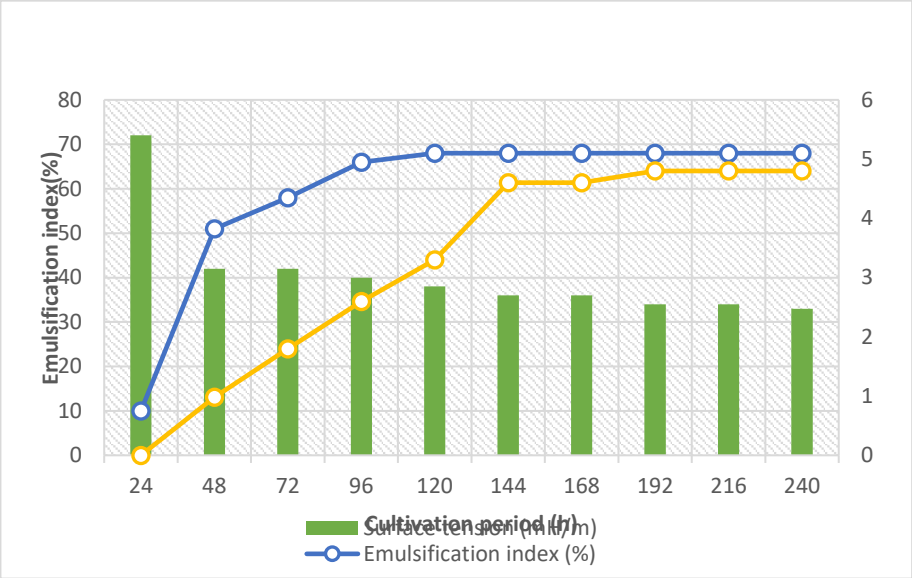

Figure 3. Dynamics of BS biosynthesis depending on the emulsification index, surface tension and hydrophobicity of cells in supernatant 2.

In the process of hydrocarbon oxidation, an important role is played by direct contact of microbial cells with the substrate, and this contact fully depends on the hydrophobic cell wall containing lipophilic compounds, which play an important role in establishing direct contact of cells with hydrocarbon droplets, as well as on the ability of microorganisms to synthesize BS capable of dissolving hydrocarbons (Abraham, Peele, Karlapudi et al. 2018). So, when cultivating the strain *Rhodococcus ruber - 3/4/3* on the PSBS medium with 2% HD, an increase in the hydrophobicity of the cell wall is observed compared to the PSBS medium without HD. According to the results of studies, the hydrophobicity of the cell surface of the *Rhodococcus ruber - 3/4/3* strain on the PSBS medium with 2% HD increased with increasing cell density. The maximum value of hydrophobicity (78%) was observed by 48 hours of cultivation, with further cultivation, the hydrophobicity of the cell walls of the strain increased, and by 144 hours of

cultivation it was 88%. When the strain was cultivated on PSBS without HD, the hydrophobicity of the cell wall of the strain was 56%.

The obtained results show the relationship between the hydrophobicity of the cell surface of the strain *Rhodococcus ruber - 3/4/3* and the formation of BS. As shown in Figure 3, by 48 hours of cultivation, a decrease in surface tension and an increase in EI is observed.

Figure 4 shows the isolation scheme of the strain *Rhodococcus ruber - 3/4/3*, the morphological and cultural features of the strain, the growth of the strain on the medium with HD, the methods for isolating BS described in this article.

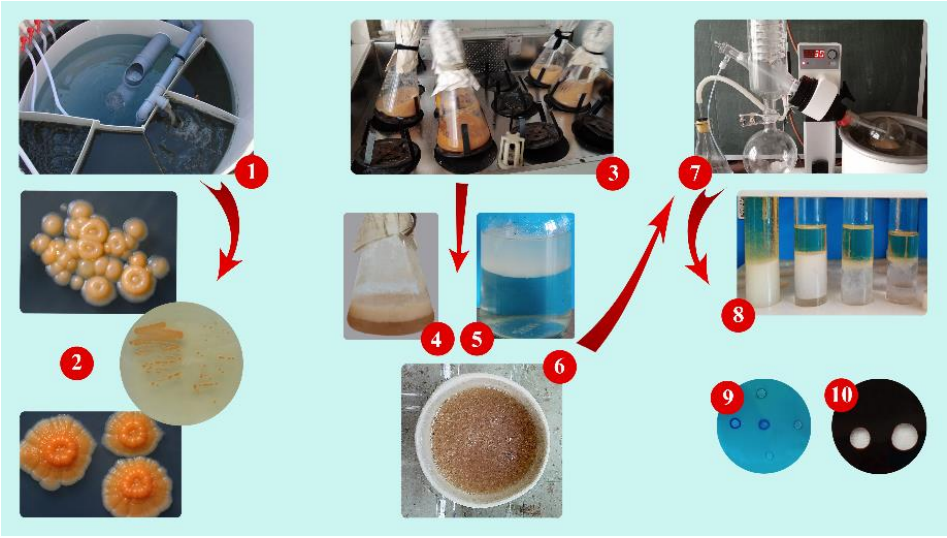

Figure 4. Isolation, characterization and properties of BS synthesized by the strain *Rhodococcus ruber - 3/4/3.* (1. Source of strain isolation (well airlift); 2. Strain *Rhodococcus ruber - 3/4/3;* 3. Strain cultivation; 4. Production media; 5. MTBE extract; 6. Crude BS; 7. Rotary evaporator; 8. Emulsification index;9. CTAB MB test;10. Oil displacement)

Thus, it was shown that the formation of a biomass layer on the surface of the medium adsorbed on the hydrophobic substrate of HD cells of the strain, the thickness of which increases with increasing cell growth, high EI, hydrophobicity of the cell walls of the strain, quantitative content of BS in supernatant 2 indicates that in the strain *Rhodococcus ruber - 3/4/3*, the main part of BS remains firmly associated with the cell wall of the strain.

Physic-chemical characteristics of BS. The crude BS was a pinkish-brown powder after evaporation of MTBE. It has been shown that crude BS is able to reduce the surface tension of water from 71.9 mN/m to 33 mN/m, CMC values are 120 mg/l. Analysis of BS by TLC during cultivation on PSBS medium with 2% n-HD and 1% glucose showed only one fraction of glycolipids with Rf = 0.68

(Fig. 4). Partially purified BS was found to contain 28.4% proteins and 64.2% carbohydrates.

CONCLUSION

This study presents the characteristics of the strain *Rhodococcus ruber - 3/4/3*, a BS producer, isolated from the activated sludge airlift of JSC "Navoiyazot" of the Republic of Uzbekistan. Based on phenotypic and genotypic identification, the strain was assigned to the species *Rhodococcus ruber*. During periodic cultivation of the strain *Rhodococcus ruber - 3/4/3*, it was shown that the process of BS biosynthesis is associated with cell growth, the maximum BS yield was 4.8 g/l for 144 hours of cultivation. The crude BS was extracted using methyl tert-butyl ether (MTBE). According to the results of TLC, one fraction of glycolipids with an Rf value of 0.68 was obtained.

It has been established that BS synthesized by the strain *Rhodococcus ruber* - 3/4/3 are predominantly cell-bound glycolipids that affect the hydrophobicity of the cell wall, and thereby facilitate the attachment and subsequent entry of hydrophobic substrates into the cell. As an active BS producer, this culture has the potential to be used in the bioremediation of oil-contaminated soils and waters.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

REFERENCES

1. Abaturov A.E. (2021) Bacterial surfactants as anti-biofilm agents. For citation: J. Health of the child. 16:94-104. doi: 10.22141/2224-0551.16.1.2021.226463

2. Abraham, Peele, Karlapudi., T.C., Venkateswarulu., Jahnavi, Tammineedi., Lohit, Kanumuri., Bharath, Kumar, Ravuru., Vijaya, R., Dirisala., Vidya, Prabhakar, Kodali. (2018). Role of biosurfactants in bioremediation of oil pollution-a review. Petroleum, 4(3):241-249. doi: 10.1016/J.PETLM.2018.03.007

3. Adu, S. A., Naughton, P. J., Marchant, R., & Banat, I. M. (2020). Microbial Biosurfactants in Cosmetic and Personal Skincare Pharmaceutical Formulations. J. Pharmaceutics, 12 (11), 1099. https://doi.org/10.3390/pharmaceutics12111099

4. Alimova B.Kh., Pulatova O.M., Sharifov M.R., Sailiev M.U., Makhsumkhanov A.A., Davranov K.D. Biosynthesis of biosurfactants by bacteria of the genus *Rhodococcus* on various carbon sources. Bulletin of Karshi State University 2022 3/1 (53) P.135-139.
5. Cappelletti, M., Presentato, A., Piacenza, E., Firrincieli, A., Turner, R. J., & Zannoni, D. (2020). Biotechnology of *Rhodococcus* for the production of valuable compounds. J.Applied microbiology and biotechnology, 104(20), 8567–8594. https://doi.org/10.1007/s00253-020-10861-z
6. Cappelletti, M., Presentato, A., Piacenza, E., Firrincieli, A., Turner, R. J., & Zannoni, D. (2020). Biotechnology of *Rhodococcus* for the production of valuable compounds. J. Applied microbiology and biotechnology, 104(20), 8567–8594. https://doi.org/10.1007/s00253-020-10861-z
7. Cooper, D. G., & Goldenberg, B. G. (1987). Surface-active agents from two bacillus species. J. Applied and environmental microbiology, 53(2), 224–229. https://doi.org/10.1128/aem.53.2.224-229.1987
8. Cooper D. G. (1986). Biosurfactants. J. Microbiological sciences, 3(5), 145–149.
9. Drews G. Microbiologists Practicum, 2nd edition / G. Drews. - Springer-Verlag, Berlin, 1974.
10. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, R.A., Smith, F., 1956. Colorimetric method for the determination of sugars and related substances. J. Anal. Chem. 28, 350.
11. Egorov N.S. Workshop on microbiology, publishing house of Moscow University 1976. http://biotex.ibss.org.ua
12. Eras-Muñoz, E., Farré, A., Sánchez, A., Font, X., & Gea, T. (2022). Microbial biosurfactants: a review of recent environmental applications. J. Bioengineered, 13 (5), 12365–12391. https://doi.org/10.1080/21655979.2022.2074621.
13. Lang S, Philp JC. Surface-active lipids in *Rhodococci*. Antonie Van Leeuwenhoek. 1998 Jul-Oct; 74(1-3):59-70. doi: 10.1023/a:1001799711799.
14. Liu K., Sun Y., Cao M., Wang J., Lu J.R., & Xu H. (2020) Rational design, properties, and applications of biosurfactants: A short review of recent advances. J. Curr. Opin. Colloid Interface Sci. 45:57–67 https://doi.org/10.1016/j.cocis.2019.12.005
15. Lowry, OH, NJ Rosbrough, AL Farr, and R. J Randall. J. Biol. Chem. *193:* 265. 1951.
16. Kim, D., Choi, K. Y., Yoo, M., Zylstra, G. J., & Kim, E. (2018). Biotechnological Potential of *Rhodococcus* J. Biodegradative Pathways. of microbiology and biotechnology, 28(7), 1037–1051. https://doi.org/10.4014/jmb.1712.12017
17. Kuyukina, M. S., Ivshina, I. B., Baeva, T. A., Kochina, O. A., Gein, S. V., & Chereshnev, V. A. (2015). Trehalolipid biosurfactants from nonpathogenic *Rhodococcus actinobacteria* with diverse immunomodulatory activities. J. New biotechnology, 32(6), 559–568. https://doi.org/10.1016/j.nbt.2015.03.006
18. Kuyukina Maria S., Ivshina Irena B. Production of Trehalolipid Biosurfactants by *Rhodococcus* Biology of *Rhodococcus* Chapter (2019) Part of the Microbiology Monographs book series (MICROMONO. V. 16)
19. Thi Mo, L., Irina, P., Natalia, S., Irina, N., Lenar, A., Andrey, F., Ekaterina, A., Sergey, A., & Olga, P. (2022). Hydrocarbons Biodegradation by *Rhodococcus*: Assimilation of

Hexadecane in Different Aggregate States. J. Microorganisms, *10*(8), 1594. https://doi.org/10.3390/microorganisms10081594

20. Nurfarahin, A. H., Mohamed, M. S., & Phang, L. Y. (2018). Culture Medium Development for Microbial-Derived Surfactants Production-An Overview. J. Molecules (Basel, Switzerland), *23*(5), 1049. https://doi.org/10.3390/molecules23051049

21. Pavlova, O. N., Adamovich, S. N., Novikova, A. S., Gorshkov, A. G., Izosimova, O. N., Ushakov, I. A., Oborina, E. N., Mirskova, A. N., & Zemskaya, T. I. (2019). Protatranes, effective growth biostimulants of hydrocarbon-oxidizing bacteria from Lake Baikal, Russia. J. Biotechnology reports (Amsterdam, Netherlands), 24, e 00371. https://doi.org/10.1016/j.btre.2019.e00371

22. Rosenberg, M. J. FEMS Microbiology. (1984). -V. 22.-P. 289–295.

23. Sharifov Sh.M., Alimova B.Kh., Pulatova O.M., Zukhridinova N.Y., Mirzaev T.Sh., Makhsumkhanov A.A., Davranov K.D. Screening of oil-degrading microorganisms producing biosurfactants. Bulletin of the Khorezm Academy of Mamun, Khiva 2021, P.100-105

24. Xin Hu, Yue Qiao, Long-Qiang Chen, Jian-Fei Du, Yue-Yue Fu, & Si Wu Lei. (2019) Enhancement of solubilization and biodegradation of petroleum by biosurfactant from *Rhodococcus erythropolis* HX-2. J. World Microbiol Biotechnol. 34(2):33. doi:10.1007/s11274-018-2415-x

25. Wittgens, A., Tiso, T., Arndt, T. T., Wenk, P., Hemmerich, J., Müller, C., Wichmann, R., Küpper, B., Zwick, M., Wilhelm, S., Hausmann, R., Syldatk, C., Rosenau, F., & Blank, L. M. (2011). Growth independent rhamnolipid production from glucose using the non-pathogenic Pseudomonas putida KT2440. *Microbial cell factories*, *10*, 80. https://doi.org/10.1186/1475-2859-10-80