Scientific Bulletin. Series F. Biotechnologies, Vol. XXVIII, No. 1, 2024
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BIOSURFACTANT PRODUCTION BY *Pseudomonas fluorescens* STRAIN

Roxana Mădălina STOICA[1], Mișu MOSCOVICI[1], Cristina BÂZDOACĂ[1], Elena Simina LAKATOS[2, 3], Lucian Ionel CIOCA[2, 4]

[1]National Institute for Chemical-Pharmaceutical Research and Development-ICCF, 112 Vitan Avenue, Bucharest, Romania
[2]Institute for Research in Circular Economy and Environment "Ernest Lupan", Cluj-Napoca, Romania
[3]Faculty of Industrial Engineering, Robotics and Product Management, Technical University of Cluj-Napoca, Cluj-Napoca, Romania
[4]Faculty of Engineering, Lucian Blaga University of Sibiu, Industrial Engineering and Management Department, Sibiu, Romania

Corresponding author email: roxym_stoica@yahoo.com

Abstract

Biosurfactants are surface-active compounds synthesized by microorganisms as secondary metabolites with important applications in medicine, cosmetics, food, oil, agriculture, and the pharmaceutical industries. In the present study, the Pseudomonas fluorescens ICCF 392 strain was screened to determine its ability to produce extracellular biosurfactants. The strain was cultivated on M44 liquid medium (5% (v/v) glycerol as a carbon source) and also, on M44 modified medium (by replacing the glycerol as a carbon source with 5% (v/v) waste cooking oil). The supernatants obtained at the end of the bioprocesses were evaluated, to confirm the ability of the strain in biosurfactant production, using the drop collaps method, oil spreading technique, and emulsification activity determination (E_{24}). The best results were obtained in the case of the M44 liquid medium. The partially purified bioactive compounds were analyzed by the TLC method, which indicated the nature of the biosurfactants produced as rhamnolipids. Therefore, our results showed that the Pseudomonas fluorescens ICCF 392 strain was efficient in biosurfactant production, using glycerol or waste cooking oil as carbon sources in the biosynthesis process.

Key words*: Pseudomonas fluorescens,* rhamnolipids, glycerol, waste cooking oil, submerged fermentation.

INTRODUCTION

Biosurfactants are natural surface-active compounds produced by a variety of microorganisms, including bacteria, yeast, and fungi (Deshmukh et al. 2023; Roy, 2017).
Among them, species of the *Pseudomonas* genus are mostly utilized in the production of this significant class of bioactive compounds (Mohanty et al., 2021; Stancu, M.M., 2017).
Biosurfactants present several advantages over synthetic ones, such as lower toxicity, biodegradability, biocompatibility, high selectivity, stability over a broad pH and temperature range (Ciurko et al., 2023), and production from cheaper substrates (Balakrishnan et al., 2022).
Also, these bioactive molecules lower the interfacial and surface tensions of liquids, also are more stable and eco-friendly (Sharma et al., 2022).
Due to these physico-chemical characteristics, biosurfactants present potential applications in the biomedical field as antibacterial, antiviral, and antifungal compounds, in the cosmetic, food, detergents, textiles, petroleum recovery, bioremediation, and agriculture sectors (Anaukwu et al., 2020; Mandalenaki et al., 2021).
Usually, biosurfactants are typically amphipathic compounds that have both hydrophilic and hydrophobic elements. While hydrophobic compounds consist of a lengthy chain of fatty acids, hydrophilic compounds are typically composed of positive, negative, or amphoteric charged ions (Kumar et al., 2021).
Based on their molecular structure, biosurfactants can be glycolipids, lipopeptides, lipoproteins, phospholipids, or polymeric surfactants (Gurkok et al., 2021).
Of these, the most widely investigated class of biosurfactants are rhamnolipids, which belong

to the glycolipids group (Sarubbo et al., 2022). Parameters that play an important role in biosurfactant production are represented by the strain used, which must be non-pathogenic, the incubation conditions (temperature, aeration and agitation, time), and the carbon and nitrogen sources (Mandalenaki et al., 2021).

Presently, the high cost of the media components required in bioprocesses, such as glucose, limits the actual production of biosurfactants (Eras-Munoz et al., 2022). As an alternative, the use of glycerol or vegetable cooking oils as substrates in the fermentation processes becomes very important, taking into account that the amount of this waste increases year by year (Silva et al., 2010). Several studies from the literature have reported that *Pseudomonas* strains (especially *Pseudomonas aeruginosa)* are able to produce biosurfactants by using a variety of inexpensive substrates as carbon sources (Praveesh et al., 2011; Cássia et al., 2017).

Therefore, in this paper, potential substrates for the production of biosurfactants by the *Pseudomonas fluorescens* strain were assessed. These substrates, including glycerol and waste sunflower oil, were utilized as carbon sources in two different liquid media.

MATERIALS AND METHODS

Biologic material

The *Pseudomonas fluorescens* ICCF 392 strain used in this study, maintained on M44 agar medium, belongs to the Collection of Microorganisms of Industrial Importance-CMII–ICCF-WFCC 232.

Fermentation media and cultivation conditions

The bacterial strain used as the pre-inoculum was cultured on M44 agar medium with the following composition % (g/v): glycerol 5.0, yeast extract 1.0, bacto-peptone 1.0, and agar 2.0, and incubated for 48-72 hours at 30°C.

In order to prepare the inoculum, 2.0 mL of pre-inoculum containing $9x10^8$-CFU/mL (McFarland Standard No. 3) was seeded into 100 mL of liquid medium in a 500 mL Erlenmeyer flask. The inoculum medium was then incubated for 24 hours at 30°C and 220 rpm on a rotary shaker.

For biosurfactant production, the following liquid media were used:

- M44 medium (without agar) % (g/v): glycerol 5.0, as a carbon source; yeast extract 1.0, and bacto-peptone 1.0, as nitrogen sources.
- M44 modified medium % (g/v): waste sunflower oil 5.0, as a carbon source; yeast extract 1.0, and bacto-peptone 1.0, as nitrogen sources.

The chemicals were purchased from Difco (USA) and Sigma-Aldrich (Germany). The culture media were prepared with distilled water, pH-adjusted to 6.5-7.0, and sterilized for 30 minutes at 115°C.

The biosurfactant production was performed in 500 mL Erlenmeyer flasks containing 100 mL of the liquid medium inoculated with 10% (v/v) of inoculum. The flasks were incubated at 30°C, 220 rpm, for 72 hours. Bacterial cell growth was determined by measuring the pH and optical density of the culture medium at 550 nm.

Detection of biosurfactant production

Three methods were used for the detection of biosurfactant production, as follows:

Drop collapse method

The drop-collapse test was performed in accordance with Ghazal et al. (2017): a droplet containing 25 µl of extracted biosurfactant was pipetted onto parafilm and allowed to flatten and spread over the surface for a few seconds or minutes. The water-stain droplet was then mixed with methylene blue, which had no effect on the droplet shape. After a period of drying, the diameter of the droplet was measured. The presence of biosurfactant in solution gave flat drops, while the absence of biosurfactant gave rounded droplets.

Oil displacement assay

After adding a biosurfactant-containing solution to an oil-water interphase, the diameter of the clear zone can be measured using the oil displacement method (Balakrishnan et al., 2022). In a Petri dish, 30 mL of distilled water were added and 1 mL of sunflower oil was added to the center of the plates. Over the oil layer, 20 µl of the culture supernatant obtained from the bacterial broth was added.

A zone of displacement in the oil was carefully observed in the Petri dishes, and the diameter of

displacement was measured and compared with the control using the uninoculated medium, after 30 seconds (Arora et al., 2015).

Emulsification activity

The production of biosurfactants by the *Pseudomonas fluorescens* ICCF 392 strain was assessed through submerged fermentations using 500 mL flasks containing 100 mL medium on a rotary shaker at 220 rpm. The strain was grown in M44 and M44 modified liquid media for 72 hours at 30°C.

After the culture broths were centrifuged for 20 minutes at 4°C and 9000 rpm, the emulsification index (E24) was calculated. In different tubes, 4 mL of the supernatant was mixed with 6 mL of heptane, octane, and sunflower oil. The tubes were then vigorously mixed for five minutes and stored for twenty-four hours (Pathak et al., 2014).

Using the formula E_{24} % = (height of the emulsified layer/total height of the liquid column) × 100, the emulsification index was determined.

The experiments were performed in triplicate.

Biosurfactant isolation

Bacterial cells were removed by centrifugation at 9000 rpm, 4°C, for 20 min.

In order to precipitate the biosurfactant, the cell-free supernatant was acidified with HCl concentrated to pH 2.0 and left overnight at 4°C.

The precipitate thus obtained by centrifugation in similar conditions was extracted using a 4-fold volume of ethyl acetate.

The lower organic phase, containing biosurfactant, was collected by using a separating funnel.

By using rotary evaporation at 40°C and low pressure, the solvent was removed.

A partially purified viscous honey-colored rhamnolipid product was collected after solvent evaporation and used for characterization (Invally et al., 2019).

Biosurfactant characterization by thin-layer-chromatography

The preliminary characterization of biosurfactants was performed by TLC analysis. Silica gel 60 F_{254} TLC plates (10 x 20 cm; Merck Millipore) were used for further analysis. Rhamnose was separated on silica gel plates using water, methanol, glacial acetic acid, and 1.2-dichloroethane (10:15:25:50, v/v/v/v) as the mobile phase.

Rhamnose spots were visualized by spraying with a thymol-EtOH-H_2SO_4conc. (0.5g, 95mL, 5mL) reagent. Plates were heated at 130°C for 10 minutes after the application of the spraying agent (Schenk et al., 1995).

Also, lipids were separated on silica gel plates using n-heptane, ethyl ether, acetic acid (70:25:5, v/v/v) as the mobile phase. Lipid spots were visualized by spraying with a 0.5% solution of vanillin in a mixture of 5 mL of sulfuric acid, 5 mL of phosphoric acid, and 90 mL of ethyl alcohol. Plates were heated at 130°C for 10 minutes after the application of the spraying agent (Avinesh et al., 2016).

RESULTS AND DISCUSSIONS

Drop collapse method

According to the drop-collapse method, an oily surface will cause a drop of liquid containing a biosurfactant to collapse and spread. The results of the collapse test revealed that the *Pseudomonas fluorescens* ICCF 392 strain was capable of producing biosurfactant on both substrates evaluated. For distilled water (control), no activity was detected. The droplets remain round, with a diameter of 0.4 mm. The biosurfactant droplets do result in collapsed droplets with a diameter of 0.8 mm in the case of media obtained using glycerol as substrate and 0.7 mm for waste cooking oil substrate, respectively, showing their effects on the reduction of surface tension (Figure 1).

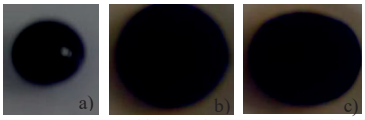

Figure 1. Detection of biosurfactant production by *Ps. fluorescens* ICCF 392 strain: drop collapse assay: a) control; b) M44 medium; c) M44 modified liquid medium

Oil displacement assay

The oil displacement method is used to measure the surface activity of a surfactant solution tested against oil; higher surfactant surface activity is indicated by a larger diameter of the displaced circle. The results of the oil displacement assay are shown in Figure 2. Both

samples produced by *Pseudomonas fluorescens* ICCF 392 strain had significantly displaced the oil layer and spread in the water; the largest diameter value of displaced circle of 7 cm was obtained in the case of media containing glycerol 5%, while the cultures obtained on media containing waste cooking oil 5% produced a displaced circle measuring 6.5 cm.

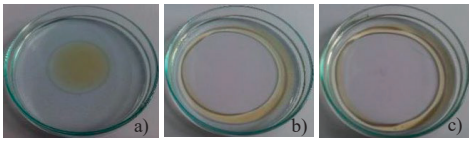

Figure 2. Detection of biosurfactant production by *Ps. fluorescens* ICCF 392 strain: oil displacement assay: a) control; b) M44 medium; c) M44 modified liquid medium

Emulsification Index

For 72 hours, bioprocesses were performed at 30°C. At the end of the process of biosynthesis, the media were centrifuged, and the ability of the supernatants to emulsify heptane, octane, and sunflower oil was assessed (Figure 3).

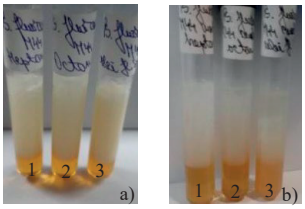

Figure 3. Emulsions obtained with 1-heptane, 2-octane, and 3-sunflower oil with the supernatants of *Pseudomonas fluorescens* ICCF 392 strain cultivated on: a) M44 liquid medium (b) M44 modified liquid medium

In Figure 3, the emulsions obtained with the supernatants of the *Pseudomonas fluorescens* ICCF 392 strain cultivated on M44 and M44 modified liquid media are presented.

Supernatants of *Pseudomonas fluorescens* ICCF 392 grown in M44 liquid medium produced stable emulsions with heptane, octane, and sunflower oil. After 24 hours, in the case of M44 liquid medium, the values of the emulsifying index were 72.46% for heptane, 75.71% for octane, and 72.58% for sunflower oil.

Regarding the M44 modified medium, the strain produced biosurfactant that emulsified heptane and octane, with an emulsifying index of 63.6% and 62.5%, respectively. Instead, regarding the emulsions obtained with sunflower oil, they registered slowly low values of the emulsifying index, at 61.3%. The emulsification index results (E_{24}%) obtained are shown in Figure 4.

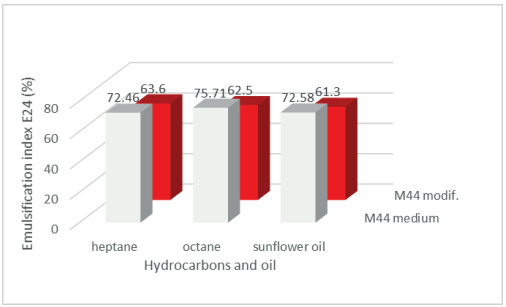

Figure 4. Emulsification index for heptane, octane, and sunflower oil obtained with supernatant of *Pseudomonas fluorescens* ICCF 392 cultivated on M44 and M44 modified liquid media

Therefore, the supernatants of the *Pseudomonas fluorescens* strain formed emulsions with heptane, octane, and sunflower oil, with the best values of the emulsification index obtained on M44 liquid medium containing glycerol at 5%, followed by those obtained using waste cooking oil at 5%.

The results show that the values of the emulsifying indices obtained are in accordance with the findings of Matátková et al. (2022), who reported an emulsification index of 70% for sunflower oil, and also with the results reported by Joice and Parthasarathi (2014), who obtained an emulsifying index of almost 70.0% for heptane. The emulsions obtained remained stable for over a month, and according to the literature, it is considered that emulsion stability is one of the most important characteristics of a biosurfactant (Anyanwu et al., 2011; Diab et al., 2016).

Biosurfactant characterization

The biosurfactants extracted were characterized by the TLC method. The analyzed samples in UV light show spots characteristic of the rhamnose compound, brown-violet-colored, identified at Rf~0.45 (Figure 5).

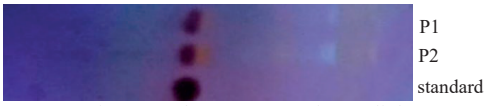

Figure 5. Rhamnose spots in UV light: P1- M44 medium; P2 –M44 modified medium; L(+)- Rhamnose - standard

Also, the biosurfactant fraction showed a positive reaction with the vanillin reagent, indicating the presence of lipid moieties. The chromatogram visualized after spraying with the identification reagent presented, in the two studied samples, multiple spots, pink-grey in color and of different intensities, at the following values: R_f~0.10; R_f~0.12; R_f~0.15; R_f~0.41; R_f~0.50; R_f~0.52; R_f~0.75; R_f~0.80; and R_f~0.92. Over time, the color of the spots changes to blue-grey and fades (Figure 6).

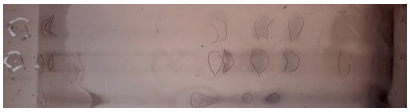

Figure 6. Spots of lipids after spraying with reagent: P1 - M44 medium; P2 - M44 modified medium; cholesterol - standard

The above results of TLC analysis demonstrated that the biosurfactant was a glycolipid composed of sugars and lipids. Similar reports of the production of rhamnolipids by *Pseudomonas* species (Rf values 0.4 and 0.37) are then in the literature (Moon et al., 2002; Rekadwad et al., 2019).

CONCLUSIONS

Our results demonstrate that the *Pseudomonas fluorescens* ICCF 392 strain is a good producer of biosurfactants on substrates containing glycerol and waste cooking oil as carbon sources at a concentration of 5%, suggesting the possibility of industrial production of biosurfactants using economically cheaper substrates. The biosurfactants isolated from the fermentation media were identified by TLC analysis as rhamnolipids and showed high emulsification activity, which makes them suitable for various industrial and environmental applications.
Thus, these preliminary results show that it is possible to reduce the costs associated with the raw materials used for biosynthesis, obtaining glycolipids with added industrial value. The biosurfactant-producing capacity of this strain will be further increased by optimization studies.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Ministry of Research, Innovation and Digitization, CCCDI-UEFISCDI, project number PN-III-P2-2.1-PED-2021-3528, 731PED/2022, within PNCDI III.

REFERENCES

Anaukwu, C.G., Ogbukagu, C.M. & Ekwealor, I.A. (2020) Optimized biosurfactant production by *Pseudomonas aeruginosa* strain CGA1 using agro-industrial waste as sole carbon source. *Advances in Microbiology 10*, 543-562.

Anyanwu, C., Obi, S.K.C. & Okolo, B.N. (2011). Lipopeptide biosurfactant produced by *Serratia marcescens* NSK-1 strain isolated from petroleum contaminated soil. *Journal of Applied Sciences Research*, 7, 79-87.

Arora, S.K., Sony, J., Sharma, A. & Taneja, M. (2015). Production and characterization of biosurfactant from *Pseudomonas spp. International Journal of Current Microbiology and Applied Sciences,* 4(1), 245-253.

Avinesh, R. B., Adarsha, G., Colin, J. B. & Munish, P. (2016). A quick colorimetric method for total lipid quantification in microalgae. *Journal of Microbiological Methods 125*, 28-32.

Balakrishnan, S., Arunagirinathan, N., Rameshkumar, M.R., Indu, P., Vijaykanth, N., Almaary, K.S., Almutairi, S.M.& Chen, T.W. (2022). Molecular characterization of biosurfactant producing marine bacterium isolated from hydrocarbon-contaminated soil using 16S rRNA gene sequencing. *Journal of King Saud University – Science,* 34 (3), 101871.

Cássia, R.F, Almeida, D.G., Meira, H.M., Silva, E.J., Farias, C.B.B., Rufino, R.D., Luna, J.M. & Sarrubbo, L.A. (2017). Production and characterization of a new biosurfactant from *Pseudomonas cepacia* grown in low-cost fermentative medium and its application in the oil industry. *Biocatalaysis and Agricultural Biotechnology,* 12, 206-215.

Ciurko, D., Chebbi, A., Kruszelnicki, M., Czapor-Irzabek, H., Urbanek, A.K., Polowczyk, I., Franzetti, A. & Jane, T. (2023). Production and characterization of lipopeptide biosurfactant from a new strain of *Pseudomonas Antarctica* 28E using crude glycerol as a carbon source. *RSC Advances,* 13, 24129–24139. doi: 10.1039/d3ra03408a.

Deshmukh, N. & Kathwate, G. (2023). Biosurfactant production by *Pseudomonas aeruginosa* strain LTR1 and its application. *Biointerface Research in Applied Chemistry,* 13(1), 1-13.

Diab, A.; Sami, S. & Diab, A. (2016). Production, characterization and application of a new biosurfactant derived from egyptian sunflower seeds. *International Journal of Science and Research,* 5, 602-612.

Eras-Munoz, E., Farre, A., Sanchez, A., Font, X. & Gea, T. (2022). Microbial biosurfactants: a review of recent environmental applications. *Bioengineered,* 13, 12365-12391.

Ghazal, M.F., Moussa, L.A., Makboul, H.E. & Fayed, S.A. (2017). Screening of some Bacilli strains for their abilities to produce biosurfactants. *Der Pharma Chemica,* 9, 6-12.

Gurkok, S. & Ozdal, M. (2021). Microbial biosurfactants: properties, types, and production. *Anatolian Journal of Biology,* 2, 7-12.

Invally, K., Sancheti, A. & Ju, L.K. (2019). A new approach for downstream purification of rhamnolipid biosurfactants. *Food and Bioproducts Processing,* 114, 122-131.

Joice, P.A. & Parthasarathi, R. (2014). Optimization of biosurfactant production from *Pseudomonas aeruginosa* PBSC1. *International Journal of Current Microbiology and Applied Sciences, 3*, 140-151.

Kumar, A., Singh, S.K., Kant, C., Verma, H., Kumar, D., Singh, P.P., Modi, A., Droby, S., Kesawat, M.S., Alavilli, H. Bhatia, S.K., Saratale, G.D., Saratale, R.G., Chung, S.M. & Kumar, M. (2021). Microbial biosurfactant: A new frontier for sustainable agriculture and pharmaceutical industries, *Antioxidants,* 10, 1472, 1-26.

Mandalenaki, A, Kalogerakis, N. & Antoniou, E. (2021). Production of high purity biosurfactants using heavy oil residues as carbon source. *Energies,* 14, 3557, 1-17.

Matátková, O., Michailidu, J., Ježdík, R., Kolouchová, I.J., Rezanka, T., Jirku, V. & Masák, J. (2022). Production and characterization of rhamnolipids produced by *Pseudomonas aeruginosa* DBM 3774: Response surface methodology approach. *Microorganisms,* 10, 1-14.

Mohanty SS., Koul Y., Varjani S., Pandey, A., Ngo, HH., Chang, JS., Wong, JWC. & Bui, XT. (2021). A critical review on various feedstocks as sustainable substrates for biosurfactants production: a way towards cleaner production. *Microbial Cell Factories,* 20, 1-13.

Moon, H.J., Lim, Y.K., Kim, H.S, Kwon, D.Y. & Chung, W.J. (2002). Glycolipid biosurfactants produced by *Pseudomonas aeruginosa* D2D2 from diesel-contaminated soil. *Journal of Microbiology and Biotechnology,* 12(3), 371-376.

Pathak, K.V. & Keharia, H. (2014). Application of extracellular lipopeptide biosurfactant produced by endophytic *Bacillus subtilis* K1 isolated from aerial roots of banyan (*Ficus benghalensis*) in microbially enhanced oil recovery (MEOR). 3 *Biotech 4*, 41-48.

Praveesh B.V., Soniyamby A.R., Mariappan C., Kavithakumari P., Palaniswamy M. & Lalitha S. (2011). Biosurfactant production by *Pseudomonas sp.* from soil using whey as carbon source. *New York Science Journal,* 4(4), 99-103.

Rekadwad, B., Maske, V., Khobragade, C.N. & Kasbe, P.S. (2019). Production of mono- and di-rhamnolipids produced by *Pseudomonas aeruginosa* VM011. *Data in brief,* 24, 103890.

Roy, A. (2017). A Review on the biosurfactants: properties, types and its applications. *Journal of Fundamentals Renewable Energy and Applications, 8* (1), 1-5. doi: 10.4172/2090-4541.1000248.

Sarubbo, L.A., Silva, C.M.G., Durval, I.J.B., Bezerra, K.G.O., Ribeiro, B.G., Silva, I.A., Twigg, M.S. & Banat, I.M. (2022). Biosurfactants: production, properties, applications, trends, and general perspectives. *Biochemical Engineering Journal 181*, 1-19. https://doi.org/10.1016/j.bej.2022.108377.

Schenk T, Schuphan I. & Schmidt B. (1995). High-performance liquid chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa. Journal of Chromatography A.*, 693(1), 7-13. doi: 10.1016/0021-9673(94)01127-Z.

Sharma, S., Verma, R., Dhull, S., Maiti, S.K. & Pandey, L.M. (2022). Biodegradation of waste cooking oil and simultaneous production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa* P7815 in batch and fed-batch bioreactor. *Bioprocess and Biosystems Engineering,* 45, 309-319.

Silva, S.N.R.L., Farias, C.B.B., Rufino, R.D., Luna, J.M. & Sarubbo, L.A. (2010). Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. *Colloids and Surfaces B: Biointerfaces,* 79,174-183.

Stancu, M.M. (2017). Polyaromatic hydrocarbons utilization by a *Pseudomonas* strain. *Scientific Bulletin. Series F. Biotechnologies*, vol. XXI, 200-205.