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Treatment of oil-containing wastewater using microorganisms immobilized on shungite

Bioremediation of oil-polluted natural objects using oil degrading microorganisms immobilized on schungite sorbents was investigated in laboratory conditions. Microorganisms isolated from soil of Botakhan oil field, located in Makat region (Atyrau) are presented for the first time. The isolated active strains of microorganisms were screened and identified (*P.stutzeri, B.atrophaeus, Isoptericola varabilis*). Immobilization of oil degrading bacteria cells on shungite sorbents from Kazakhstan ("Bakyrchik" field) and Russia ("Zazhegino" field) was performed. Sorption and destructive activity of bacteria strains have been studied. The biosorbents proposed here are shown to provide effective adsorptive and biological purification of wastewater from oil.

Keywords: immobilization, adsorption, biosorbent, association of microorganisms, shungite, oil.

Introduction

It is well known that oil producing and oil refining companies have made a significant impact on the environment. In oil extraction areas it is observed a change in chemical and microbiological composition of ground and surface waters and soil as a result of their contamination with petroleum products, surface-active substances, other chemical reagents.

Kazakhstan is among the top ten oil producing countries in the world which largely causes related problems of environmental pollution with oil and oil products. The most serious such oil pollution problems appear in Kyzyl-Orda, Atyrau, Mangistau, West Kazakhstan and Aktobe regions where large oil-contaminated areas are located. To address and solve these problems, significant efforts of the researchers from different countries were made. Among different methods to combat pollution an important place belongs to biological methods, including bioremediation and phytoremediation [1-4], the creation of microbial consortia of destructive microorganisms, including immobilized ones [5-7]. Currently in great demand are so-called "bacterial cocktails", in a wide range offered by biotech companies in Europe, USA and Japan. Nevertheless, in real practice the use of foreign bacterial drugs is ineffective because of the variations in climate and environmental conditions. In addition, uncontrolled introduction of microbial populations into open ecosystems, frequently of unknown composition, may pose a significant danger to the functioning of soil biocenosis, and for the health of the people involved in technological process. Therefore, the development of domestic biological products for purification and restoration of natural objects Kazakhstan is an urgent task.

In connection with the above mentioned, we set the objective to carry out identification and screening of active strains of oil-oxidizing microorganisms in order to study the adsorptive and destructive properties of biocatalysts.

Currently, much attention is paid to new, highly efficient technologies using biosorbents which combine advantages of both sorption and biodestructive methods of liquidation of oil spills. Biodestructive sorbents localize oil pollution and break down petroleum products adsorbed by the biological method. Such combination of methods allows to achieve an extremely high purification efficiency under optimal conditions.

Preliminary stages of biodestructive methods include a selection of microbial destructors which are supposed to be: capable of degrading various oil components, genetically stable, viable during storage time, rapid growth after storage, high extent of enzymatic activity and growth in the environment, the ability to compete with local microorganisms, etc.

Currently a wide range of microorganisms are used for the destruction of petroleum hydrocarbons in the environment including bacteria *Pseudomonas, Mycobacterium, Micrococcus*, etc. and yeast *Candida* [8]. Greater attention should be paid to the associations of microorganisms [9]. Developing in associations, germs enter into complex relationships. The most active of bacteria destroy more complex hydrocarbons.

Under natural conditions, the vast majority of microorganisms live, breed and exhibit various biochemical activity in the fixed position being attached to the mineral particles of soil, sediments of lakes, rivers, seas, to the roots or ground parts of plants. Therefore, to create optimal conditions for the growth of microbial destructors introduced into water, and to ensure their long vital activity in it, one usually uses preliminary immobilization of cells on insoluble carriers.

The main issue for immobilizing cells on a carrier is the selection of the most efficient carrier since microorganisms exhibit greater selectivity for the adhesion to various carriers in respect of the quantity of microbial cells being attached to the sorbent, and to the strength of bonds formed [10].

Immobilized cells due to their viability represent more stable inductors of redox reactions. In addition, immobilized microorganisms as compared with free cells are more potent (2-3 times) and more resistant to environmental factors such as temperature, pH, xenobiotics.

Thus, picking up a suitable pair ‘carrier - microbial cell’ with desirable properties meeting the technological requirements, you can get highly effective biosorbents for their use in purification of water bodies from various pollutants.

As a source for producing a number of carbon-containing materials (carriers for microorganisms) can serve shungite rocks available in Kazakhstan in quantities sufficient for industrial use. In this connection, the development of fundamental technological concepts for creation of new carbon-containing products from Kazakhstan raw materials is of particular interest.

Shungite rock composed of fine crystalline silicate and amorphous carbon materials, is unique in their composition and structure natural composite, with a number of promising technological properties. They are of interest as a composite adsorbent, catalyst or catalyst carrier possessing properties of both carbon and silicate materials. There is an ongoing study of the possibility of using shungite as sorption material for purification of wastewater from oil products and phenols [11].

Researchers explain the unique properties of shungite by its unusual structure. Schungite carbon forms in the rock matrix in which are uniformly distributed particulate silicates with an average size of about 1 micron. Schungite rock properties are determined by two factors: first, the properties of carbon schungite, second, the structure of rock, the relationship between carbon and silicates.

Given the complex mineralogical and chemical composition of this carbon-containing material, the synthesis of materials of given composition and properties, becomes an urgent problem of both scientific and practical importance.

Materials and Methods

The objects of study were active strains of microorganisms isolated from oil contaminated soil of “Botakhan” field, Atyrau region: bacterial cultures AT1, AT3, AT4, the association of microorganisms 1:1:1 (AT1 + AT3 + AT4) and schungite sorbents from “Bakyrchik” and “Zazhegino” fields.

The isolated strains were immobilized on the two types of schungite sorbents from “Bakyrchik” (Kazakhstan): K AT-1, K AT-3, K AT4 and from “Zazhegino” (Russia): R AT-1, R AT-3, R AT-4.

The following well-known techniques were used in this investigation: cell growth analysis by seeding on solid nutrient media; the determination of oil-oxidizing activity of free and immobilized cells of destructive strains, measurements of morphological and cultural properties of microorganisms, genetic identification of bacteria on the basis of nucleotide sequence analysis of 16S rRNA gene, fluorometric method (Fluorat-02) [12] for quantitative oil determination in water. Selection of the most promising strains was performed according to criteria such as high oxidizing activity under normal conditions, and the ability of strains to complement each other in their capacity for biodegradation of petroleum hydrocarbons.

Morphological study of microorganisms was performed using trinocular microscope Micros TX 300 models with built-in video camera.

DNA was isolated by KateWilson method [13]. Qualitative DNA evaluation was performed by DNA electrophoresis method [14], while quantitative DNA analysis was conducted by spectrophotometry using a NanoDrop spectrophotometer at a wavelength of 260 nm. When DNA of high purity is isolated, it shows values of optical density of 1.83 to 2.12 at 260/280 nm.

Sequencing reaction was conducted using BigDye ® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following manufacturer's instructions, with subsequent fragmentation on an automated Genetic Analyzer 3730xl DNA Analyzer (Applied Biosystems). The nucleotide sequences for 16S rRNA gene of three identifiable strains were analyzed and integrated into the joint sequence in the software SeqScape 2.6.0 (Applied Biosystems). After that the terminal fragments (nucleotide sequences of the primers, fragments with a low quality score) were removed, which resulted in the nucleotide sequence of more than 650 bp length. Nucleotides were identified by the algorithm BLAST in the Gene Bank [14].

Shungite samples used as sorbents were sterilized, dried in an oven to dry weight. To obtain a suspension of microbial biomass, the microorganisms were cultured in a nutrient agar medium for 24 hours at a temperature of 30°.

Optical density of the cell suspension was measured with a spectrophotometer UNICO.

Results and discussion

Preliminary studies were conducted on synthetic wastewater which was subject to microbial purification from organic pollutants, which included: adsorption of microbial cells, obtaining biocatalysts based on the adsorbed cells, study of the destructive properties of the biocatalysts.

These studies resulted in selection of the microbial strains, which demonstrated active growth in an oil medium.

To produce effective biocatalysts it is necessary to isolate active destructive microbial strains directly from the oil-contaminated areas. We conducted a study of microflora in soil of Botahan oil field to isolate the active strains of oil-oxidizing microorganisms. From the isolated cultures three strains revealed the destructive ability in relation to crude oil. Genetic identification of the isolated active destructive strains was held at National Center of Biotechnology in Astana.

As an alternative to the classical biochemical identification may serve modern molecular genetic techniques. In the 1980s, a new standard for bacterial identification was introduced, which allows to identify microorganisms, with no information about its biological features. C. Woesec with coworkers found that phylogenetic relationships of bacteria may be identified by comparing the highly conserved part of the genetic code, which marked the beginning of a rapidly developing universal identification system. High nucleotide sequence identity of 16S rRNA gene as compared with other rRNA genes, allowed its use as a standard genetic marker for identification and taxonomic classification of bacterial species [15].

Identification of three strains was conducted by determining the direct nucleotide sequence for the fragment of 16S rRNA gene, with further determination of the nucleotide identity with the sequences deposited in the GeneBank international database. The strain names and the sources of isolation are given in Table 1.

Table 1 - Isolation sources and DNA concentration.

Sample	Strain	Isolation source	Concentration ng/ul	260 /280
1	AT1	Soil	2173,5	1,99
2	AT3	Soil	591,3	1,91
3	AT4	Soil	759,9	2,03

DNA isolation experiments resulted in DNA samples prepared with high concentration and good quality. Using polymerase chain reaction (PCR) method a fragment of 16S rRNA gene was amplified, with molecular weight of about 1,000 base pairs (bp) (Fig. 1) for all identifiable strains. Products of PCR amplification were used to determine the nucleotide sequence.

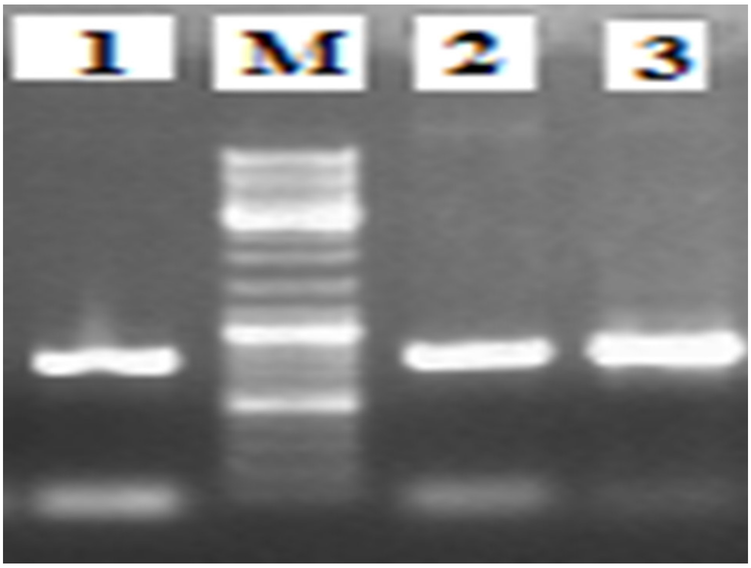

Figure 1 - Electrophoregrams of the products of PCR amplification of the 16S rRNA gene fragment of DNA:
Note: 1-3 – samples, numbering according to bp; M – molecular weight marker (Fermentas)
(100 – 1000 bp, from 100 to 1000 bp in 100 bp increments)

After removing terminal fragments (nucleotide sequences of the primers, fragments with low quality) the nucleotide sequences of the length over 650 bp were obtained and identified by the algorithm BLAST in the Gene Bank [14].

When using the international identification database NCBI [16] to identify nucleotides, species with maximal identity were chosen. Identification results are shown in Table 2.

Table 2 - Identification results of nucleotide sequences in International Database.

Strain name	Accession Number (GeneBank)	Strain name by identification results	Coincidence rate, %
AT1	NC_018028.1	*Pseudomonas stutzeri*	99
AT3	NC_014639.1	*Bacillus atrophaeus*	100
AT4	NC_015588.1	*Isoptericola variabilis*	97

It is officially recognized that the sequence identity of 97% for 16S rRNA gene represents a general threshold value of the species [17]. As it is seen from the results given in Table 2, identified strains have maximum identity.

Thus, the isolated strains of bacteria - oil destructors are identified by us as representatives of the species *Pseudomonas* stutzeri - AT1, *Bacillus atrophaeus* - AT3, *Isoptericola variabilis* - AT 4.

There were investigated physical and chemical properties of shungite based carbon-mineral sorbent, which are shown in Table 3.

Table 3 - Physical and chemical properties of carbon sorbents.

Indicator	Shungite sorbent of "Batyrchik" field	Shungite sorbent of "Zazhegino" field
Bulk density, g/cm^3	0,74	0,70
Humidity, %	0,17	0,33
Ash content, %	6,15	10,05
Specific surface, m^2/g	42,83	11,671
Specific pore volume limit at complete filling, cm^3/g	0,018	0,005
Iodine adsorption activity, %	24,43	18,31
Average pore size, nm	1,716	1,718

Harmlessness of schungite based carbon-mineral sorbent for the environment, its availability (made from local raw materials and has a low cost unlike other popular carbon sorbents) allowed the use of schungite sorbent as a carrier for cell immobilization of oil-oxidizing microorganisms with the purpose of purifying natural objects from oil.

Immobilized microorganisms have a number of benefits along with free cells, thus using immobilized cells of microorganisms in purification of contaminated ecosystems high efficiency of bioremediation may be achieved.

Figures 2 and 3 show the adsorption dynamics of the association made from strains AT1, AT3 and AT4 and monocultures on shungite sorbents from Zazhegino and Bakyrchik fields.

As can be seen from Fig. 2, after 6 hours from the beginning of adsorption, 90% of AT1` strain cells was adsorbed, while by the end of this experiment this number changed to 84%. At the same time cell strains AT3 and AT4 showed a much lower adsorption values equal to 50% and 64% respectively, as compared to AT1 strain, but further experiment showed increased adsorption: 65% for AT3 and 92% for AT4. Adsorption activity revealed by association reached 91% during the first 6 hours, and by the end of the experiment this value gradually rose to 95%.

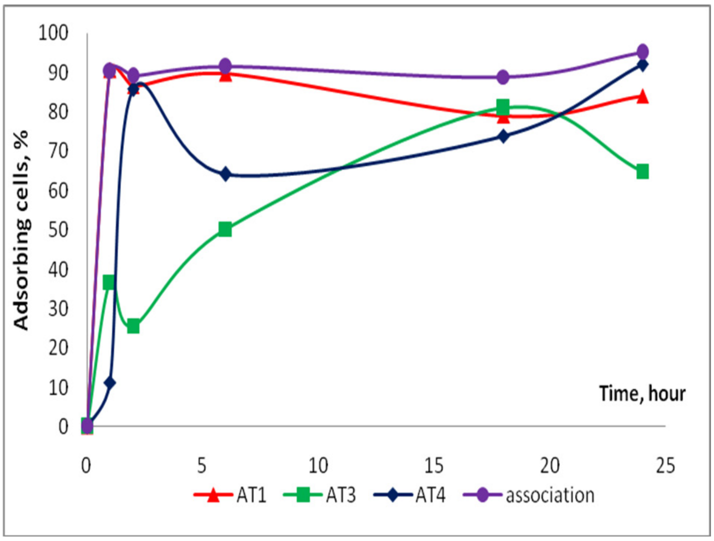

Figure 2 - Adsorption dynamics of microbial cells on "Zazhegino" sorbent.

The adsorption dynamics of the cells on Bakyrchik sorbent, as can be seen from Fig. 3, is high. At the beginning of the experiment, up to 6 hours, the cells are adsorbed in the following amounts: association - 97%, AT1 strain - 94%, AT3 strain - 93%, AT4 strain - 90% of the adsorbed cells. These parameters do not change significantly throughout the time of the experiment, reaching after 24 hours 95% adsorption for AT1 strain, 98% for AT3 strain, 95% for AT4 strain and 98% for the association.

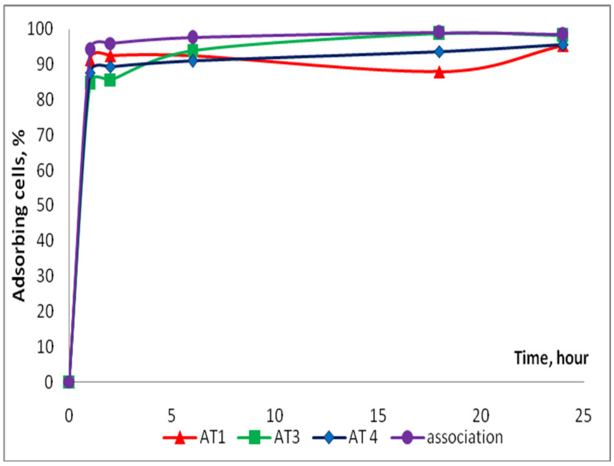

Figure 3 - Adsorption dynamics of microbial cells on “Bakyrchik” sorbent

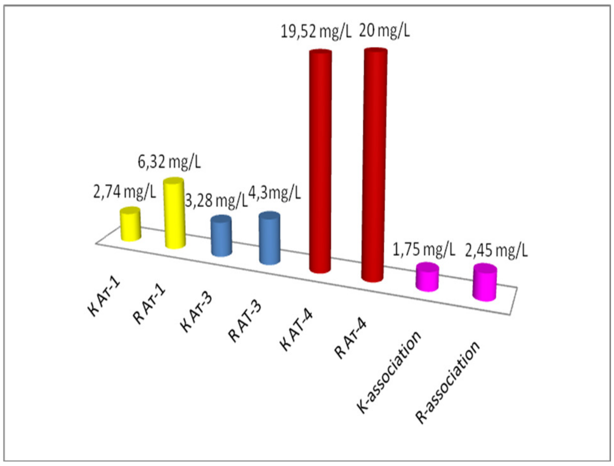

Figure 4 - The residual amount of oil in water after treatment.

Thus, we have determined that association representing a destructor consisting of strains of microorganisms AT1, AT3 and AT4, revealed active adsorption on sorbents Zazhegino and Bakyrchik: adsorption rates equal to 95% and 98% respectively, which is higher than the results shown by monocultures of microorganisms.

In recent years, an interest in the application of microorganisms for the treatment of industrial wastewater has been observed. The mechanism for purification of oil-contaminated biological objects with single-celled organisms are biosorption and interaction with metabolites [18].

Improving the efficiency of biological products can be achieved using cells - destructors immobilized on different carriers. This is due to the fact that the attachment of cells to solid surfaces provides high concentration of microbial cells in the area of action, prevents their leaching, protects from the effect of high concentrations of toxic oil components and makes it possible to increase the specific destructive activity of microflora.

We studied the microbial degradation of organic substances by the active strains of microorganisms isolated from “Botahan” deposit, Atyrau region. Crude oil was used as the organic substance. Mass concentration of oil measured on fluid analyzer “Fluorat-02” was equal to 4081.25 mg/l.

The experiment was performed during 30 days in a rotary shaker at 220 rpm and room temperature. Initial oil concentration was equal to 4081.25 mg/l. Significant reduction of oil products in water, as it is shown in fig.4, demonstrates the possibility of using microorganisms immobilized on shungite for purification of oil-contaminated water bodies.

As seen from Fig. 4, R AT-1 and R AT-3 biocatalysts made from sorbent “Zazhegino” (Russia) show higher destructive power when compared to R AT-4. The concentration of oil products in water on the 30th day dropped from 4081.25 mg /L to 6.32 mg/L (R AT-1) and 4.3 mg/L (R AT-3) respectively. High destructive activity of R-association (2.45 mg / L) exceeds the activity demonstrated by all three biocatalysts based on “Zazhagino” sorbent: R AT-1, R AT-3, R AT-4.

As for the biocatalysts made from “Bakyrchik” sorbent (Kazakhstan), they showed the same results as biocatalysts based on “Zazhegino” sorbent (Russia).

Conclusion

Screening and identification of the active strains of microorganisms (*P.stutzeri, B. atrophaeus, Isoptericola variabilis*), isolated from soil of Botahan oil field (Makat region, Atyrau oblast), was performed. Cell immobilization of oil-oxidizing bacteria on different sorbents (RAT-1, RAT-3, RAT-4, KAT-1, KAT-3, KAT-4) has been studied. The results of using microbial associations showed that biosorbents based on association strains reveal higher destructive activity in relation to oil as compared to monocultures. The attempt was made to purify oil-contaminated water using microorganisms immobilized on shungite. Significant reduction of oil products in water after microbial treatment may be further developed to serve as the basis for future industrial application.

References

1 Shatalov A.A., Novikov A.D., Janenko A.S. Biodegradacija neftjanyh zagrjaznenij morskimi obligatnymi nefteokisljajushhimi mikroorganizmami // Mat. II-go Moskovskogo mezhdunarodnogo kongressa «Biotehnologija: sostojanie i perspektivy razvitija». – M. - Ch. 2. - 2003. – 220 s.

2 Wilkinson S., Nicklin S. // Biotransformations: Bioremediation Tecchnology for Health and environmental Protection / Stapletion Amsterdam; London; New-York; Oxford; Paris; Tokio. 2002. 69-100 p.

3 Pleshakova E.V., Matora L.Ju., Turkovskaja O.V. Nefteokisljajushhij shtamm Dietzia maris i vozmozhnosti ego ispol'zovanija dlja bioremediacii zagrjaznennoj pochvy // Vestnik MGOU. Serija Estestvennye nauki. – 2010. – № 4. – S. 82-89.

4 Dubrovskaya E., Pleshakova E., Turkovskaya O. Using molasses for stimulation of the degradative and activities of the microbial community in soil contaminated with oil shale liquid fuel // Soil Contamination: New Research / Ed. A.N. Dubois. – USA: Nova Science Publishers, 2008. – P. 121-138.

5 Wuyep P.A. Chuma A.G., Awodi S. and Nok A. J. Biosorption of Cr, Mn, Fe, Ni, Cu and Pb metals from petroleum refinery effluent by calcium alginate immobilized mycelia of Polyporus squamosus. Scientific Research and Essay, 2009. - Vol. 2, № 7. - P. 217-221

6 Sidorov A.V., Morozov N.V., Gicareva E.V. Biodegradacija neftesoderzhashhih prirodnyh i stochnyh vod konsorciumom uglevodorodokisljajushhih mikroorganizmov // Pjatyj mezhdunarodnyj kongress po upravleniju othodami i prirodoohrannymi tehnologijami VjejsTek – 2007. – Moskva, 2007. – S. 341-342.

7 Pirog T.P. Ispol'zovanie immobilizovannyh na keramzite kletok nefteokisljajushhih mikroorganizmov dlja ochistki vody ot nefti // Shevchuk T. A., Voloshina I. N., Grechirchak N. N. // Prikladnaja biohimija i mikrobiologija. - 2005. - T. 41. - № 1. - S. 58-63.

8 Sidorov D.G., Borzenkov I.A., Ibatullin R.R. i dr. Polevoj eksperiment po ochistke pochvy ot neftjanogo zagrjaznenija s ispol'zovaniem uglevodorodokisljajushhih mikroorganizmov // Prikladnaja biohimija i mikrobiologija. - 1997. – T. 33. - № 5. – S. 497-502.

9 Baryshnikova L.M., Grimennov V.G., Arinbasarov M.U., Shkidchenko A.N., Boronin A.M. Biodegradacija nefteproduktov shtammami-destruktorami i ih associacijami v zhidkoj srede // Prikladnaja biohimija i mikrobiologija.-2001.-T.37. – № 5. – S. 542-548.

10 Zhubanova A.A., Baubekova A.S., Abisheva N.K., Kajyrmanova T.K. Immobilizacija kletok drozhzhej i bakterij na zauglerozhennye prirodnye nositeli // Vestnik KazNU. Ser.jekol.- 2001. - № 2 (9). – S. 46-50.

11 Anufrieva S.I., Isaev V.I. i dr. Ocenka vozmozhnosti ispol'zovanija prirodnogo materiala - shungita dlja ochistki neftesoderzhashhih stokov. // Trudy mezhdunarodnogo simpoziuma. -Petrozavodsk, 2000. - S. 156-161

12 Leonenko I.I., Antonovich V.P., Andrianov A.M., Bezluckaja I.V., Cymbaljuk K.K. // Metody opredelenija nefteproduktov v vodah i drugih obektah okruzhajushhej sredy (obzor) // Metody i obekty himicheskogo analiza. - 2010. - T.5. - №2. - S. 58-72.

13 Wilson K. Preparation of genomic DNA from bacteria. Current Protocols in Molecular Biology. Editors (Editors Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., et al.). - New York: Wiley, 1987. - 650 p.

14 Dudikova G.N., Jakusheva T.V., Kudrjakova A.V., Zholdybaeva E.V., Shevcov A.B. Geneticheskaja identifikacija molochnokislyh bakterij na osnove analiza nukleotidnoj posledovatel'nosti gena 16SrRNA // Biotehnologija. Teorija i praktika.- 2012.- №3. – S. 55 – 64.

15 Kolbert C.P., Persing D.H. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens // Current Opinion in Microboiligy. – 1999. – Vol. 2. – P. 299 – 305.

16 National Center for Biotechnology Information (NCBI) http: // www.ncbi.nlm.nih.gov/

17 Stackebrandt E., Goebel B.M. Taxonomic note: a place for DNA–DNA reassociationand 16S rRNA sequence analysis in the present species definition in bacteriology // International Journal of Systematic Bacteriology. – 1994. – Vol. 44. – P. 846–849.

18 Hence M., Armojes P., Lja-Kur-Jansen J., Arvan Je. Ochistka stochnyh vod. Pod redakciej d-ra him. nauk S.V. Kaljuzhnogo. - M.:, Mir. 2006. – S. 256-257.