Potential biodegradable bacteria and their identification

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Abstract. The article describes soil bacterial isolates isolated from technogenic zones of Kyrgyzstan, possessing high oil-oxidizing and bioemulsifying activity, capable of decomposing petroleum hydrocarbons in the temperature range of 4 - 39°C at pH 4 - 9 in the presence of 18% salt. Our research has confirmed the possibility of bioremediation of the damage caused to soils as a result of chemical pollution of soils (lands) due to spillage of oil products on the territory of the oil depot in Balykchy city of Kyrgyzstan. The used bacterial consortium consisting of spore-forming bacteria of Bacillus genus: B1-025-4(2), B1-05-3(2), B1-025-5 had high oil-degrading activity both at low (4°C) and moderate (18-25°C) temperatures. In this work, special attention was paid to the molecular genetic properties of 4 bacterial strains isolated from oil-contaminated and different types of soils. The maximum biodegradability of these strains for many hydrocarbons (hexadecane, benzene, naphthalene, diesel fuel, gasoline) was also noted. Based on physiological-biochemical and molecular genetic analysis, the isolated petroleum destructor bacteria were found to belong to Bacillus pumilus (strains B1-05-3(2) and B1-025-4(2), B1-025-5) and Bacillus amyloliquefaciens (strain H-5-2). These selected strong bacterial strains are of great interest for biotechnological applications as effective microorganisms for environmental cleaning. Keywords: bacteria-destructors, effective strains, oil-contaminated soil, bioremediation, identification, molecular methods.

1 Introduction

Recently, the most promising method of cleaning oil-contaminated soils, both from the economic and environmental point of view is considered [1-3] biotechnological approach based on the use of various groups of microorganisms characterized by increased ability to biodegrade components of oil and petroleum products [4-6].

The ability to utilize hard-to-degrade substances of anthropogenic origin has been found in many organisms. This property is provided by the presence of specific enzyme systems in microorganisms [7, 8], which carry out the catabolism of such compounds. Since microorganisms have a relatively high potential for destruction of xenobiotics, show the

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ability to rapid metabolic reorganization and exchange of genetic material, they are given great importance in the development of ways of bioremediation (biopurification) of contaminated objects.

The use of any biopreparation, which has active forms of microorganisms in its composition, requires the creation and strict implementation of the original technology, which must be adjusted in each soil and climatic zone. In this case, the most effective biopreparations are considered to be based on natural hydrocarbon-oxidizing microorganisms, including various taxonomic and physiological groups [9].

Microbial preparations produced in a wide range by biotechnological companies in Europe, USA and Japan cannot be used in all territories of other countries, which differ sharply and significantly in climatic and other natural conditions. Therefore, it is expedient to use local effective microorganisms where bioremediation procedures are carried out in the environment polluted by oil and other toxic elements. However, the use of natural microbial biodegradation community requires comprehensive knowledge of microorganisms included in their composition.

The aim of the study is to identify strains of effective oil-oxidizing bacteria using physiological-biochemical and molecular-genetic methods for use in biopurification of polluted ecosystems.

2 Materials and methods

Bacterial isolates and objects of study. Destructor bacteria were isolated from samples of oil-contaminated and different types of soils of technogenic zones of Kyrgyzstan (Table 1).

Table 1. Location of isolation of bacteria-destructors

Laboratory Isolates	Soil type	Altitude	Source of isolation, geographic origin
B1-05-3(2)	Light gray soils	1600	Balykchy city, oil depot, Issyk-Kulsk obl.
H-5-2	Mountain black soils	2700	Ak-Tyuz tailing. Kemin district
B1-025-4(2)	Light gray soils	1600	Balykchy city, oil depot, Issyk-Kulsk obl.
B1-025-5	Light gray soils	1600	Balykchy city, oil depot, Issyk-Kulsk obl.

The isolation and primary screening of hydrocarbon-oxidizing bacteria was carried out from soil samples collected from the above-mentioned sites. IPA media were used for bacteria cultivation. Luria-Bertani (LB) agarized media were used as complete media.

The cultural, morphological and physiological-biochemical properties of the obtained cultures were characterized as described in [10].

Bacterial genomic DNA isolation and amplification. Bacteria were grown for 24 hours (lag phase of growth) at 27°C on LB liquid medium and collected in centrifuge tubes. DNA was isolated using the Qiagen DNeasy Blood and tissue kit according to the standard protocol provided by the manufacturer. DNA integrity was determined in a 1.0% agarose gel in 1X TAE (Tris, Acetate and EDTA) buffer as described by Sambrook et al. [11]. The 16S rRNA marker (Table 2) and a PCR program consisting of an initial denaturation at 95˚C for 5 min, 35 cycles of denaturation at 95˚C for 40 sec, annealing at 55˚C for 1 min and elongation at 68˚C for 2 min, and a final elongation at 68˚C for 4 min were used.

The quality of PCR products was monitored by horizontal gel electrophoresis with 1.0% agarose, stained with ethidium bromide, and visualized using a UV transilluminator.

The amplified PCR product was purified using QIAquick PCR purification kit (Qiagen, Germany) and the amplicons were sent to Macrogen DNA Sequensing Service (Korea, Seul).

The raw sequence data were combined into a consensus sequence for each marker gene using the MEGA version 6 program [12]. Consensus sequences were used as queries in GenBank database searches using the BlastN algorithm [13, 14].

Table 2. PCR primers used in this study

Marker gene	Primer designation	Primer sequence	Reference
16S rRNA	27F	5′- AGAGTTTGATCCTGGCTCAG	5
	1492R	5′- GGTTACCTTGTTACGACTT	

Phylogenies were reconstructed: (a) from p-distance matrices using the Neighbor Joining-(NJ) method as implemented in the MEGA 6 software tool with pairwise removal of alignment gaps and missing data; b) with the Maximum Likelihood (ML) method implemented in the PhyML software tool [15] using the Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution [16] according to a gamma distribution model based on the model [17] allowing eight rate categories. The confidence bounds of the tree topology were investigated in a nonparametric bootstrap analysis on 1000 pseudo-repeats [18].

Statistical analysis. Molecular analyses, including bootstrapping statistics for phylogenetic tree topologies, were performed using the MEGA 6 and PhyML software package and for NJ and ML trees, respectively.

3 Results and discussion

Degrading oil in associations of active bacterial strains designated as B1-05-3(2), H-5-2, B1-025-4(2), and B1-025-5 were previously characterized by culture-morphological and biochemical, then molecular methods (Figs. 1, 2).

Hydrocarbon-oxidizing bacteria were isolated from oil-contaminated soil (OC) at the oil depot in Balykchy town, Issyk-Kul region (coordinates: 42°27'13.44" N; 76°12'12.91" E; altitude - 1630m), where a major accident occurred in the 1990s, when about 600 tons of oil products, mainly diesel fuel and kerosene, leaked out of worn pipes (Fig.1).

Further, laboratory semi-production tests were continued using bacterial strains with different variants in bioremediation of oil-contaminated soils. As a result, four most effective bacterial strains belonging to the genus Bacillus were selected: B1-05-3(2), H-5-2, B1-025-4(2) and B1-025-5 as active candidates with oil-oxidizing and bioemulsifying properties capable of decomposing petroleum hydrocarbons in the temperature range of 4-39°C at pH values of 4 - 9 in the presence of 18% salt (Fig. 2).

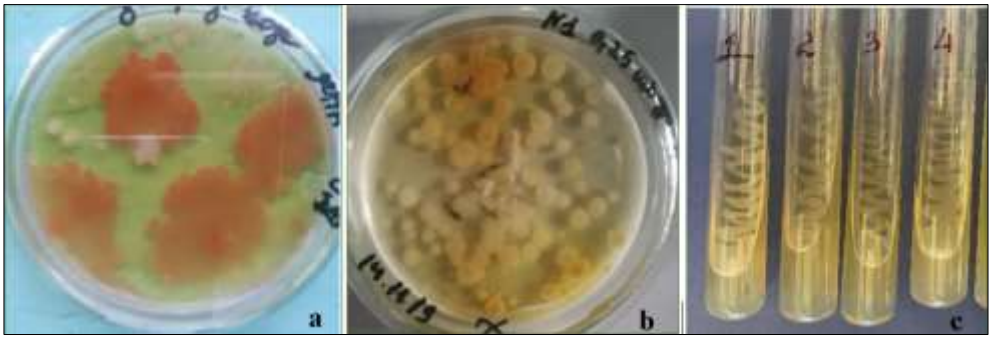

Fig. 1. Hydrocarbon-oxidizing bacteria: a, b - bacterial colonies grown on solid nutrient medium; c - pure bacterial cultures.

Cultural, morphological and biochemical properties of oil-degrading bacterial strains: *B1-05-3(2), H-5-2, B1-025-4(2)* и *B1-025-5*. All strains were able to grow on liquid and dense media containing NaCl in the amount of 20 g/l, at pH from 5.5 to 8.5. Growth processes in all bacterial strains were observed in a wide temperature range (from 4°C to 30°C), on the basis of which they were categorized as psychroactive, psychrotolerant single and in pairs. The cells are gram-positive. Spores are formed. Aerobes. Oxidase- Catalase-positive.

Strain B1-05-3(2) forms colonies up to 1.2-1.5 mm in size, rounded, shiny, smooth, convex, with an even edge, homogeneous structure, soft consistency. The color of colonies varied from light yellow at the beginning of growth to bright orange at aging. Bacilli of medium size: 0.9-1.0*1.5-3.0 μm. Cells Gram-positive. Aerobe, facultative. Oxidase- and catalase-positive (Fig. 2 a).

Strains B1-025-4(2) and B1-025-5 form colonies of medium size (about 1.5-2.5 mm), light beige with a grayish tint, smooth, shiny, rounded shape with even edges, weakly convex, homogeneous structure, soft consistency and mucilaginous. With aging, colonies become larger (d = 4-5 mm), rough, with the appearance of a bulge in the center. The cells are gram-positive. Spores form. Aerobes. Oxidase- and catalase-positive. Microscopy of smears showed motile straight bacilli, single and in pairs (Fig. 2b, c).

Also, when creating an association of oil-oxidizing bacteria, all the necessary requirements to the preparation for more effective degradation of contaminants in soils were met: regional cultures of bacteria isolated from soil samples taken in areas of oil pollution were used; strains that grow well at 4-10°C on medium with increased concentration of NaCl (15-18%) were selected; strains that do not show antagonism to indigenous microbiota were selected; the association was composed of a minimum number of strains that most completely oxidize oil.

It is known that mixed cultures of microorganisms can interact with each other through cooperation, i.e. intermediates obtained as a result of degradation of oil hydrocarbons by some microorganisms can serve as substrates for other bacterial strains.

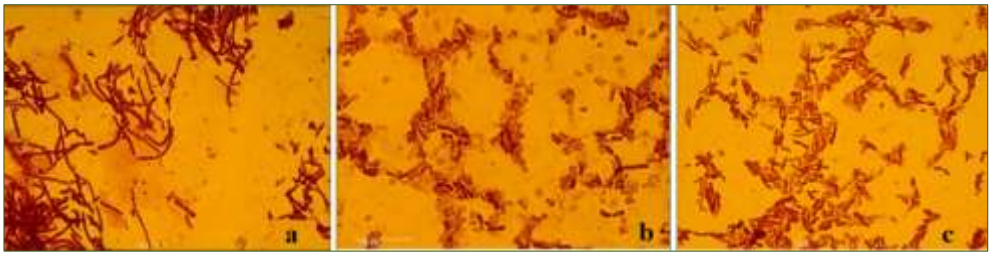

Fig. 2. Cells of oil-degrading bacteria on phase-contrast microscope at x900 magnification growing at elevated salt and pH concentrations: a- B1-05-3(2); b- B1-025-4(2) and c- B1-025-5. Scale: 1-5 - 10 μm.

Such a relationship of associations was possible in our studies, since the introduction of 4 bacterial strains into contaminated soil resulted in active destruction of kerosene (Table 3).

Thus, as a result of our research it was shown that the amount of petroleum products in soil samples with a high level of pollution (3100 mg/kg soil) after the introduction of individual strains, decreased to a low level (from 600 to 1200 mg/kg soil); especially in associations of bacterial strains up to 400 mg/kg soil after 90 days: the reduction in the amount of petroleum products in the soil is from 61.3 to 87.1%.

Also, it should be noted that the high index was given by strain H-5-2 singly, the destruction of oil in the soil almost the same level with the association of strains, i.e. amounted to 600 mg/kg soil (Table 3). This is most likely due to their synthesis of active

secondary metabolites and the presence of unique enzyme systems. The research results presented in this work on finding methods of bioremediation of oil-contaminated soils can be used as environmentally safe methods of restoration of northern ecosystems.

Table 3. Oil degradation in soil treated with liquid biopreparation based on bacterial strains

№	Experimental variants	Oil products content, mg/kg		Oil degradation, %
		At the beginning of the experiment	At the end of the experiment	
1	OCS + strain **B1-05-3(2)**	3100,0	1100	64.2
2	OCS + strain **H-5-2**	3100,0	600	79.1
3	OCS + strain **B1-25-4(2)**	3100,0	900	71
4	OCS + strain **B1-025-5**	3100,0	1200	61.3
5	OCS + assoc. strains	3100,0	400,0	87.1
6	Control (OCT)	3100,0	2890,0	6.7

Note: Oil-contaminated soil – OCS; assoc. strains - B1-05-3(2) + H-5-2 + B1-25-4(2) + B1-025-5

According to numerous publications, there are several characteristics of the genus Bacillus that are more advantageous than other microorganisms in the biodegradation of hydrocarbons. Bacillus species exhibit a range of physiological abilities that allow them to live in a wide range of habitats, including many extreme environments such as desert, sand, hot springs, and arctic soils. They can also be thermophilic, psychrophilic, acidophilic, alkaliphilic, halotolerant or halophilic and are able to grow at pH values, temperatures and salt concentrations where few other organisms can survive. They are very resistant to extreme environmental conditions such as low or no nutrient availability, desiccation, irradiation, H_2O_2 and chemical disinfections [19]. They are frequently found in oil-contaminated environments [3, 9], [20-23]. The key and fundamental factor in the biodegradation rate of hydrocarbons in soil or liquid phase, is based on the survival of microorganisms in diesel medium after inoculation. Bacillis can utilize the hydrocarbon as a carbon and energy source for growth in the diesel medium.

Thus, Gram-positive bacteria, particularly Bacillus are of interest in both environmental bioremediation strategies and biotechnology. Table 4 provides a list of bacterial species from the genus Bacillus capable of utilizing hydrocarbons as a carbon source.

Table 4.: Hydrocarbon-degrading strains of the genus Bacillus

Strain	Description	References
Bacillus lichenformis DHT	A halotolerant and thermotolerant strain capable of degrading a wide range of hydrocarbons and producing biosurfactant as a by-product	[1]
Bacillus subtilis	Uses gasoline fuel as a hydrocarbon and energy sources	[4]
Bacillus lichenformis	Thermophilic bacteria were evaluated for degradation of Maya crude oil, a type of Mexican heavy oil, using a bubbler column reactor	[7]
Bacillus cereus	Potential crude oil degradants isolated from a refinery area in Kaduna, Nigeria	[8]
Bacillus pumilus 28-11	A naphthalene degrading strain isolated from oil sludge and capable of producing biosurfactant	
Bacillus subtilis DM-04	Crude oil-degrading bacteria were used in a consortium shake flask study	[22]
Bacillus cereus	The strain was capable of degrading PAHs up to 250ppm and	[24]

CPOU13	the optimum pH for degradation was 6-8. The strain degraded 73.46% of phenanthrene, anthracene (85.76%) and pyrene (47.88%) within 14 days	
Bacillus pumilus JL_b	Used to test the effectiveness of bio-additives for diesel degradation, in contaminated soils with/without added nutrients with fertilizers	
Bacillus cereus DRDU1	Extracted from an automobile engine and widely used in hydrocarbon degradation studies	[25, 26]
Bacillus cereus JMG-01	Anthracene-degrading strain with 98% substrate degradation (500ppm) over 21 days of incubation	[27]
Bacillus cibi and *Bacillus megaterium*	It was used in a study of a bacterial consortium that showed an outstanding ability to degrade sludge in liquid medium (90.7% reduction of the aliphatic fraction, 51.8% of the aromatic fraction)	[28]
Bacillus pumilus KS2	The strain isolated from oil fields in Sivasagar (Assam district), India was capable of degrading 80.44% TPH during 4 weeks of aerobic incubation.	[29]
Bacillus sp.	One of the isolated bacterial species from soil and bottom sediments of hypersaline environments capable of degrading PAHs	[30]
*Bacillus sp.*EgeB.6.2i	Showed 60% and 33% degradation of chrysene and naphthalene with over 30% emulsifying activity	[31]
*Bacillus sp.*Ege B.1.4ka	Showed 36% and 55% chrysene degradation and naphthalene degradation.	[32]
Bacillus stearothermophilus	Able to grow obligately on crude oil at high temperatures (optimally at 60°C) and grow best on medium-chain alkanes	[33]
Bacillus subtilis 22BN	The strain was isolated based on their ability to utilize n-hexadecane and naphthalene simultaneously while producing a surfactant compound (rhamnolipid) at a concentration of 1.5-2.0 g/L.	[34]
Bacillus subtilis A1	Biosurfactant-producing and alkane-degrading strain. The biodegradation efficiency of the strain was about 87% in a short period of time (7 days)	[35]
Bacillus subtilis C9	Obtaining a lipopeptide-type biosurfactant, and showed rapid degradation of alkanes to C19	[36]

Bacterial identification based on molecular genetic methods of 16s RNA fragment. PCR amplification, sequencing, and raw data assembly revealed a partial sequence (497 bp) encoding the 16S rRNA gene of strains B1-05-3(2), B1-025-4(2), and B1-025-5. This sequence was found to be identical (i.e., showing 100% nucleotide sequence similarity with 100% sequence coverage) to the Genbank database of records representing the genus Bacillus, species Bacillus pumilus, and species Bacillus pumilus. i.e., showing 100% nucleotide sequence similarity with 100% sequence coverage) to the Genbank database of records representing the genus Bacillus, species *Bacillus pumilus* or *Bacillus zhangzhouensis*. The redundancy-corrected reference dataset created for phylogenetic reconstruction consisted of 105 annotated Bacillus genomes, i.e. 99 genomes representing the species Bacillus pumilus or its putative synonyms *Bacillus zhangzhouensis*. In the NJ phylogeny generated for this dataset based on combining two MLSA marker sequences (Fig. 3), clades representing species B. velezensis, B. amyloliquefaciens, and B sonorensis

appeared well separated, obtaining at least 99% bootstrap support. Several bacterial strains including *B. pumilus* have demonstrated the ability to efficiently degrade diesel fuel and used motor oil in a liquid environment [9]. In a study of PAH degradation by bacterial consortia [37] noted that *B. pumilus* was one of the members of the consortium, but these authors did not determine whether this bacterium was responsible for the degradation of any particular PAH. Khanna et al. [22] reported that *B. pumilus* (PK-12, MTCC 1002) can metabolically uptake 64% of pyrene from the growth medium.

Iuliani et al. [38] recently showed that *B. pumilus* C 15 is able to degrade PAHs (polyaromatic hydrocarbons) pyrene and phenanthrene because the strain possesses the dioxyrene genes nidA and nahAc, which are responsible for PAH uptake.

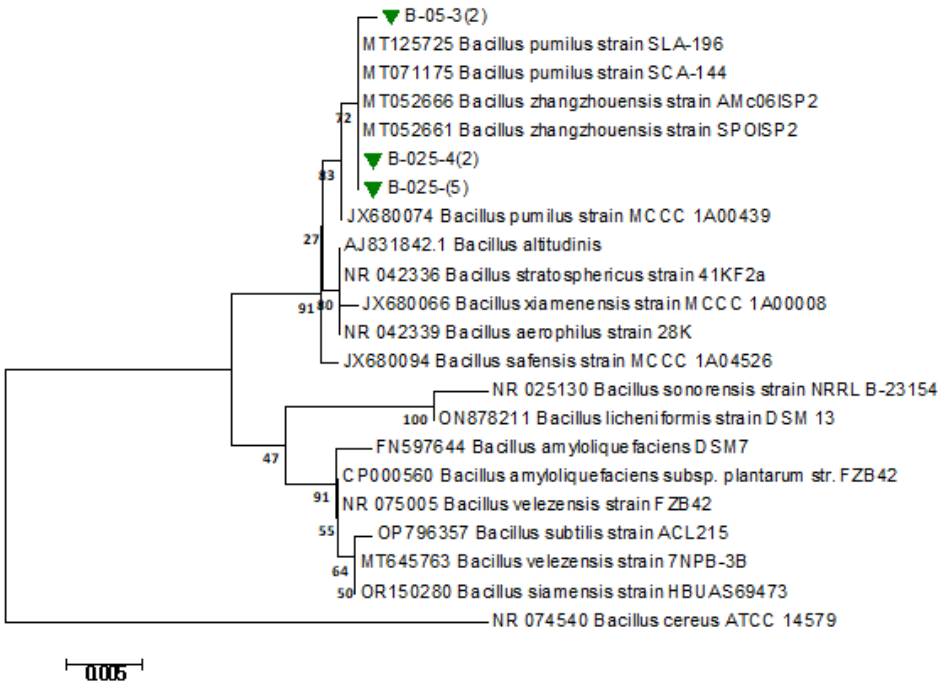

Fig. 3. Maximum likelihood (ML) phylogeny of bacteria belonging to the *Bacillus pumilus* operational group reconstructed from pooled 16S rRNA marker sequences. Terminal branches are labeled with genus, species and strain designation, and the studied isolates are highlighted with figure. Numbers on the inner branches indicate the percentage of bootstrap support. The size bar corresponds to 0.005% sequence divergence along the horizontal branches.

Strain H-5-2 - from the collection of the laboratory, which received [39] earlier a patent of the Kyrgyz Republic under number 815 for the destruction of heavy metals, in particular mercury and lead in associations were also included in the experiment variants in bioremediation of oil pollution in soil. This strain of spore-forming bacteria were isolated from soil samples taken from the territories of the tailing dump (42°52'46" N; 76°08'03" E; Altitude (m) 2205) of the Ak-Tyuz mining complex of Kyrgyzstan.

Thus, PCR amplification, sequencing and raw data assembly revealed a partial sequence (497 np) of the 16S rDNA encoding gene of strain H-5-2 and was found to be identical (i.e. showing 100% nucleotide sequence similarity with 100% sequence coverage) to the Genbank database of records representing the genus Bacillus, species *Bacillus amyloliquefaciens* (Fig.4).

Phylogenetic analysis of 16S rDNA gene showed that strain H-5-2 has high homology with bacterial sequences with strain CP000560.2 *Bacillus amyloliquefaciens subsp plantarum str* FZB42.

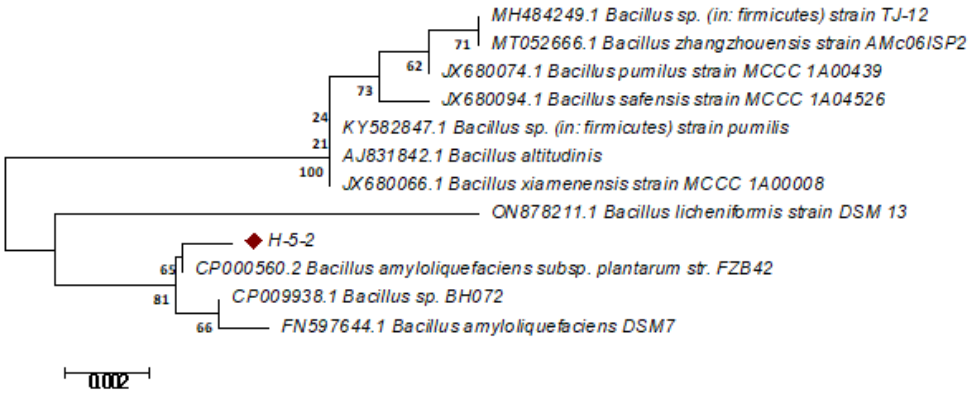

Fig. 4. Maximum likelihood (ML) phylogeny of bacteria belonging to the operational group *Bacillus amyloliquefaciens* reconstructed based on pooled 16S rRNA marker sequences.

Bacillus amyloliquefaciens belongs to the genus Bacillus, unicellular organisms, short bacilli in the form of long chains. In general, *Bacillus amyloliquefaciens* have been identified as degradants of petroleum hydrocarbons [18, 33] and are known to degrade naphthalene and pyrene [24].

Our proposed strain H-5-2, identified as *Bacillus amyloliquefaciens,* not only has accumulative properties of heavy metals [39] and oil product destructors, but also has a high antagonistic potential against phytopathogenic pathogens (Fig. 4). *Bacillus amyloliquefaciens* produces a broad-spectrum antifungal protein, designated as bacyamine, which is among the few bacterial antifungal proteins. Batsiamin causes membrane permeabilization in fungi. The antifungal protein found dynamic activity against *Botrytis cinerea, Rhizoctonia solani, Valsamali, Mycosphaerella arachidicola* and some other fungal species [40].

Morphological and cultural characteristics of strain H-5-2: bacilli: 0.8-1.0*2.5-4.0 μm, arranged singly and in chains, motile. Peritrichia. Gram-positive; spores 0.7-1.0*1.1-1.5 μm, oval, central or paracentral. On meat and peptone broth (MPB), rapid cloudiness occurs, followed by a delicate film, rings, sometimes flakes. On potatoes, the thick soft layer is whitish creamy with a slight pinkish cream color. On MPA it forms dirty-white to cream-colored colonies, 13-15 mm in diameter, with irregular edges and a rough, dry surface. It forms yellowish-green fluorescence on oblique agar (Fig. 5, b-c).

Physiological and biochemical properties. Chemoorganotroph, aerobe, facultative, catalase-positive. Ferments glucose, sucrose, glycerol. Hydrolyzes starch. Indole is not formed. Nitrates are reduced. Optimum temperature for growth is 25-28ºC. Grows in a range of pH values 6.8-9. Optimum pH 7.0-8.0 for growth.

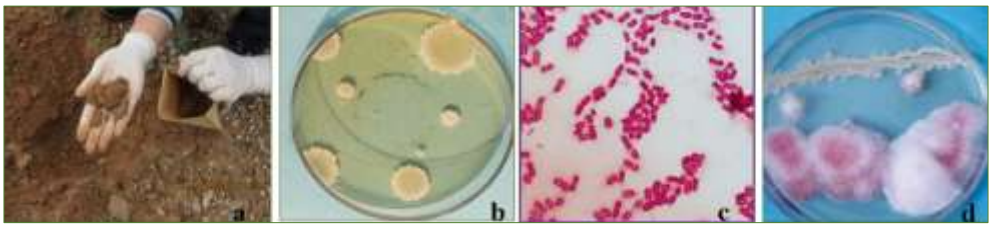

Fig. 5. Culture-morphological (b, c) and some biological properties (d) of soil isolate (a) of oil-degrading strain H-5-2.

4 Conclusion

The isolated mixed bacterial consortium showed higher biodegradation rates from 61.3 to 87.1 % as a result of synergistic interactions between bacteria of associations of the same genus, which can lead to complete degradation of toxic petroleum hydrocarbons to non-toxic end products. Optimal growth conditions for active strains were found at temperatures of 4 - 39°C, at pH values of 4 - 9 in the presence of 18 % salt. Some bacteria besides degradation of different types of petroleum products, also have potential antagonistic properties against phytopathogens at the same time.

Besides in the present work, phylogenetic analysis based on 16S rDNA gene sequence marker was described and identified as *Bacillus pumilus* and *Bacillus amyloliquefaciens*. The above results are in agreement with several publications reporting that these bacteria are able to degrade different types of petroleum products and can be further used for remediation of soil ecosystems heavily contaminated with petroleum products.

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