УДК 577.15

ISOLATION OF SOIL BACTERIA FOR BIOREMEDIATION OF HYDROCARBON CONTAMINATION

Ilyina A.*, Castillo Sanchez M.I., Villarreal Sanchez J. A.*, Ramirez Esquivel G., Candelas Ramirez J.

*(Corporacion Mexicana de Investigacion en Materiales S.A. de C.V. (COMIMSA); Blvd. Oceania 190, Frac. Saltillo-400, C.P. 25290, Saltillo, Coah., Mexico Tel: 52-844-411-32-00 (Ext. 1145); *Universidad Autonoma de Coahuila, Facultad de Ciencias Quimicas, Departamento de Biotecnologia; Blvd. V. Carranza e Ing. J. Cardenas V., C.P. 25280, Saltillo, Coahuila, Mexico, Fax: 52-844-415-95-34, E-mail: anna_ilina@hotmail.com)*

Bacterial strains were isolated from sites impacted by spill of petroleum hydrocarbons for the development a product (COBE-10) applicable in soil bioremediation. Initially, 82 bacterial strains were isolated in selective agars (agar diesel, combustion oil and petroleum). After monitoring absorbance change of mineral media (containing petroleum as sole carbon source) inoculated by isolated strains, 30 strains were selected. The strains were evaluated for their potential to degrade the hydrocarbons of petroleum in soil, artificially contaminated under laboratory conditions. Based on DTPH/day, finally 6 strains were selected. Seven carrier materials were tested to select suitable vehicle for final formulation of COBE-10. *Ex -situ* evaluation of the developed product (COBE-10) was performed in field with soils contaminated with diesel and refinery wastes. The field test showed a high efficiency of biopreparation COBE-10.

Life in our planet is sustained in a fragile biological balance; microorganisms play an important role on nutritional chains, that are an important part of this biological balance [1]. Adapting several abilities, microorganisms have become an important influence on the ecological systems, making them necessary for superior organisms life in this planet. Ability of microorganisms to transform and degrade many types of pollutants in different matrixes (soil, water, sediments and air) has been widely recognized during the last decades [2, 3].

Soil contamination with hydrocarbons causes extensive damage of local ecosystems since accumulation of pollutants in animals and plants tissues, may cause progeny's death or mutation [4]. In Mexico, an endless number of contaminated sites exists as a result of more than 60 years of oil petroleum activity; in recent years this problem has motivated researches to recover these contaminated sites [2].

Microorganisms survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche. Contaminants are often potential energy sources for microorganisms [1]. Bioremediation, a process that exploits the catalytic abilities of living organisms to enhance the rate or extent of pollutant destruction, is an important tool in attempts to mitigate environmental contamination [3, 5]. Bioremediation achieves contaminant decomposition or immobilization by exploiting the existing metabolic potential in microorganisms with catabolic functions derived through selection, or by the introduction of genes encoding such functions. The effectiveness of bioremediation is often a function of the extent to which a microbial population or consortium can be enriched and maintained in environment. When few or no indigenous degradative microorganisms exist in a contaminated area and practically does not allow time for the natural enrichment of suitable population, inoculation may be a convenient option [5].

The goal of the present work is to isolate from hydrocarbon contaminated soils the bacterial strains to assess their potential for bioremediation and develop a bioproduct useful for soil inoculation.

MATERIALS AND METHODS

Soils contaminated by different petroleum hydrocarbons were collected from 3 different places (Table1), by simple soil sampling method [6] at different depths (minor of 3 m). Microorganism isolation was carried out using selective mediums (agars containing petroleum, diesel and heavy fractions of refined petroleum) as unique carbon source. The selective medium used for isolation contained 15 g of agar-agar, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of Na_2HPO_4, 1L of sterilized water and 10 ml of carbon source (petroleum hydrocarbons). After dilution of soil samples (10^{-2}–10^{-12}), selective agar media were inoculated and incubated at 25°C. For the bacterial strain selection, the following rules were considered: colonies of microorganisms grown within 48–72 hr period and colonies with the bigger size at the end of incubation period (12 days).

In the next step, the test tubes containing liquid mineral medium with 3% v/v of petroleum as sole carbon source were inoculated by isolated strains. Four test tubes per strain, were shaken at 250 rpm for three weeks at 25–

27°C. The absorbance change (turbidity) of the mineral medium was measured in HACH spectrophotometer at 540 nm once a week. Absorbance change was the evaluation criteria for microorganism adaptation in the used media. In some cases degradative activity caused changes in petroleum during the incubation period, these changes did not allow microbial growth kinetics evaluation.

For the pathogenicity evaluation of the selected strains, some microbiological tests were performed [1]. Gram positive microorganisms were cultivated in S-110 agar and manitol. Gram negatives were inocluated in ENDO agar, Salmonella-Shigella agar, EMB-agar, eosin-blue of methylene and Mc Conkey agar [1].

The final microorganisms selection was performed using the soil fertilized with a 200:1 hydrocarbon-nitrogen ratio, 800:1 hydrocarbon-phosphate ratio, using $(NH_4)_2SO_4$ and $Na_2HPO_4.7H_2O$, respectively [7]. Then, the soil was sterilized and petroleum was atomized into the soil until it reached 3% w/w concentration. Soil was distributed in petri plates, inoculated with a dilution of 2 ml of the original strain in 10 ml of sterilized water and incubated for 15 days at 25–27°C. Three commercial products (A, B and C) were used as positive controls. Their activity was compared against the isolated strain′s activity. Uncontaminated soil was used as blank. Further, a soil sample with the same petroleum content but without any strain was used as experimental control. After 15 days total petroleum hydrocarbons (TPH) were measured by EPA 418.1 method [8].

Table 1

Sampling place features

Sample indentification	Features
A	Samples from a contaminated soil with refinery wastes. Two years of contamination. Sampling depth 0.15 m. (SITE 2).
B	Samples from a site contaminated with petroleum. 5 years of contamination. Sampling depth 1.00 m. (SITE 1).
C	Samples from site contaminated for 1 year by gasoline from a damaged pipeline. Sampling depth 0.15 m. (SITE 3).
D	Samples from a contaminated soil with refinery wastes. Two years of contamination. Sampling depth 1.00 m. (SITE 2).
E	Samples from site contaminated for 1 year by gasoline from a damaged pipeline. Sampling depth 1.00 m. (SITE 3).
F	Samples from a site contaminated with petroleum. 5 years of contamination. Sampling depth 2.00 m. (SITE 1).
G	Samples from a contaminated soil with refinery wastes. Two years of contamination. Sampling depth 2.00 m. (SITE 2).

Table 2

Primary isolation results

Samples keys	Agar-Petroleum	Agar-Diesel	Agar-Combustion oil	Number of isolated strains per sample
A	4	4	1	9
B	5	5	3	13
C	6	4	0	10
D	3	4	2	9
E	3	5	3	11
F	4	11	4	19
G	2	5	4	11
Isolated strains per medium	27	38	17	82

To select the carriers for microorganisms, several materials were tested: perlita, medium and bulky vermiculite, TBK, PGX, sand and brand. The commercial materials were selected according to their price, availability in the market and physical and chemical properties (apparent density, real density, porous fraction, pH, humidity and ability to absorb water or oil) [9, 10].

Using the isolated microorganisms and selected materials the bioproduct COBE-10 was formulated. The bioremediation test was performed at field [11]. One cubic meter of soil from an area freshly contaminated with diesel and refinery wastes, was treated with: a) water; b) COBE-10 and c) commercial product A. The samples were used to form mounds on a high density polyethylene liner to avoid leaching. Total petroleum hydrocarbons (TPH) were measured by EPA 418.1 method [8].

RESULTS AND DISCUSSION

Initially, 82 bacterial strains were isolated from 7 samples of soil originating from three different places contaminated with petroleum hydrocarbons on Northwest of Mexico (Table 1).

Isolation was carried out using the traditional microbiological technique with petri dishes containing selective agars with hydrocarbons (petroleum, diesel and heavy fraction of refined petroleum) as the sole source of carbon (Fig. 1). Results demonstrated (Table 2) that soil sample (F) which showed higher contamination age yielded more number of colonies [5]: 11 colonies grown in diesel media, 4 colonies in petroleum media and 4 colonies in combustion oil media (Table 2).

Table 3

TPH values in soils contained petroleum measured on 15th day after inoculation with bacterial strains. ($TPH_{Initial}$ = 30,000 ppm; $TPH_{control\ on\ 15\ days}$ = 25,280 ppm. 100% is equivalent to TPH value of control after 15 days of test).

Key	TPH after 15 days	Δ TPH compared with control	Δ TPH?S per day	% Hydrocarbon removing
Control	25.280	0	-	0
PROD, A	17.536	7.744	516.25	30.63
PROD, B	18.142	7.138	475.89	28.24
PROD, C	18.612	6.668	444.50	26.37
C4-7C	17.604	7.676	511.75	30.37
F4-6C	19.150	6.130	408.64	24.25
A4-8A	18.881	6.399	426.57	25.31
F5-6A	19.083	6.197	413.12	24.51
F3-7C	19.957	5.323	354.84	21.05
B4-8F	19.285	5.995	399.67	23.71
G3-6B	20.294	4.986	332.42	19.72
D4-9A	21.571	3.709	247.24	14.67
D5-9A	20.630	4.650	310.00	18.39
E5-7A	20.495	4.785	318.97	18.93
A3-11A	21.437	3.843	256.21	15.20
F5-6B	20.428	4.852	323.45	19.19
F5-11A	20.630	4.650	310.00	18.39
G5-7A	20.966	4.314	287.59	17.06
E4-9A	21.235	4.045	269.66	16.00
F4-11B	21.773	3.507	233.79	13.87
B5-7B	22.513	2.767	184.47	10.95
E3-7A	21.437	3.843	256.21	15.20
G4-7A	21.033	4.247	283.10	16.80
G5-6A	21.437	3.843	256.21	15.20
F4-6B	20.832	4.448	296.55	17.60
A3-6A	22.109	3.171	211.37	12.54
G4-6B	21.706	3.574	238.27	14.14
B5-8C	21.706	3.574	238.27	14.14
D3-9A	23.185	2.095	139.64	8.29
C3-7C	23.454	1.826	121.71	7.22
D3-8A	22.446	2.834	188.96	11.21

Fig. 1. Test tubes with mineral media containing 3% v/v petroleum applied in Assay for selection of strains. Compare the FIRST TUBE (Control) with the other tubes inoculated with the bacterial strains. Solubility of petroleum in water can be observed in tubes inoculated with bacterial strains

Table 4

Characteristics of tested carrier materials

Materials	Apparent density g/cm^3	Real density g/cm^3	% porous space	pH	Oil abs. ml/gr	Water abs. ml/gr	% Humidity
Medium Vermiculite	0.12	2.79	95.49	5.71	7.00	5.00	0
Perlita	0.06	0.80	91.75	8.57	10.00	5.00	0
TBK	0.13	1.30	89.39	4.00	6.00	4.30	28
PGX	0.14	2.71	93.00	5.58	5.00	4.00	20
Bulky Vermiculite	0.13	1.63	91.72	7.53	7.00	5.00	10
Sand	1.58	1.37	27.01	8.41	0.70	0.25	0
Brand	0.40	1.50	72.80	6.71	3.00	2.50	14

Table 5

Results obtained in the field test

Key	Treatment identification	% TPH Remaining in tested soil		
		Initial	4° Week	8° Week
1	CONTROL	100	50.6	55.7
2	COBE-10	100	30.3	9.3
3	COMMERCIAL PRODUCT (A)	100	38.2	38.3

Based on the monitoring of absorbance change in the mineral medium (with 3% petroleum as sole source of carbon), 30 strains were selected. In the tubes with selected strains absorbance increase was related with the microbial growth as well as with the increase of water solubility of petroleum hydrocarbons as a result of transformation of petroleum due to the bacterial degrading activity. This phenomenon is demonstrated in Fig. 1. It can be observed, at the end of the experiment, that in the control tube, first of the presented tubes (first of the presented tubes), petroleum phase is located on top of culture media. While, in tubes inoculated with some isolated strains, hydrocarbons were mixed with the aqueous phase.

After tests for evaluating pathogenicity, 3 strains were excluded. Common biochemical tests were performed to identify the genus of isolated strains.

The strains were evaluated for their potential to degrade the hydrocarbons of petroleum in soil, artificially contaminated under laboratory conditions. Based on ΔTPH/day (Table 3), 6 strains were selected. Table 3 shows that the activities of the 6 selected strains were similar to the activity of commercial products (A, B and C) used in this assay as positive control. The genus of those strains were identified as *Bacillus sp., Rhodococcus sp., Providencia sp.* and *Citrobacter sp.*

Seven carrier materials were tested to select suitable support and vehicle for final formulation of COBE-10 (Table 4). The actual and apparent densities are two important parameters related to such characteristics as weight and volume and defined the management of final bioproduct during its preparation and field application [10]. Another important parameter is the material's water and liquid oil absorption, since the final bioproduct will be in contact with these two types of liquids. Based on the properties of selected bacterias, materials with pH near to 7 are preferred. Table 4 shows results for the tests applied on carrier materials.

Using 3 selected carriers and the 6 isolated strains the bioproduct COBE-10 was formulated. *Ex-situ* evaluation of the developed product (COBE-10) was performed in the field with soils contaminated with diesel and refinery wastes [11]. The field test showed a high efficiency of biopreparation COBE-10 (Table 5). The analysis of data obtained in the control assay demonstrates that without treatment the contamination was diminished by 50%. This effect could be attributed to hydrocarbons volatilization by the periodic movement of soil as well as other physical factors [3, 5].

The Table 5 shows that after 8 weeks of resting, 91% of contamination was removed. Whereas, in the control, it was removed only 44% and 62% in treatment with a commercial bioproduct, commonly used in Mexico for bioremediation.

Thus, using the bacterial strains isolated from the regional soils and the carriers selected for bacterial inoculation and transportation, the bioproduct COBE-10 was developed. In the field test, COBE – 10 removed the hydrocarbon contaminants better than the commercial bioproduct. Based on the obtained results we conclude that COBE-10 has good prospects for application in bioremediation and is competitive with the common commercial products available in the market.

REFERENCES

1. *Madigan M.T., Martinko J., Parker M., Brock J.* // Biologia de los Microorganismos. 1998. **8.** P. 726.
2. *Saval S.* // Biotecnologia. 1998. **3.** P. 71.
3. *Autry A. R., Ellis G. M.* // Enviromental Progress 1992. **11**. P. 318
4. *Alvarez P., Vogel M.* // Biodegradation. 1991. **2.** P. 43.
5. *Kalyuzhnyi S.V.* Vestnik Moskovskogo Universitets. Khimiya. 2000. **41.** (6, Supp.). P. 15.
6. American Society for Testing and Materials. 1998. Designation: D4-700-91.
7. *Stapleton R., Savage D., Sayler G., Stacey G.* // Appl. Environ. Microbiol. 1998. **64**. P. 4180.
8. Secretaria de Comercio y Fomento Industrial. 1985. Norma Mexicana NMX-AA-015-1985.
9. *Ansorena M.J.* Sustratos propiedades y caracterizacion. Madrid, 1994. P. 79
10. *Ilyina A, Villarreal S.J.A., Rivera R. E., Garza G.Y., Rodriguez M.J.* // Vestnik Moskovskogo Universitets. Khimiya. 2000. **41.** (6, Supp.). P. 135.
11. PROFEPA. 1999. Restauracion De Suelos Contaminados. Memorias del grupo de Trabajo sobre Restauracion de Suelos Contaminados. P. 1.

Поступила в редакцию 25.10.02

УДК 577.15

ПОЛУЧЕНИЕ ПРЕПАРАТА НА ОСНОВЕ БАКТЕРИЙ, ВЫДЕЛЕННЫХ ИЗ ПОЧВЫ, ДЛЯ БИОРЕМЕДИАЦИИ НЕФТЯНЫХ ЗАГРЯЗНЕНИЙ

А. Ильина*, М.И. Кастилю Санчес, Х.А. Винареаль Санчес*, Г. Рамирес Эскивель, Х. Кандэлас Рамирес

*(Мексиканская корпорация по исследованию материалов (COMIMSA); *кафедра биотехнологии, химический факультет; Университет штата Коауила, Мексика)*

Получен микробиологический препарат (КОБЕ-10) на основе бактериальных штаммов, выделенных из почв, загрязненных нефтяными отходами. Он использован для биоремедиации почв. Использование селективных сред на основе агара и углеводородов нефти (агар-нефть, агар-дизельное топливо и т.д.) позволило выделить на начальном этапе работы 82 бактериальные культуры. На основании оценки способности роста культур в жидкой минеральной среде, содержащей нефть в качестве единственного источника углерода, были отобраны 30 штаммов. Количество штаммов было уменьшено до 6 после сравнения их активности в процессе деградации углеводородов нефти, добавленных к почве в лабораторных условиях. Разработанный микробиологический препарат был использован для биоремедиации почвы, загрязненной дизельным топливом и отходами нефтеперерабатывающей промышленности в полевых условиях. Полученные результаты показали, что КОБЕ-10 является эффективным биопрепаратом, пригодным для обезвреживания почвы, загрязненной углеводородами нефти.