Journal of Multidisciplinary Engineering Science and Technology (JMEST)
ISSN: 2458-9403
Vol. 3 Issue 7, July - 2016

ISOLATION AND IDENTIFICATION OF BACTERIA AND FUNGI RESISTANT TO CRUDE OIL

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***Abstract*— Recently, has been studied the isolation of crude oil tolerant microorganisms and his capacity of degradation of crude oil in contaminated soils, such bacteria, yeast and fungi. Therefore, in this work, we isolate different microorganisms, which grow in presence of petroleum as the sole carbon source and we determined the activity of alcohol oxidase by a colorimetric method, in different cell fractions. Bacteria found were *Serratia marcescens, Pseudomonas aeruginosa*, *Enterobacter aerogenes and Escherichia coli,* the yeast, *Candida albicans*, and the fungi *Aspergillus niger*. In addition, they have good activity of alcohol oxidase in the cytosolic fraction with different substrates. It was concluded that this microorganisms could be used for decontamination of aquatic habitats polluted with petroleum.**

***Keywords*— Isolation, Microorganisms, Crude oil, alcohol oxidase,**

I. INTRODUCTION

Oil spills are a major source of contamination of soil and water, presently the use, more cross-border transportation of both crude oil and its derivatives, spill containers, breaks in underground pipes and various industrial processes, makes spills hydrocarbons are increasingly frequent, causing risks to human health by inhalation of fumes and ingestion of those hydrocarbons which are dissolved in water and dermal contact, which occurs mainly in recreational activities, as some of its components are considered carcinogenic and teratogenic [1]. Also, petroleum exploration, production, transportation and usage result in adverse effects for marine life, land, atmosphere and humans, [1], and there is a relationship between polycyclic aromatic hydrocarbons (PAHs) and hypertension [2], and it causes tolerance develops to the presence of this compound, inducing selectivity and reduction of microbial diversity in different ecological niches contaminated, and oil tolerant microorganisms develop and use different specialized responses (enzymatic and physiological) to grow in the presence of this contaminant [3]. These conditions favor the variations in the indigenous population of the microorganisms, and naturally made chemical degradation of the oil present in water and soil.

In recent years, various technologies have emerged in order to manage oil residues and effluents contaminated with hydrocarbons, for example, soil washing, vapor extraction, encapsulation and solidification/stabilization, are available to remediate hydrocarbon-contaminated environments. However, these methods are expensive and may only be partly effective [4]. Bioremediation is one of the most

extensively used methods because of its low cost and high efficiency [5]. Biodegradation of hydrocarbons by natural populations of microorganisms is the main process acting in the depuration of hydrocarbon-polluted environments. The mechanism has been extensively studied and reviewed [5]. In many reports, bacteria have been identified as more efficient crude oil degraders than yeast. On the other hand, there is scanty information that yeast and fungi are better crude oil degraders than bacteria [6, 7]. Additionally, a consortium of symbiotic bacteria or supporting materials can be used to enhance the biodegradation process as described [8, 9]. Many microorganisms are capable of using hydrocarbons as the only carbon and energy source; however, when the number of carbon atoms in the hydrocarbon chain is increased to a certain amount, some bacteria and fungi are capable of metabolizing the hydrocarbonated chains [3, 5, 6, and 7].

It has been shown that the growth of microorganisms requires carbon sources derived from petroleum hydrocarbons. Saturated compounds and aromatic with one to five benzene rings are used as energy sources; however, the aromatic rings with more than five, resins and asphaltenes are difficult to degrade by its recalcitrance [1]. The selection of microorganisms through successive tests of population growth in oil-rich pure cultures is an efficient strategy to evaluate the adaptation and survival of strains tolerant to high concentrations of oil. The results of the laboratory tests confirm the selection of the most tolerant and adapted strains. Success in the following stages, both in greenhouses and in soil and contaminated water depends on the quality of the selection and environmental conditions [10]. It is very important to the subsequent evaluation of microorganisms using petroleum hydrocarbons as an energy source, to demonstrate the efficiency of bioremediation technologies in soil and exposed to toxic concentrations of oil water [11]. Recently, isolation has been studied tolerant microorganisms and their degradability, from contaminated with the same sites as the bacteria *Rhodococcus aetherivorans* and *E. wratislaviensis* [12], *Streptomyces* spp [13] and *Pseudomonas aeruginosa* [14], the yeast *Candida tropicalis* and *Candida albicans* [15, 16, and 17] and the filamentous fungi *Penicillium* sp and *Aspergillus* sp [11] *Trichoderma asperellum* [18], and *Mucor rouxii* [19]. Therefore, the aim of this work was the isolation and identification of bacteria and yeast tolerant crude oil from five areas nears to gas stations.

II. Experimental

A. Microorganisms and crude oil resistance test

Some crude oil resistance bacteria, yeast and filamentous fungi was isolated from areas nears to gas station in San Luis Potosí, México, in petri dishes containing modified Lee's minimal medium [LMM, 20] [0.25% KH_2PO_4, 0.20% $MgSO_4$, 0.50% $(NH_4)_2SO_4$, 0.50% NaCl, 0.25% glucose] supplemented with 1mL of crude oil; the pH of the medium was adjusted and maintained at 5.3 with 100 mMol/L citrate-phosphate buffer. The cultures were incubated at 28°C for 3 days for bacteria and 7 days for yeast and filamentous fungi. The colonies obtained were purified by successive spread in the same culture medium, and for subsequent identification, were seeded in duplicate in the following selective media: Iron Agar Kligler, SIM (Sulfide Indol-Mobility) and OF (oxidation-fermentation) for *Pseudomonas* and as Biggy Agar and germ tube test for yeast [21], all biochemical tests was also performed for the identification of Enterobacteriaceae (API 20 E) [22].

The fungal strain was identified based on their morphological structures such color, diameter of the mycelia, and microscopic observation of formation of spores [23]. Crude oil-resistant tests of the isolated strains were performed on liquid LMM containing the appropriate nutritional requirements and different concentrations of crude oil, and determining the dry weight.

B. Assesment of the isolated strains

The bacteria were conserved in COPAN: Transystem Culture SWAB Transport System. Sabouraud Dextrose Agar (SDA) media, and LMM media, containing the specified amounts of hydrocarbons as carbon sources were used to cultivate the fungi. Fungal strains were maintained in agar slant tubes, and spores were obtained after growth in SDA medium as described.

The resistance was tested by inoculating 1 x 10^6 bacteria, yeasts or spores/mL in Erlenmeyer flasks of 250 mL containing 100 mL of LMM, containing 1.0 mL of crude oil as carbon source, incubating at 28°C at 100 rpm for 3 days for bacteria and 7 days for yeast and fungi. The supernatant was harvested in a graduated tube, previously heavy, and centrifuged at 3000 rpm/10 min, discarding the supernatant. The cell package was dry at 80°C for 12 h, and the tube was weight, and determining by difference the dry weight of the strains, comparing with control grown under the same conditions without adding crude oil. All experiments were performed at least 3 times by duplicate.

C Preparation of Cell Free Extracts

Liquid cultures (100 mL) were propagated in 250 mL Erlenmeyer flasks inoculated with bacteria, yeast and/o spores at a final concentration of 1 × 10^6/mL, with and without 1.0 mL of crude oil, and incubated in a reciprocating water bath shaker at 28°C for 3 days for bacteria, and 7 days for yeast and fungi. The cells were collected, washed twice with sterile distilled water, and suspended in buffer breach 8.5 (50 mM Tris-HCl [pH 8.5] containing 1mM phenylmethylsulfonyl fluoride [PMSF]), and dissolve in dimethylsulfoxide. A volume of about 10 mL of cells was mixed with an equal volume of glass beads (0.45–0.50 mm diameter) and disrupted in a Mini-Bead Beater homogenizer (Biospec Products) for four periods of 30 s. The homogenate was centrifuged at 3000 g for 15 min to remove cell walls and unbroken cells. The cell wall-free

supernatant (crude extract) was centrifuged at 25 000 rpm for 45 min at 4°C; the resulting pellet, a mixed membrane fraction, was discarded and the supernatant (cytosolic fraction) was saved for enzymatic determinations [16].

C. Enzyme assay

Alcohol oxidase activity was measured according to Janssen, et al. [24]; the enzymatic assays were performed at 25°C in reaction mixtures of 1.0 mL total volume containing 780 µL of reactive A, made of 1.2 mL of 0.2 M potassium phosphate buffer (pH 7.5); 10 µL of 1.0% o-dianisidine dissolved in 0.025 M HCl, 5 µL of 3% peroxidase (0.01% final concentration), 150 µL of 0.2 M potassium-phosphate buffer, 15 µL of substrate (crude oil, methanol ethanol, propanol, butanol, pentanol or hexadecanol) and 50 µL of cell free extract (100–200 µg protein). The reaction was started by the addition of substrate and development of colour measuring the absorbance at 460 nm in a Beckman DU-650 spectrophotometer. Specific activity was expressed as µg H_2O_2/min/mg protein.
Protein was measured by the Lowry´s method [25] with bovine serum albumin used as the standard.

III. Results and Discussion

A. Identified microorganisms of the analized samples

From the different samples analysed from five areas nears to gas stations, we isolated and identified 17 colonies of bacteria, yeast and fungi, and they can grow in the presence of crude oil (Table No. 1), the most frequent was *Pseudomonas aeruginosa* (45.4%), followed by *Escherichia coli* (18.18%), *Serratia marcescens, Enterobacter aerogenes, Candida albicans* and *Aspergillus niger* (9%) (Figure No. 1). It is indicating that isolated microorganisms developed resistance and perhaps the degradation mechanism of crude oil in an environment contaminated with it, which coincides with a variety studies because from different sources, have been isolated different microorganisms with the capacity of resistance and crude oil degradation [3, 4, 5, 6, 7, 8, 15, and 16].

B. Growth in dry weight of the different strains

Incubated in the presence of 1.0 mL of crude oil, for 3 days for bacteria and 7 days for the yeast and fungi, the growth was determined by dry weight; found that the five strains of *P. aeruginosa* grew up better in the presence of hydrocarbon, with an increase between 0.89-4.15 times with 1000 µL of crude oil, *S. marcescens* and *E. aerogenes* with 400 µL of crude oil 1.66 and 1.57 times), *C. albicans* and *A. niger* with 600 and 500 µL of crude oil 1.82 and 1.59 times of growth) (Figure 2, 3, 4, 5, and 6). In the literature was found that 96% of bacteria isolated from liquid resources (lakes, rivers, and lagoons), present ability to grow and emulsify petroleum hydrocarbons [5], and the results obtained in this study showed that the microorganisms grow efficiently in the liquid medium added with different concentrations of crude oil, besides the emulsifying the medium. These results are similar to those obtained with *Rhodococcus erythropolis*, *Achromobacter xylosoxidans,* and *Brevundimonas diminuta* [26], Endophytic bacteria [27], *P. aeruginosa* and C. *albicans* [16], *C. tropicalis* [28], *Serratia marsescens* [17], *Bacillus cereus* [29], and *M. rouxii* [19]. The survival of the fungi and bacteria in these conditions suggests that they may have the ability to use aliphatic and aromatic compounds such as carbon source and/or electron donor [5, 29].

TABLE I. Identified microorganisms of the analized samples

Fuel station	Identified microorganism	Total
1	*Pseudomonas aeruginosa* *Serratia marcescens*	2 1
2	*Pseudomonas aeruginosa* *Enterobacter aerogenes*	2 1
3	*Pseudomonas aeruginosa* *Escherichia coli*	2 1
4	*Pseudomonas aeruginosa* *Candida albicans*	2 1
5	*Escherichia coli* *Pseudomonas aeruginosa* *Aspergillus niger*	3 1 1

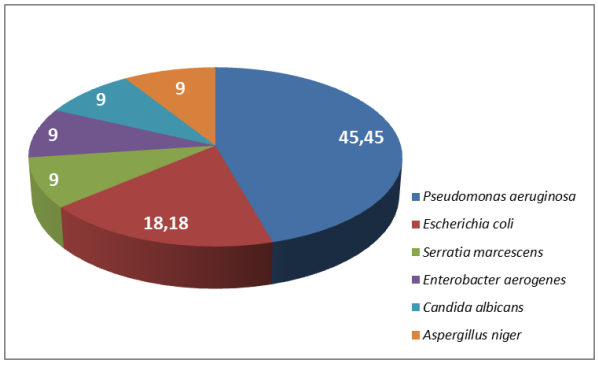

Figure 1: Frequency (%) of microorganism isolated in dry weight

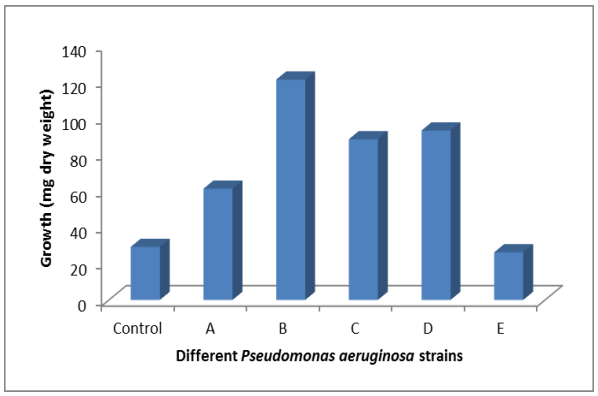

Figure 2: Growth in dry weight of *Pseudomonas aeruginosa* strains, with 1 mL of crude oil. 3 days of incubation. 28°C. 100 rpm. LMM 1X 10^6 cellules/mL.

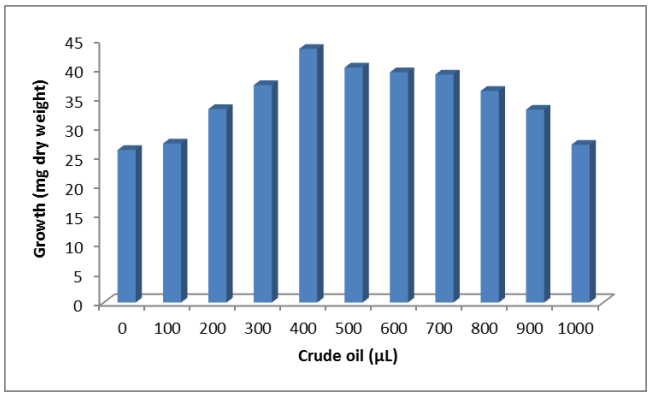

Figure 3: Growth in dry weight of *Serratia marcescens*, with different concentrations of crude oil. 3 days of incubation. 28°C. 100 rpm. LMM 1X 10^6 cellules/mL.

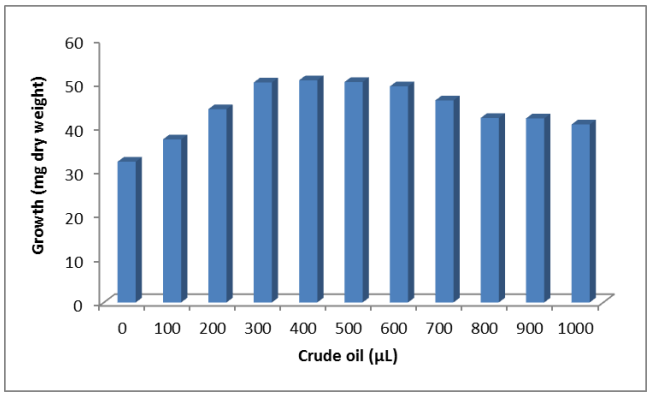

Figure 4: Growth in dry weight of *Enterobacter aerogenes*, with different concentrations of crude oil. 3 days of incubation. 28°C. 100 rpm. LMM 1X 10^6 cellules/mL.

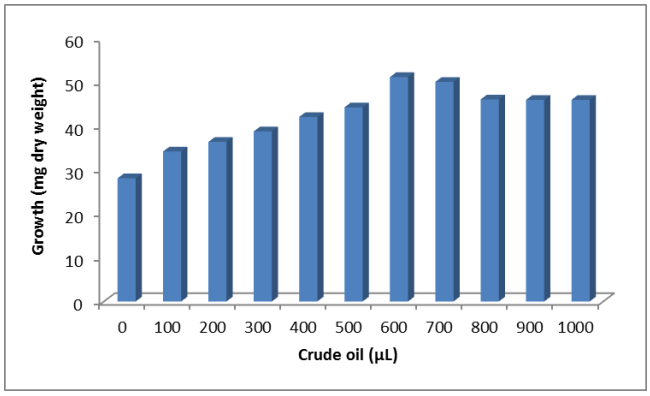

Figure 5: Growth in dry weight of *Candida albicans*, with different concentrations of oil crude. 7 days of incubation. 28°C. 100 rpm. LMM 1X 10^6 yeast/mL.

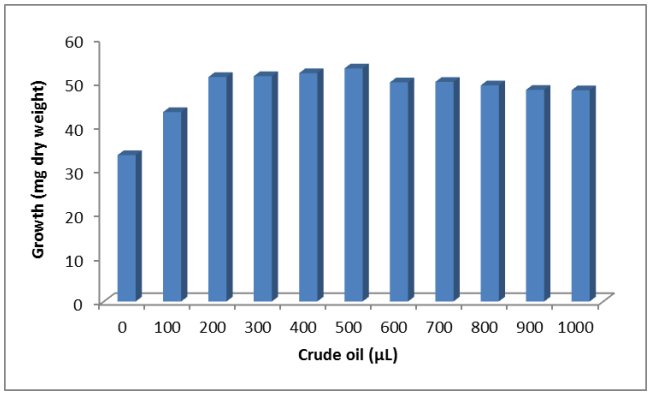

Figure 6: Growth in dry weight of *Aspergillus niger*, with different concentrations of crude oil. 7 days of incubation. 28°C. 100 rpm. LMM 1X 10^6 spores/mL.

C. Alcohol Oxidase Activity in Different Subcellular Fractions

We also analysed the activity of alcohol oxidase in subcellular fractions (crude extract, FMM and supernatant of 25 000 rpm) with different substrates (crude oil, methanol and ethanol). The strains *P. aeruginosa* isolated areas nears to gas stations B (this show most growth), *C. albicans* (only yeast) and *A. niger* (only fungi), grown in the presence and absence of crude oil (see Methodology). Enzyme activity with these substrates was detected mainly in the cytosolic fraction, and little in the MMF and cell walls (date not shown), and Table 2 shows the levels of specific activity of the three strains used, *P. aeruginosa* and *C. albicans* shown higher specific activity when grown in the presence of methanol, crude oil, and ethanol as substrate (338.4, 207.4, and 110.74, and 247.3, 182.6, and 162.1, for *P. aeruginosa* and *C. albicans*, respectively), and *A. niger* show higher activity with crude oil (54.3). The results found in this study are similar to those reported by for the fungus YR-1 isolated from petroleum contaminated soils, although they use different substrates [30], being the main enzyme inducer methanol [31], and for alcohol dehydrogenase NAD +dependent with methanol, ethanol, and hexadecanol as substrates [32], with activity of alcohol oxidase of *Pseudomonas aeruginosa* and *C. Albicans* [16], for contaminated soils of different fuel station in San Luis Potosí, S.L.P. México [17], for the activities of different enzymes (dehydrogenase, catalase, urease and polyphenol oxidase) of a *Rhodococcus* strain isolated from the activated sludge in oil field [33], and *M. rouxii* lead-resistant, isolated from polluted air with industrial vapors, near smelting plant in San Luis Potosí, México [19].

TABLE 2. Alcohol Oxidase Activity in *P.aeruginosa*., C. albicans and A. niger, Growth With and Without Crude Oil

Substrate	***Crude oil (mL)***	***Specific activity (mg H_2O_2/min/mg protein)***		
		P. aeruginosa	*C. albicans*	*A. niger*
Crude oil	0	14.3	88.4	10.2
Crude oil	1	207.4	182.6	54.3
Methanol	0	4.3	76.2	16.3
Methanol	1	338.4	247.3	47.3
Ethanol	0	21.0	93.1	11.3
Ethanol	1	110.7	162.1	37.2

IV. CONCLUSIONS

We isolated different strains of microorganism, resistant to crude oil with the potential for degrade crude oil. In the presence of crude oil as a carbon source, present a great alcohol oxidase activity, with

methanol, ethanol, and crude oil as substrate, whereby, this strain can be used to remove crude oil present in contaminated water and soils.

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