CAPÍTULO 10

ISOLATION OF A *Mucor* sp., STRAIN RESISTANT TO CRUDE OIL AND THEIR ALCOHOL OXIDASE ACTIVITY

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ABSTRACT: Recently, has been studied the isolation of oil tolerant microorganisms and his capacity of degradation from places contaminated with oil, such bacteria, yeast, and fungi. In this work, we isolated a fungal strain (*Mucor* sp.), resistant to heavy metals, from the environment near a smelting plant. The strain can grow in the presence of petroleum as the sole carbon source. Furthermore, the fungal strain shows good activity of alcohol oxidase in the cytosolic fraction with different substrates. It was concluded that this microorganism could be used for decontamination of aquatic habitats

polluted with petroleum.
KEYWORDS: Isolation, oil, microorganism, degradation, alcohol oxidase

RESUMEN: Recientemente, se ha estudiado el aislamiento de microorganismos tolerantes a petróleo y su capacidad de degradación de éste, a partir de lugares contaminados con el mismo, como bacterias, levaduras y hongos. En este trabajo, se aisló una cepa fúngica (*Mucor* sp.), resistente a metales pesados, a partir del medio ambiente cercano a una planta de fundición. La cepa puede crecer en presencia de petróleo como única fuente de carbono. Además, la cepa fúngica muestra una buena actividad de alcohol oxidasa en la fracción citosólica con diferentes sustratos. Se concluye que este microorganismo podría ser utilizado para la descontaminación de hábitats acuáticos contaminados con petróleo.
PALABRAS CLAVE: Aislamiento, petróleo, microorganismos, degradación, alcohol oxidasa

1 | INTRODUCTION

Consumption of petroleum have grown exponentially, so is its impact on the environment. In the old era of petroleum, environmental effects of petroleum operations were insignificant probably because of the small population, very small scale of production, utilization of simple tools and low petroleum usage. Petroleum was then a good mineral resource without adverse consequences. Today, 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].

In recent years, various technologies have emerged 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 [3]. Bioremediation is one of the most extensively used methods because of its low cost and high efficiency [4]. 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]. The utilization of n-alkanes by yeast and fungi as a sole carbon and energy source has been reviewed [5, 6].

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 [7]. Additionally, a consortium of symbiotic bacteria or supporting materials can be used to enhance the biodegradation process as described [8, 9].

Many microorganisms can use 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 can metabolize the hydro carbonated chains [3, 5, 6, and 7]. The first step in hydrocarbon biodegradation is catalysed by the protein complex Cytochrome P-450 followed by the action of alcohol oxidase [10]. Alcohol oxidase catalyse the oxidation of alcohols to the corresponding aldehyde, which, in turn, is converted into the corresponding carboxylic acid [11]. The reactions catalysed by Cyt-P 450 and alcohol oxidase are a special point for bioremediation chemistry. So far, most of the studies regarding the role of alcohol oxidase in hydrocarbon metabolism have been made on bacterial strains, and fungi. In several cases, these enzymes from eukaryotic origin with physiologic roles related to hydrocarbon metabolism have been reported [12].

In this work, we describe the growth in presence of crude oil of a fungus, *Mucor* sp., resistant to zinc, lead, and copper, isolated from the air collected near smelting plant in San Luis Potosí, México, as well as the activity of alcohol oxidase, with different substrates, present in cell-free extracts of the microorganism.

2 | EXPERIMENTAL

2.1 Microorganism and crude oil resistance test

A lead-resistant filamentous fungus was isolated from polluted air with industrial vapors, near smelting plant in San Luis Potos, México, in petri dishes containing modified Lee's minimal medium (LMM, with 0.25% KH_2PO_4, 0.20% $MgSO_4$, 0.50% $(NH_4)_2SO_4$, 0.50% NaCl, 0.25% glucose (13)] supplemented with 500 mg/L $PbCl_2$; 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 7 days. The strain was identified based on their morphological structures such color, diameter of the mycelia, and microscopic observation of formation of spores [14]. Crude oil-resistant tests of the isolated strain, filamentous fungus *Mucor* sp., were performed on liquid LMM containing the appropriate nutritional requirements and different concentrations of crude oil and determining the dry weight.

2.2 Assessment of the isolated strains as filamentous fungi

Sabouraud Dextrose Agar (SDA) and LMM media, containing the specified amounts of hydrocarbons as carbon sources were used to cultivate the fungus. Strains were maintained in agar slant tubes, and spores were obtained after growth in SDA medium as described. Liquid cultures (400 mL) were propagated in 1 L Erlenmeyer flasks inoculated with spores at a final concentration of 1×10^6/mL and incubated in a reciprocating water bath shaker at 28°C for the different time periods (see next section). To obtain aerobic mycelium, spores were inoculated in LMM media supplemented with 1.0 mL of crude oil, methanol, ethanol, propanol, butanol, pentanol and hexadecanol, and the cultures were incubated aerobically [15].

2.3 Preparation of Cell Free Extracts

Liquid cultures (100 mL) were propagated in 250 mL Erlenmeyer flasks inoculated with 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 different time periods. Mycelium 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.

2.4 Enzyme Assays

Alcohol oxidase activity was measured according to Janssen, et al., [16]; 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 [17] with bovine serum albumin used as the standard.

3 I RESULTS AND DISCUSSION

3.1 The effect of incubation time and pH

Incubated in the presence of 1.0 mL of crude oil, for 7 days the growth was determined by dry weight, found that *M. rouxii* sp., grew up better in the presence of hydrocarbon, presenting an increase of 1.26 times with 400 and 600 µL of crude oil (Figure 1). 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 [18], and the results obtained in this study showed that the fungus grow efficiently in the liquid medium added with 1.0 mL of crude oil, besides the emulsifying the medium. These results are like those obtained with *Rhodococcus erythropolis*, *Achromobacter xylosoxidans,* and *Brevundimonas*

diminuta [19], endophytic bacteria [20], *Pseudomonas aeruginosa* and C. *albicans* [21], *Candida tropicalis* [22], *Serratia marsescens* [23], and *Bacillus cereus* [24]. 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 [4, 24].

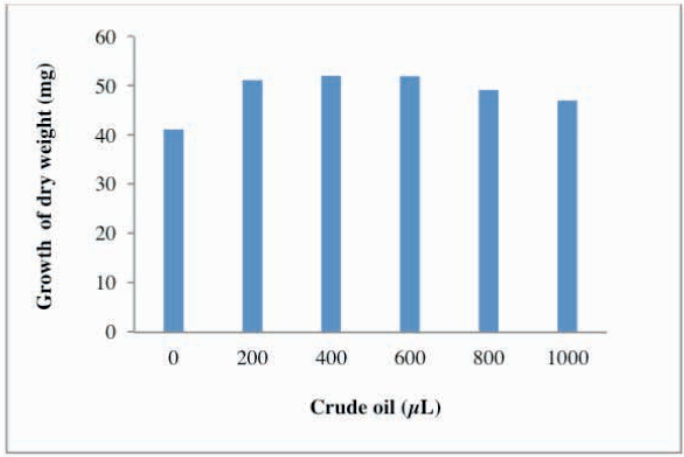

Figure 1: Growth in dry weight of *Mucor* sp., with different concentrations of oil crude. 7 days of incubation. 28°C. 100 rpm. LMM 1X 10^6 spores/mL.

3.2 Specific Growth Rate and Generation time

The appearance of alcohol oxidase activity as a function of incubation time in growth medium with 1.0 mL of crude oil was estimated. Figure 2 shows that in the enzyme production reached it is maximum after 32 h and then declined, whereas growth increased afterward. The pattern of induction of alcohol oxidase activity by crude oil observed is reminiscent with the report for YR-1 strain, a filamentous fungus isolated from petroleum-contaminated soils, with hexadecanol as carbon source [25], for bacteria and yeast petroleum resistant, isolated from different river of “Huasteca Potosina” (San Luis Potosí, S.L.P. México) [21], and contaminated soils of different fuel station in San Luis Potosí, S.L.P. México [23].

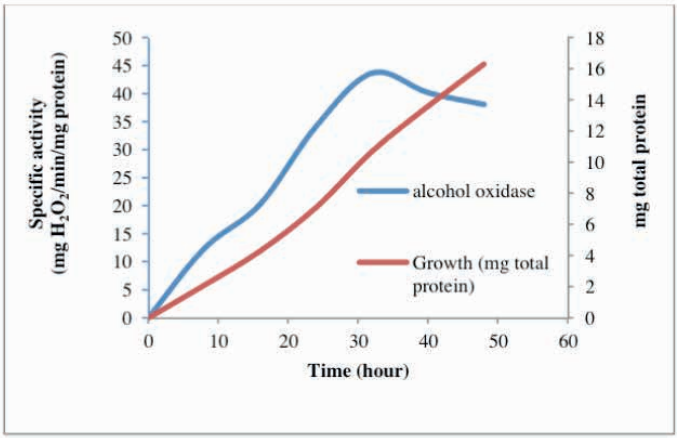

Figure 2: Growth kinetics of *Mucor* sp., strain, and appearance of alcohol oxidase activity as a function of incubation time, with 1.0 mL of crude oil as carbon source and substrate. 1 X 10^6 spores/mL. 28°C. 100 rpm.

3.3 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 ethanol, propanol, butanol, pentanol or hexadecanol). The fungal strain of *Mucor* sp., grow 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 (Figure 3), and Table 1 shows the levels of specific activity of the strain used, being higher when grow in the presence of crude oil as the carbon source, and methanol, crude oil, and ethanol as substrate (48.8 and 47.7, and 33.3, respectively). 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 [25], being the main enzyme inducer methanol [10], and for alcohol dehydrogenase NAD^+ dependent with methanol, ethanol, and hexadecanol as substrates [26], with activity of alcohol oxidase of *P. aeruginosa* and *C. Albicans* [21], for contaminated soils of different fuel station in San Luis Potosí, S.L.P. México [23], for the activities of different enzymes (dehydrogenase, catalase, urease and polyphenol oxidase) of a *Rhodococcus* strain isolated from the activated sludge in oil field [27],and for the bacteria *S. marcesens* and *P. aeruginosa,* and the yeast *C. albicans* (28).

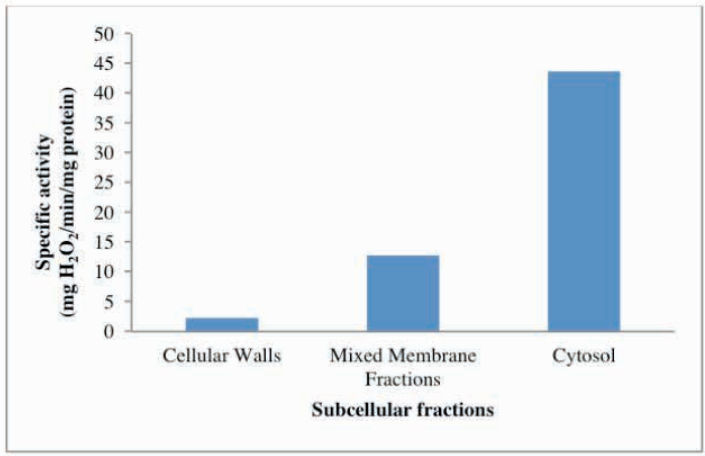

Figure: 3 Activity of alcohol oxidase in different subcellular fractions. 32 h of incubation. 28°C. 1 X 10^6 spores/mL.

Substrate	Crude oil (mL)	Specific activity (mg H_2O_2/min/mg protein)
Crude oil	0	9.71
Crude oil	1	43.7
Methanol	0	12.8
Methanol	1	48.8
Ethanol	0	13.5
Ethanol	1	33.3
Propanol	0	8.36
Propanol	1	13.3
Butanol	0	10.7
Butanol	1	12.5
Pentanol	0	17.2
Pentanol	1	22.47
Hexadecanol	0	18.3
Hexadecanol	1	26.3

*1 X 10^6 spores/mL. 28°C.100 rpm. 32 h of incubation.

TABLE 1 - **ALCOHOL OXIDASE ACTIVITY IN *Mucor* sp., GROWTH WITH AND WITHOUT CRUDE OIL***

4 I CONCLUSION

We isolated a fungal strain, resistant to zinc, lead, and copper, 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 this contaminant from contaminated water and soils.

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