



NUTRITIONAL REQUIREMENT OF *ENTEROBACTER CLOACAE* FOR BIODEGRADATION OF HYDROCARBONS

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ABSTRACT

Nineteen bacterial isolates were obtained have the ability to degrade the hydrocarbon waste from different sample at Al - Daura refinery which included, tanks , soils , refinery operation stages and waste accumulation area. The microbial flora were isolated on agar medium and choose the active isolate in analysis and grown on hydrocarbon waste and identified by biochemical tests , API 20 E system and VITEK 2 system . The results showed that the active isolate in degradation of hydrocarbon waste is *Enterobacter cloacae* .Optimization growth condition that related to hydrocarbon waste degradation nutrient, nutrient concentration, aeration were determined .Results showed that nutrient is NPK, nutrient concentration 0.50 % and used shaker incubator at 250 rpm.

KEYWORDS: *Enterobacter cloacae* , Bioremediation , optimization growth.

INTRODUCTION

Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products [1]. Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution [2]. Microorganisms can be isolated from almost any environmental conditions. Microbes can adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. The main requirements are an energy and a carbon source. Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards. Natural organisms, either indigenous or exogenous (introduced), are the prime agents used for bioremediation [3]. Bioremediation is a treatment for the cleanup of hazardous waste using microorganisms to break down or change *contaminants* into less toxic or nontoxic substances. The term bioremediation has been introduced to describe the process of using biological agents to remove toxic waste from environment. Bioremediation is the most effective management tool to manage the polluted environment and recover contaminated soil [3]. The aim of this study isolation and diagnostic of *Enterobacter cloacae* and the optimum environmental condition for the analysis of nutrient and aeration bioremediation of hydrocarbon.

MATERIALS AND METHODS

Collection of Samples

For isolation of hydrocarbons degrading bacteria, sixteen samples were aseptically collected from different sites at Al-Daura refinery , these includes: tanks, soils, refinery operation stages, waste accumulation area, sludge ,water

pollutant with oil . The samples were put in clean glass bottles and sterilized, samples were reserved in the refrigerator until used to isolate microorganisms.

Isolation and identification

Hydrocarbons degrading bacteria were isolated by spreading 0.1 ml of liquid samples (0.5 gm soil was mixed in 4.5 ml sterile saline) on either nutrient agar or /and macconkey agar and incubated at 37 C for 24-48 hr. Colonies developed were then transferred to the other agar media for cultural characteristics, form and shapes of colonies as well as colonies were microscopically examined using stained cells and using API 20E system (Biomérieux ,A.A.; France) [4].

Screening of bacterial isolates

The ability test of isolated on the degradation of hydrocarbon waste

The ability of these isolates to degrade hydrocarbon waste, 250 ml from brain heart infusion broth containing 1% of petroleum waste was dispensed 5 mls and inoculated with loopful of cells and incubated at 37°C for 3 days. Optical density was measured at 600 nm for growth determination. Four isolates were chosen and then incubated for 9 days in the shaker incubator at 50 rpm/min. and absorbance was measured at t 600nm to select efficient degrading one. as described by [5].

Analysis of Heavy Metal

Chemical analysis of heavy metals was done by ISSC laboratory (Ibn Sina Statc Company Laboratory /Ministry of Industry and Minerals) by Atomic absorption spectrophotometer , the metals include (Zn^{+2} , Fe^{+2} , Mg^{+2} , Sn^{+2} , Cr^{+2} , Cu^{+2} , Pb^{+2} , Ni^{+2}) Were determined in liquid waste (sludge) before and after remediate [6].

Effect of different concentration of nutrients

(100 ml) of Brain heart infusion supplemented with (0.25%, 0.50%, 0.75%, 1.0%) gm different concentrations

of NPK nutrients ,and 1% hydrocarbon waste(sludge) then inoculated with 10^{-6} bacterial inoculums, pH (7.0) was adjusted and incubate in shaker incubator at speed 75 rpm/ min and 37°C for 6 days. Then the biomass and surface tension and the optical density were estimated.

Effect of aeration

100 ml of Brain heart fusion broth was supplemented with 1% hydrocarbon waste (sludge) and 0.50% gm nutrient NPK, then inoculated with 10^{-6} bacterial inoculums. To determine effect of aeration, cultures were incubated either as static or on shaker incubator at speed 75 rpm/ min at 37°C ,then speed increased to (150, 200, 250) rpm/ min at 37°C , 1 day. static incubation was at 37°C for 6 days. Dry weight, surface tension and optical density duration were then estimated.

RESULTS AND DISCUSSION

Isolation and Identification

Nineteen isolated were obtained capable of hydrocarbons degradation using selective enrichment techniques. Most of these isolates were identified as gram negative (89.5%) depending on cultural and biochemical test suggested by [7] as shown in table (1) . While the number of isolates gram positive stain only two isolate (10.5%). On the basis of the morphological and biochemical properties, this bacterium was identified as *Enterobacter cloacae* [9]

TABLE 1: Biochemical identification of *Enterobacter cloacae*

Result	Biochemical test
Gram stain	-
Cell shape	Shorts rods
Surface	Smooth,mucoid
Elevation	Flat
Pigment	Cream
Spore	-
Oxidase	-
Catalase	+
Citrate	+
Indole	-
MR	-
VP	+
Blood hemolysine	-
Motility	+
Lactose	+
Kligler	+
Urease	-

Screening bacterial isolates

Bacterial isolates were screened for their ability to degrade petroleum hydrocarbons. Results shown in Figure (1) indicate that a variations in degradation ability were found among these isolates. Maximum degradation was obtained with isolates [18] which is selected for further studies and identified as *Enterobacter cloacae* .

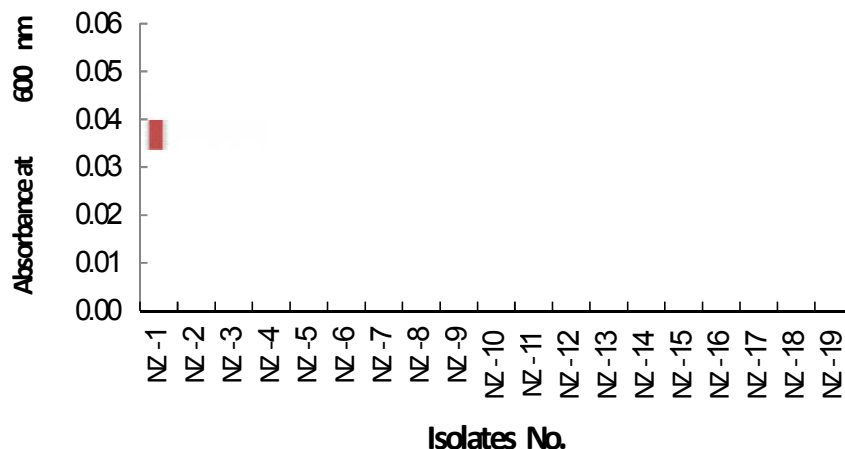


FIGURE 1: Screening bacterial isolates for their ability to degrade petroleum hydrocarbons.

TABLE 1: The percentage of heavy metal before and after remediate with *Enterobacter cloacae*.

Heavy Metal	Concentration (µg/L)		
	Before	After	% Removal
Zn ⁺²	393	7	98%
Fe ⁺²	960	6	99%
Mg ⁺²	1100	480	56%
Sn ⁺²	0.1	0	100%
Cr ⁺²	5	0.1	98%
Cu ⁺²	66	7	89%
Pb ⁺²	15	0	100%
Ni ⁺²	89	6	93%

Analysis of Heavy Metal

The results shown in Table (1) that the percentage of metals (Zn, Fe, Mg, Sn, Cr, Cu, Pb, Ni) before analysis were (393, 960, 1100, 0.1, 5, 66, 15 and 89 µg/L) respectively , and after inocubation with *Enterobacter cloacae* isolate the percentage of metals were (7 , 6 , 480 , 0 , 0.1, 7 , 0 and 6 µg/L) respectively.

The results from this study clearly demonstrated that *Enterobacter cloacae* was decreased concentration of Pb⁺², Sn⁺² from 15, 0.1 µg/L respectively to Nil in the broth media after ten days. Other results show that *Enterobacter cloacae* removed about 98%, 99%, 56%, 98%, 89% and 93% respectively of Zn⁺², Fe⁺², Mg⁺², Cr⁺², Cu⁺² and Ni⁺². these results agree with Kumar et al., (2010) show the heavy metals Zn, Pb, Cu, Cr, and Ni were determined in liquid waste , the acclimated microorganisms were used to remediate the waste biosorption process . *pseudomonas sp.* and *Bacillus sp.* reduce Cu (68% and 56%) and Ni (65% and 48%) respectively. Recent studies have demonstrated that

microbes might be used to remediate metal contamination by removing metals from contaminated water . This is primarily accomplished either by biosorption of metals or enzymatically catalyzed changes in the metal redox state (Kumar et al., 2010).

Effect of different concentration of nutrients

Fig (2) demonstrated that the best concentration give the high activity in degrading of hydrocarbon and moderate growth is 0.50 % , when measure the bacterial growth by absorbance (O.D 600nm) in the one, four and six day the absorbance are 1.880, 1.979 and 1.995 respectively .

When used the concentration 0.25 % , the low of growth rate in the six day and the results in the first, four and six day are 1.790, 1.920 and 1.860 , respectively . the concentration 0.75, 1 % shows inhibition of growth rate in the four and six day , when measure the absorbance the results were in the one, four and six day is 1.908, 1.999 and 1.999 for concentration 0.75% and 1.888, 1.999 and 1.999 for concentration 1%.

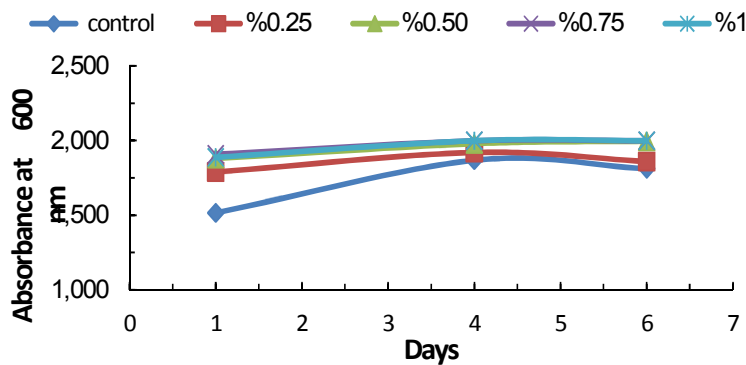


FIGURE 2: Effect of different concentration of nutrients on degradation of hydrocarbon by *Enterobacter cloacae* isolate.

Figures (3) and (4). illustrated the biomass and surface tension values for different concentration (0.25, 0.50, 0.75 and 1)% in the concentration 0.50 % gave the highest biomass 6.75 gm/l and surface tension 50 mN/m after six days , than other concentration (0.25, 0.75 and 1) % gave 4.5 ,5.25, 6.20 gm/l biomass and gave 48 ,45, 48 mN/m surface tension respectively .

From this results explain the rapid growth bacterial increased in low concentration from nitrogen source because it needs to growth and composition of biomass but high concentration lead to inhibition of growth and production [12].

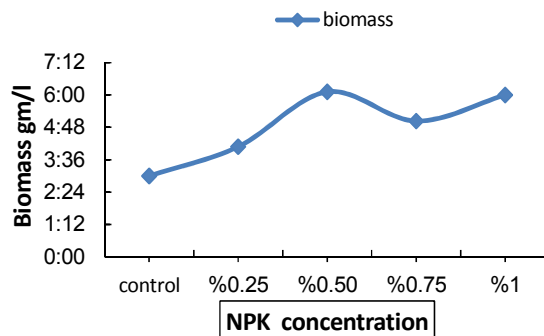


FIGURE 3: Effect of different concentration of nutrients on production of biomass by isolate *Enterobacter cloacae*

Nutritional requirement of *Enterobacter cloacae*

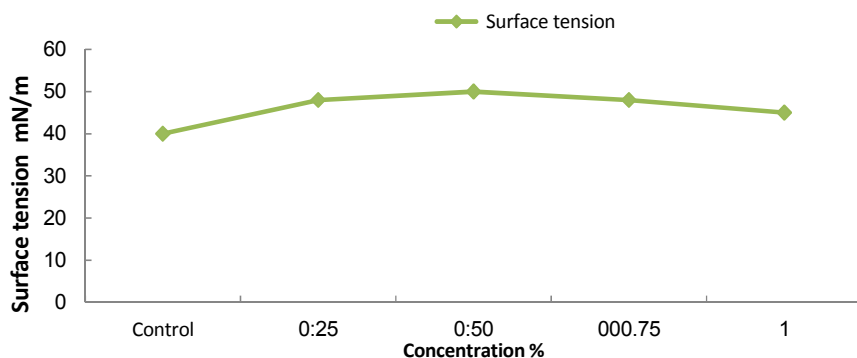


FIGURE 4: Effect of different concentration of nutrients on surface tension by isolate *Enterobacter cloacae*.

These results agree with the study of (13). In that the remediation efficiencies using samples of oily sludge with NPK fertilizer was used as the nutrient supplement at varying concentrations. Nutrient amendment was shown to enhance the rate of oil removal in the following order: 0.2% NPK > 0.075% NPK > no NPK. However, the combined effects of nutrient addition and agitation proved to be the most effective treatments.

Effect of aeration

Experiment was conducted to study the oxygen requirement for *Enterobacter cloacae* for complete degradation of hydrocarbons.

Fig (5) demonstrated the analysis of hydrocarbon in shaker incubator is best from static incubation, the rate of bacterial growth when measuring the Absorbance at 600nm is higher in cultivate found in shaker incubator at (75 rpm), in first day reach to 1.860, and after six day 1.999, either in static incubator was the higher growth reached 1.784 in the second day.

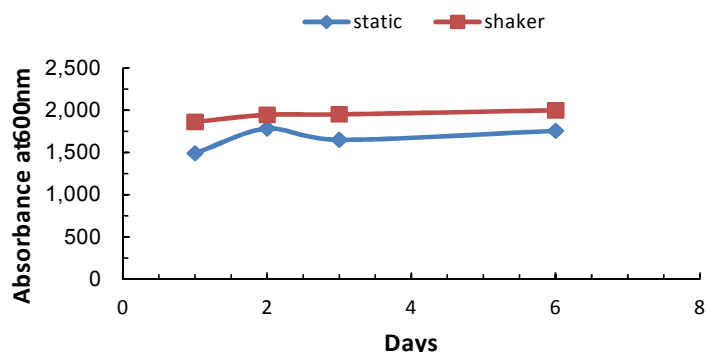


FIGURE 5 : Influence of aeration on degradation of hydrocarbon (sludge) by *Enterobacter cloacae* (37C° , 75 rpm/min , 6 days , 0.50 % sludge, 0.50 % NPK)

Figure (6) illustrated the biomass and surface tension values for shaker and static incubator, after 6 days the biomass was 6.48 gm/l and surface tension 46 mN/m in shaker incubator , either in static incubator the biomass was 1.50 gm/l and surface tension 45 mN/m after 6 day,

the results from this study clearly demonstrated that the aeration has an influential role in speed and efficiency of the degradation process, and the shaker incubator is best from static , these results agree with the study of [14].

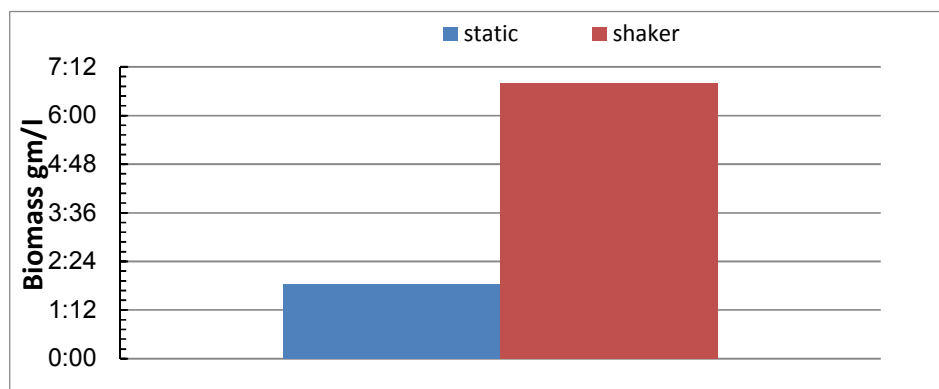


FIGURE 6 : Influence of shaker and static incubator on production of biomass by *Enterobacter cloacae*.

Aerobic conditions are generally considered necessary for extensive degradation of oil hydrocarbons in the environment since major degradative pathways for both saturates and aromatics involve oxygenases [15]. In the study of [14] showed The presence or provide oil waste pool with air is an important factor for the success of the bioremediation, the continuous stirring of the media helps to increase the dissolved air in the media , which stimulate analysis bacterial enzymes ,the molecule of hydrocarbon become ready for the consuming by the process of metabolism. Also, the continuous agitation is necessary to increase the surface area of the oil droplets exposed to bacteria and increased exposure to oxygen.

It was noted phenotypic changes in growth media , as it seemed turbid color after a day or two day of incubation, it indicate the emulsification of oil in the media by the secretion of isolates *Enterobacter cloacae* of emulsifier , working fragment of oil into small droplets , so increase surface area exposed to the action of bacteria , production emulsifier evidence on the ability of bacteria in consuming

of hydrocompound compound , is the speed and degree of emulsifying an indicator of bacteria efficiency [16].

Effect of different speeds of shaker incubator .

When used different speeds of shaker incubator at (150,200 and 250) rpm . The results showed when increased the speed of shaker incubator increased in growth rate when measured Absorbance at 600nm , in 150 rpm is 1.814 , 200 rpm is 1.861 , 250 rpm is 1.946 as figure 7, because when increase speed provide high quantity of oxygen for cultivated media . either biomass was 6.30 gm/l in 250 rpm and surface tension is 66 mN/m , and in the speed 200, 150 rpm was 4.30 , 3.70 respectively. So the best speed of shaker incubator is 250 rpm, these results agree with [17] which used a strain of *Pseudomonas alkanolytica* had been examined to degrade oil spill is surface water such strain which is olated from petroleum hydrocarbon contaminated soil has the ability to utilize a variety of hydrocarbons substrate , the cultivation was carried out at 30C° in shaker incubator with 250 rpm.

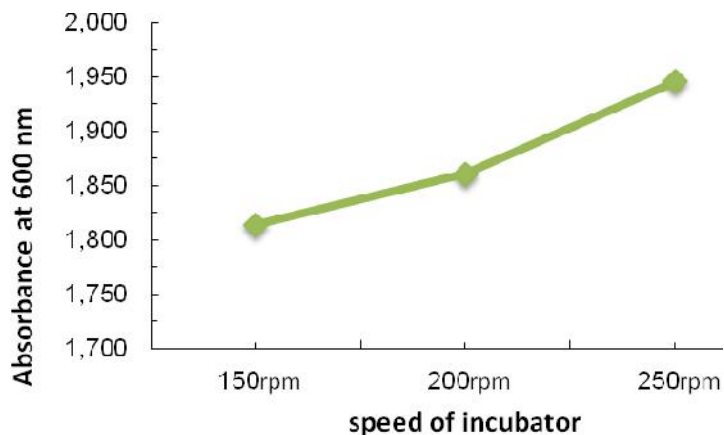


FIGURE 7 : Effect of different speed of shaker incubator on degradation of hydrocarbon (sludge) With different speed.

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