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RESEARCH ARTICLE

Octadecane biodegradation by *Bacillus* spp. isolated from contaminated oily soil

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Abstract

Octadecane is an alkane hydrocarbon with the chemical formula $CH_3(CH_2)_{16}CH_3$ and the efficiency of *Bacillus* spp. strains isolated from a petroleum contaminated soil for the biodegradation of octadecane hydrocarbons, is studied after cultivation on MSM and MMSM medium, stirred at 150 rpm, for 27 days, at 30ºC. Results showed that the number of viable count of *Bacillus* spp. was increasing up to 22 days of incubation at 30ºC, then it decreased gradually and reached to 8×10^8 and 2.8×10^8 cell / ml at period 25 and 27 days of incubation, respectively. Dry biomass was increased and reaching maximal density to 3.16gm / l, about 7.2gm / l of the bioemulsion was produced during the first 27 days and isolate have the ability to degraded 62.11% of the weight of octadecane used in the experiment.

INTRODUCTION

Saturated aliphatic hydrocarbons, such as octadecane, may be incompatible with strong oxidizing agents like nitric acid. Charring of the hydrocarbon may occur followed by ignition of un reacted hydrocarbon and other nearby combustibles. In other settings, aliphatic saturated hydrocarbons are mostly un reactive. They are not affected by aqueous solutions of acids, alkalis, most oxidizing agents, and most reducing agents. When heated sufficiently or when ignited in the presence of air, oxygen or strong oxidizing agents, they burn exothermically to produce carbon dioxide and water.

Amphipathic surface-active molecules consisting of both hydrophobic and hydrophilic domains, can facilitate the growth of microorganisms on hydrocarbons by increasing the permeability and hydrophobicity of the cell surface and⁄or by alkanes transfer including emulsification⁄solubilization of the substrate (1; 2; 3 and 4).

Some compounds in hydrocarbons may not be degraded by organisms (5), while others may be degraded and broken down into carbon dioxide, water and cell mass (6).

Surfactants are surface active agents with wide ranging properties including the lowering of surface and interfacial tensions of liquids. Surface tension is defined as the free surface enthalpy per unit area (7), and is the force acting on the surface of a liquid leading to minimization of the area of that surface.

A clear correlation exists between surface active agent production and alkane utilization by the degrading organism. However, different modes of uptake have been proposed for different microorganisms for the growth on hydrocarbons, sometimes more than one mechanism occurring simultaneously, for example, microorganisms can direct contact with big hexadecane droplets and emulsified small droplets in the different growth phase (8 and 9).

Oil pollution from industrial sources and other activities are hazardous to terrestrial and marine ecosystems. Petroleum is a complex mixture of aliphatics, aromatics, resins and asphaltenes. According to (10), that have reported biodegradation of petroleum oil by *Achromobacter*, *Arthrobacter Acinetobacter*, *Alcaligenes*, *Bacillus*, *Flavobacterium*, *Nocardia*, *Pseudomonas* and *Rhodococcus*.

Bioremediation of oily waste water is treatment technology that use of microorganisms or their enzymes to reduce the concentration or toxicity of hydrocarbon contaminants into less toxic forms (11 and 12).

In another hand (13) has reported that nearly 100 species of bacteria, representing 30 microbial genera, had hydrocarbon oxidizing properties, many species and genera have been found to have this ability. In general, *Bacillus* spp. has been identified as petroleum hydrocarbon degraders (14 and 15) and is known as naphthalene and pyrene degraders (16 and 17). *Fusarium* spp., F092 degraded 89% of n-octadecane (from about 13-125 mg L^{-1}) at 60 days. The metabolites produced during the degradation of n-octadecane were also investigated in saline liquid culture (18). There is no single strain of bacteria with the metabolic capacity to degrade all the components found within crude oil. In nature, biodegradation of a crude oil typically involves a succession of species within the consortia of microbes present. A combination of bacterial strain with broad enzymatic capabilities is required for active extensive degradation of crude oil (19).

Materials and Methods

Culture media

Mineral Salt Medium (MSM)

This media was used for growth of bacterial isolate that used in the experimental which consists of (gm/l): NaCl 1gm. KH_2PO_4 1gm. Na_2HPO_4 1gm. NH_4NH_3 0.5gm. $(NH_4)_2SO_4$ 0.5gm. $MgSO_4.7H_2O$ 0.2gm. $CaCl_2.2H_2O$ 0.02gm. $FeCl_3$ 0.002gm. $MnSO_4.2H_2O$ 0.002gm., which all dissolved in distill water and pH has been justify to 7 and sterilizes by autoclave (20).

Modified Mineral Salt Medium (MMSM)

This media was used to encourage oil emulsion process which leads to oil degradation. This media consist of (gm / l): KH_2PO_4 4gm. NH_4NH_3 4gm. $MgSO_4.7H_2O$ 0.2gm. $CaCl_2.2H_2O$ 0.01gm., which all dissolved in distill water and pH has been justify to 7 and sterilizes by autoclave (21).

Isolation of bacteria

Samples of oily polluted soil were heat-treated (80ºC for 10 min) to kill all vegetative cells and individually placed on nutrient agar plates. After 24hr of incubation at 30ºC, colonies were recovered and selected as gram positive bacilli cells, then streaking on fresh nutrient agar.

Viable count of bacteria isolate

A series of dilutions were done to calculate the count of isolate after the inoculation directly (Zero time) up to 27 days. The viable count was calculated for growing bacteria colonies by Italian Colony Counter (22).

Account of Biomass

After bacterial isolate was incubated on the activation medium for 24hr, 50ml of sterile liquid MSM was inoculated by bacterial isolate, then added 0.5ml of octadecane. Flasks were stirred at 150 rpm, for 27 days, at 30ºC. Biomass yield was determined after the elimination of the fatty fraction present in the cultivation medium, then culture was precipitate by centrifugation at 12000 rpm for 30min, then pellet was collecting and extracted with a mixture of acetone and hexane 1: 3 to rid it from hydrocarbons which adherent with it. Cells were dried at 105ºC for 24hr in oven. Finally, dry weight method was used to estimate the weight of the biomass (23).

Estimate the amount of bioemulsion

Fifty ml of sterile liquid MMSM has been inoculated by bacterial isolate (2%) and 1% octadecane, then incubated at 150 rpm, for 27 days, at 30ºC. The emulsion amount was estimated by expelled culture by centrifuge at 12000 rpm for 30min, then adjusted the supernatant to acidic value (pH 2) and left for 24hr at 4ºC. Extraction of emulsion was done by a mixture of chloroform and methanol solvents. Finally, before estimating the weight, dry the bioemulsion at 45ºC (24).

Measuring the quantitative loss of octadecane

Mineral salt medium was inoculated by bacterial isolate (2%) and 1% octadecane, then incubated at 150 rpm, for 27 days, at 30ºC. Supernatant was prepared by centrifugation the culture at 12000 rpm for 30min, then adjusted the supernatant to acidic value (pH 2). Octadecane extracted by adding chloromethane solvent, second stage was extracting the product through filter paper contains anhydrous sodium sulfate as a dryer factor, then solvent with octadecane was evaporated, calculated the remaining weight and the lossing of octadecane as:

$$\text{Percentage Rate of Remaining octadecane (PRRR) (PRR)} = \frac{\text{Remaining weight of octadecane}}{\text{Weight of octadecane in control sample}} \times 100$$

100 – (PRR) = Percentage of biodegraded octadecane (25).

Results and Discussion

Morphological changes in octadecane culture

The result was showed superficial changes in the layer of octadecane due to bacterial growth after incubation period for 27 days at 30ºC, the layer lossed its strength and emulsification was appeared, compared with control sample (Fig 1). Biodegradability, generally low toxicity, changing surface active phenomena, such as lowering of surface and interfacial tension, wetting and penetrating action, hydrophilic and hydrophobic action, microbial growth enhancement and antimicrobial action (26).

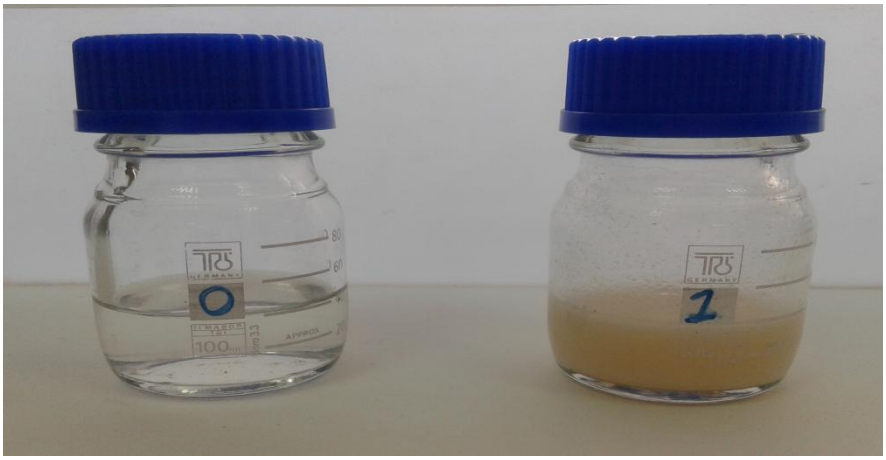

Figure (1) Morphological changes of octadecane which degraded by *Bacillus* spp. after incubation for 27 days at 30ºC on MSM medium.
(Right): treating sample, (Left): control.

Increasing growth of isolate

Survival of microorganisms in octadecane medium after their inoculation is a key deciding factor in the rate of biodegradation of hydrocarbons either in soil or in liquid phase (27). Increasing numbers of bacteria with the progress of time is considering one of the methods that used to determine the extent of their ability to adaptation and degradation of crude oil compounds (28). Table (1) showed that the number of viable count of *Bacillus* spp. was increasing up to 22 days of incubation at 30ºC, then it decreased gradually and reached to 8×10^8 and 2.8×10^8 cell / ml at period 25 and 27 days of incubation, respectively. From the microbial point of view, they metabolizing or consuming the oil to provide the energy and materials that needed to live and grow (29).

Table (1) Viable count of *Bacillus* spp. along the period of 25 and 27 days for incubation on MSM medium with octadecane

Incubation time (period / days)	Viable count cell / ml
Zero time	9.3×10^3
1	2.5×10^5
3	4×10^5
6	1.1×10^6
9	3×10^6
13	8×10^6
16	4.5×10^7
19	5.8×10^8
22	5.3×10^9
25	8×10^8
27	2.8×10^8

Biomass of isolate

Bacterial strains utilized crude petroleum oil hydrocarbons as sole source of carbon and energy, which was evident from the increase in bacterial dry biomass, by breaking down into simple carbon compounds that are used to make the sugars, fats, and proteins needed for growth and energy production, ultimately the byproducts become carbon dioxide and water (30).

In this study, biomass was increased with incubation time in all cases, reaching maximal density that ranged from 9.3×10^3 to 2.8×10^8 cell / ml for 27 days, with dry biomass 3.16 gm / l (Table 2).

Quantitative calculate of bioemulsion

The biosynthesis of bioemulsion by *Bacillus* spp. was done by cultivation on MMSM. However, It is interesting to note that about (7.2gm / l) of the bioemulsion was produced during the first 27 days (Table 2) (31), also observed that biosurfactant production by *Bacillus subtilis* 21332 started during the exponential phase and was continued during the stationary growth phase. Production of biosurfactant is related to the utilization of available hydrophobic substrates by the producing microbes from their natural habitat, presumably by increasing the surface area of substrates and increasing their apparent solubility (32 and 33).

Degradation count of octadecane

Results were showed that the *Bacillus* spp. have the ability to degraded 62.11% of the weight of octadecane used in the experiment after incubation period for 27 days at 30ºC (Table 2). This may be due to production of emulsion materials and to the presence of bacterial enzymes (35).

Table (2): Biomass, quantity of bioemulsion and percentage degradation of octadecane due to bacterial growth after incubation period for 27 days at 30ºC**.**

Biomass	Quantitative of bioemulsion	Percentage of degradation
3.16 gm	7.2 gm / l	62.11

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