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Enhancement of Crude Oil Biodegradation by Immobilized Bacterial Consortium in Small Batch and Continuous Bioreactor Modes

Soha Farag[1]*, Nadia A. Soliman[2], Yasser R. Abdel-Fattah[2]

[1]Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt.

[2]Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt.

OIL SPILLS besides the discarding of oil refineries byproducts directly into the environment is a major source of nature pollution which contributes to environmental contamination and health problems. Firstly for solving this problem oil degrading bacterial consortium *(Pseudomonas* sp. sp48 and *Bacillus cereus* M12 which were isolated from soil samples from Bahary area, SidiKerir branch, Alexandria, Egypt, respectively) was immobilized with different carriers, where rice straw was selected as the best one for oil degradation. Conditional optimization for immobilized bacterial consortium has been explored by testing various amounts of preculture (2, 3, 4, 5.5 and 7%), different incubation time with rice straw (1-5 h), enumeration of viable cells immobilized and different oil concentrations (1, 2, 3, and 4%). The degradation of crude oil (1% concentration) reached to 61.6% due to biological action alone while it raised to 97.14% by binary actions of biological and physical after 6 days incubation time and applying the optimum conditions (4% bacterial consortium preculture and 3 h incubation time with the carrier). Finally application study has been carried out using the ideal model conditions in small batch bioreactor and continuous to treat oil contaminated water in an open system. The results showed that the crude oil degradation biologically only and biologically/ physically reached to 56.2% and 87.2%, respectively. Whereas the continuous addition of 1% oil concentration for three times the removal reached to 81.27%. This technique of immobilization produces a promising result for solving the oil pollution problem due to the collaborating effects for both physical and biological actions.

Keywords: Biodegradation, Crude oil, Bacteria, Immobilization, Bioreactor.

Introduction

Crude oil is a very important mineral resource vital to everyday life. However, crude oil spillage is one of the most serious forms of water and land pollution. Oil spillage is the accidental discharge or pouring of crude oil into the environment. It involves the contamination of any part of the environment with any liquid hydrocarbon. These spills endanger public health, imperil drinking water, devastate natural resources, pollute beach and shorelines, and disrupt the economy [1].

Biodegradation of crude oil is not a new technology for combating oil pollution. Isolation of active oil-degrading bacteria from the marine environment was conducted as early as 1960s [2]. There are many challenges to recover oil-polluted sediment or water by inoculating oil-degrading bacteria like nutrient deficiency which was identified as one of the limiting factors in oil degradation [3, 4]. Although nutrients-enrichment helps the bioremediation, it is not the only compel in oil degradation. Factors such as inhibitive metabolites and the ability of the oil degrading microorganisms are among other reasons that retard biodegradation [5]. Biodegradation involved a series of complicated biochemical reactions which cannot be done by an only one

*Corresponding author e-mail: sohafarag88@yahoo.com
Tel.: (203)4593418; fax: (203)4593407.
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organism. Importance of the bacterial consortium for bioremediation was highlighted in many reports [6]. Ito et al [7] reported a consortium of symbiotic bacteria, which are capable of degrading microbial-resistant turbine engine oil; and Li et al [8] reported a microbial consortium of bacteria–fungi complex capable of degrading more than 50% of aged polycyclic aromatic hydrocarbons in soil and slurry. Radwan et al [9] reported the superior performance of oil-consuming microbial consortia in the Arabian Gulf.

The most important limiting factor is the inhibition effect of high oil concentration on the microorganism. According to William [10], who reported that the using of carriers, mainly in the sea or in non-accessible areas, such as marshes or flooded zones is the most common practices for oil spill remediation or oil recovery. Synthetic products such as polypropylene, polyurethane, foam, polyethylene terephthalate and teflon are the most used materials due to their low cost, high availability and high oil-sorption capacities. Inorganic mineral products as perlite, vermiculite, dolomite and graphite are also used [11, 12]. Finally the agriculture wastes as corn leaves, cane leaves, rice straw and wood chips which economically cheap, renewable supports and high oil sorption capacities will be extensively studied in our work for oil removal.

Oil carriers can adsorb and concentrate floating petroleum and prevent its migration to shorelines and beaches. If oil carriers were immobilized with oil degrading bacteria, bioremediation may occur in situ or ex situ [13].

The use of immobilization technique involving carrier materials for the delivery of microbial cells to natural ecosystems is an attractive option. Carrier materials are generally intended to provide protective niche to microbial inoculants, either physically, through the donation of a pore space or protective surface, or nutritionally, through the donation of a specific substrate. An optimum carrier should provide favorable conditions for survival and activity of the inoculant cells [14]. The carrier should, additionally, be nontoxic, nonpolluting, have a constant quality and be locally available at low price [15- 17]. Furthermore carrier inert surfaces in cultures of degrading microorganisms and immobilized cells have been shown to encourage crude oil degradation [18, 19].

Immobilized microorganisms could degrade crude oil at a higher initial concentration and for a longer period. In addition, these cells were also protected from harmful effects of toxic wastes. In some previous studies, immobilized cells could be stored for long periods without losing their degrading abilities. The adsorbed oil materials may even be extracted and the adsorbents reused [20].

Immobilization of oil degraders on carriers may therefore solve the problem of dilution of microbes and nutrients in open water. Preventing migration of floating oil, bioremediation or removal of the oil from the sea will significantly decrease this exposure and facilitate the clean-up of an oil spill. Hence, the use of suitable carrier immobilized with oil degraders may assist in the protection of coastal environment [13].

Consequently, this study aims to demonstrate the efficiency of bacterial consortium immobilized on different carriers in oil degradation. The optimum immobilization conditions for the highest degradation are being to assess. As well applying the immobilization technique in a bioreactor to resembling the nature for application in sea water oil spill.

Materials and Methods

Development of carrier materials

The used carrier materials were divided into two categories, the first one is natural product which economically cheap and renewable supports, have not any benefit in our life as corn leaves, cane leaves, rice straw and wood chips, the other one is a synthetic carrier as foam and wax. The natural carriers were cut before using into small pieces 3-5 mm then sterilized by autoclaving at 121 °C for 20 min. but wax and foam were put in oven at 40 °C for 24 h.

The needed amounts of tested carriers were determined individually by measuring the amount of carrier that covers the surface of Erlenmeyer flask 250 mL (50 cm^2) filled with 50 mL natural sea water (NSW). The amount of preculture needed to saturate the carrier was determined by immerging each carrier in a constant volume of preculture then determine the amount of adsorbed preculture.

Based on previous statements the used weight of tested carrier (gram)/preculture volume (mL) was (0.5/3.5) for rice straw; (0.3/5) for corn leaves; (0.5/2.5) for cane leaves; (0.8/2) for wood chips; (0.06/1) for foam and (1.5/1) for wax.

Immobilization on different carriers

The bacterial isolates *Pseudomonas* sp. (sp48) and *Bacillus cereus* (M12) which previously identified as marine oil-degrading bacterial isolates and kept in GeneBank under accession numbers (ac: KP202717& KP202718) respectively were used in this study. The isolates were cultured in LB medium has the following composition (g/L): 5 g NaCl, 10 g peptone, 5 g yeast extract, pH was adjusted to 7 with either HCl/NaOH. The flasks were incubated for 24 h at 30 °C on a rotary shaker operated at 200 rpm. The growth of the bacteria was determined spectrophotometry at 600 nm. The resulted culture was used in subsequent experiments.

Each carrier material was placed into a 250 mL flask containing 50 mL of *Pseudomonas* sp. (sp48) culture (constant growth OD_{600nm}=1.2). Afterwards, the flasks were incubated at room temperature under shaking (100 rpm) to allow cells to attach to the carrier material (wet formulation) for constant time 3 h. The wet formulation was removed from the flask and rinsed with sterile LB to remove any cells that were not sufficiently adhered to the carrier material. The same method was repeated for strain M12.

Evaluation of oil bioremediation using immobilized bacteria

In this experiment, bacterial pre-cultures were prepared by allowing *Pseudomonas* sp. (sp48), *Bacillus cereus* (M12) strain to grow individually till constant $OD_{\lambda 600nm}$ (1.2). Also a consortium of them (sp48 and M12) at equal volumes ratio (1:1%; $OD_{\lambda 600nm}$ = 1.2) was prepared. The bacterial preculture at concentration 4% were incubated individually with different carriers (corn leaves, cane leaves, rice straw, wood chips, foam and wax) for 3 h at room temperature, then the immobilized bacteria was inoculated to 50 mL natural sea water containing 1% crude petroleum oil and incubated at 30 °C, 200 rpm in shaker incubator for 6 days. The similar experiment was performed without using the bacteria to know the ability of carrier to adsorb oil physically at the same time. Two controls were prepared, the first one contains free bacterial cells kept under the same former conditions, but the second contains 1% of crude oil only in natural sea water.

To know the biological effect of immobilized bacteria without interference with physical action of carrier, the flask which contains the immobilized bacteria on the carrier was extracted by dichloromethane then sieved by filtration using a mesh sieve then crude petroleum oil consumption was determined gravimetrically as percentage. In case of physical test the carrier which doesn`t contain bacteria was sieved first then the decanted natural sea water was extracted. The test was done in replica and the average result was taken. Crude petroleum oil consumption was determined gravimetrically in percentage as described previously by Law et al [21] and Giorgio et al [22] in which the residue oil of each sample was extracted with 100 ml dichloromethane. Then the residue was then transferred into round bottom flasks and concentrated by rotary evaporator (Ika-Heizbed HB-250). Initial weight of the round bottom flasks were weighted after it has been cleaned and dried. After removing dichloromethane, the round bottom flasks were dried at 50°C in oven for an hour and then put into desiccator for another hour before weighing with a 3 decimal points balance. The weights difference of flask equals the weight of residual oil. The percent of oil removal was calculated using equation:

$$\text{removal } \% = (W_c\text{-}W_s/W_c)*100$$

where, W_c = Weight of control (g/L) and W_s = Weight of sample (g/L).

Conditional optimization for immobilized bacterial consortium

Various amounts of bacterial consortium preculture (2, 3, 4, 5.5 and 7%) and different incubation period with rice straw 1-5 h (one hour interval) were tested in order to optimize the parameters affecting oil degradation by bacterial consortium added to natural sea water supplemented with 1% crude oil.

Firstly 0.5 gm of carrier material (rice straw) covers the surface area of flask is saturated with 3.5 mL of bacterial consortium preculture in 50 mL medium. Accordingly, different amounts of culture (2, 3, 4, 5.5 and 7%) were incubated with rice straw for 3 h afterwards, the free cells were removed by decantation. After that 50 mL natural sea water contains 1% crude petroleum oil was added to the immobilized bacteria and incubated at 30 °C, under shaking (200 rpm) for 6 days.

To optimize the incubation period, the carrier material was incubated with the bacterial consortium preculture (OD_{600nm}=1.2) for various periods of time. The rice straw (0.5 gm) were placed into the consortium culture (4%; 2 mL

culture: 1.5 mL distal water; in 50 mL medium) and incubated to allow the attachment at different time 1, 2, 3, 4, and 5 h, thus the immobilization of the cells on the rice straw was allowed. Then the immobilized consortium transferred to the experimental flasks containing natural sea water supplemented with 1% of crude oil and incubates at 30 °C and 200 rpm in shaker incubator for 6 days.

Enumeration of viable cells immobilized on rice straw

To count the attached viable cells, 0.1 g of the formulation sample was suspended in 5 mL LB medium. The suspension was agitated vigorously using a vortex mixer for 3 min to dislodge the immobilized cells. Serial dilutions were made from the supernatant and aliquots of 0.1 mL were spread on LB plates. The plates were incubated at 30 °C until colonies appeared (24–48 h). The colonies were counted to assess the number of viable bacterial consortium cells immobilized on the carrier material. The attachment efficiency was calculated as the fraction of the total viable cells that was immobilized [23]. This was carried out to evaluate the viable cells immobilized on rice straw at different incubation time based on the previous experiment.

Evaluation of immobilized bacterial consortium efficiency

These experiments were performed to determine the crude oil removal efficiency of carrier materials (rice and foam) with the bacterial consortium (biologically and physically together) and biological effect alone using bacterial consortium (immobilized cells). The oil removal efficiency was determined by adding 4% preculture which immobilized and incubated with carrier (0.5 gm of rice or 0.06 gm of foam) for 3h then added to 250 mL flask that contained 50 mL of natural sea water with different concentrations of crude oil 1, 2, 3, and 4%. The control of this experiment is the natural sea water with the same different concentrations of crude oil. All flasks were shaken at 200 rpm and 30 °C in shaker incubator for 6 days. Crude petroleum oil consumption was determined gravimetrically in percentage as described previously by Law et al [21] and Giorgio et al [22].

Follow up crude oil degradation

Bacterial consortium was cultured in LB medium for 24 h till OD_{600} 1.2, then used at 4% for immobilization on rice straw (0.5 gm) for 3 h. The immobilized consortium was transferred to the experimental flasks containing natural sea water supplemented with 1% crude oil then incubated at 30 °C under shaking (200 rpm) for 12 days. Two flasks were withdrawn each day and the average result of crude oil biologically removed was determined.

Examination of immobilized cells using Scanning Electron Microscope (SEM)

Three samples were prepared to examine under SEM, rice straw carrier without addition of oil, bacterial consortium immobilized on rice straw after 3h incubation time without oil addition, and rice straw carrier with added oil, where bacterial size was measured in the second sample. The samples were sputter coated with gold (Bal-Tec SCD005 Sputter Coater) and examined using a JEOL JSM 6390LV Scanning Electron Microscope.

Application study in a bioreactor

A small scale reactor (Glass beaker v: 2 L, diameter: 10 cm, height: 15 cm) was used to apply the optimized conditions to treat oil contaminated water in open systems.

In this stage, an equivalent weight of carrier and oil similar to flask experiment were used to cover the surface area of the reactor in order to obtain comparable results. The reactor containing 300 mL natural sea water, 1% oil (800 µl), and 0.8 gm of carrier (rice straw) per 80 cm^2 reactor surface area. The reactor was placed on the bench shaker for approaching the sea waves, at room temperature (25 °C) and using natural sea water for resembling the nature for application in sea water oil spill. Water lost in the reactor via evaporation was replaced daily.

A similar reactor was used by Mang et al [24] whose studies were carried out in a series of identical glass-made vessels which had a total volume of 7 L. Each reactor received 5 L of 25% slurry, 600 rpm, 25-30 °C, 15% (v/v) oil, aeration rate up to 2 mg/L.

Batch bioreactor optimization

All biodegradation experiments in this study were carried out in a series of identical glass-made vessels which had a total volume of 2 L as described before. The immobilized bacterial consortium on rice straw was prepared at optimum conditions to know its biological effect in oil degradation. The experiment was carried out at different times (6, 9 and 12 days) and carrier weights (0.8, 1.2 and 1.6 gm) at constant incubation time (6 days), where 0.8 gm of carrier material (rice straw) covers the

surface area of reactor (80 cm^2).

This experiment was performed with immobilized bacteria to know the ability of carrier to adsorb oil physically and degraded biologically at the same time. Two control reactors were prepared, one contains free bacteria (without immobilization) in the same condition, and the other contains 1% crude oil only in natural sea water.

Continuous bioreactor

This experiment was carried out to verify the efficiency of immobilized bacterial consortium on rice straw for repeated oil removal. The small scale reactor (2 L) was prepared and filled with 300 mL natural sea water, 1.6 gm rice straw immobilized with bacterial consortium (*Pseudomonas* sp. sp48 and *Bacillus cereus* M12) and 1% crude oil (800 µl) according to reactor surface area (80 cm^2). The bioreactor was placed on a bench shaker at room temperature (25 °C) where, 1% crude oil was added every 3 days to the bioreactor for three times. The test was done in replica and the average result of crude petroleum oil consumption was determined.

Results and Discussion

Investigation of the best carrier used in immobilization

The percentage of oil degradation using *Pseudomonas* sp. sp48 /or *Bacillus cereus* M12 immobilized at different carriers was represented in Table 1, the results show that the best carriers for immobilization of tested bacteria were rice straw, cane leaves then corn leaves (natural products), where the oil removal reached to 46%. The synthetic carriers (foam and the wax) not support the biological degradation of oil throw the immobilization process. On the other hand the foam and wax showed higher efficient for oil removal physically rather than biologically. The percentages of oil removal physically reach 98% and 96% when using foam and wax, respectively.

Polyurethane foam (PUF) can adsorb and concentrate Arabian light crude (ALC), possibly increasing substrate limitation and product inhibition within PUF as reported by Nawaz et al [25]. Cell division was also slower in the matrix and this could also decrease uptake of substrates and metabolites leading to slow degradation rate [25]. So the foam is efficient for physical removal of crude oil not as carrier for biodegradation.

It was found in this study the immobilized experimental bacteria is most potent than free bacteria as shown in Table 1, the percentage of oil removal reaches to 28.8, 36.8 and 39.4% when using strains sp48, M12 and bacterial consortium, respectively at 1% oil concentration while it reaches to 46.73, 45.016 and 61.04% when immobilized on rice straw at the same conditions. These results illustrate that the bacterial consortium of sp48 and M12 strains are most potent for oil degradation than individual bacterium. Besides the best carrier for oil degradation is rice straw. The beneficial of employing mixed cultures consortium in bioremediation attributed to the effect of synergetic interactions among microorganisms of the consortium. It is possible that one bacterial species of the consortium removes the toxic metabolites produced by other bacterial species, that would delay activities and/or one species is able to degrade compounds others are not able to degrade or partially degrade them [26].

TABLE 1. % of oil consumption using sp48 (*Pseudomonas* sp.) and M12 (*Bacillus cereus*) immobilized individually and its consortium on different carriers.

Different carriers	% of oil consumption			
	Physically*	Biologically** by immobilized		
		sp48	M12	Bacterial consortium
Rice straw	88.049	46.73	45.016	61.04
Cane leaves	84.96	45.131	42.95	53.67
Wood chips	55.6	29.54	37.39	41.5
Corn leaves	81.44	33.147	39.96	60.26
Foam	98.03	15.5	12.54	18.42
Wax	96.37	0	0	0
Free cells	-	28.8	36.8	39.4

*Physically: means adsorptions of oil on the surface of carrier.

**Biologically: means degradation of oil by bacteria immobilized on carrier without the effect of oil adsorption on the surface of carrier.

The characteristics of suitable carrier materials should preferably include the following; the carrier should not be soluble, exhibit high stability, not readily degraded, be a natural product, easily available and economical [16, 27]. And these characteristics were found in the selected natural carriers specially rice straw which represents as an agriculture waste and makes a serious problem in the environment by burning. This became a controversial source of visible pollution because of the high visibility and potential hazard of the smoke. Hence, rice straw was selected in the immobilization of bacterial consortium in subsequent experiments in this work.

There are several studies reporting that immobilized cells showed a faster degradation rate than free cells [28-31]. The better and faster degradation rate observed was most likely due to the high immobilization efficiency of the cells onto the immobilization material and the high attraction between the hydrophobic immobilization material and the substrates. These lead to improving availability of the substrates for the cells and a better contact between the substrates and the immobilized cells, synergistically increasing the degradation rate [18, 29-33].

Investigation the optimal immobilization conditions

By testing different consortium preculture concentrations, it was found the % of oil degradation increased to 59.8% with the increasing the bacterial preculture concentrations 2, 3, and 4%. On the other hand by increasing the culture concentration over 4%, the % of oil degradation reaches to steady state as shown in Fig. 1. From these results the preculture concentration 4% (2 mL culture: 1.5 mL distal water; in 50 mL medium) was selected for the further work. Similarly Arafa et al [33] studied different inocula intervals (2%, 4% and 6%) and found that the biodegradation rate of oil sludge reaches to maximum amount 89.8% using 6% inoculum size of bacterial consortium after 12 days.

On the other hand by studying different bacterial incubation time with rice straw, the results showed that when the carrier material was incubated for 3 h, the % of oil degradation reaches to 54%. No significant increase in oil degradation was observed at longer incubation time (Table 2). The optimum incubation period necessary for the development of a well-established formulation was determined to be 3 h. Similar observation was reported by Farag et al [32] by using isolate AQ1 (*Candida tropicalis*) immobilized by thin and thick wood chips the % of oil consumption by immobilized AQ1 at incubation time 3 h reached to 74 and 64.2 respectively.

Furthermore Quek et al [13] reported that a well-established formulation could be generated by incubating polyurethane foam with a bacterial culture for 96 h. A longer incubation period is inconvenient and less economical for the generation of a formulation. Other results showed that F9 cells could stably attach and be immobilized onto the carrier material (chitosan) within 4 h [23]. The electronic attraction between the negatively charged bacterial surfaces and the positively charged chitosan might explain why a shorter incubation period was sufficient for attachment. This shorter incubation period may make this compound carrier material advantageous in future scale-up for industrial production [23].

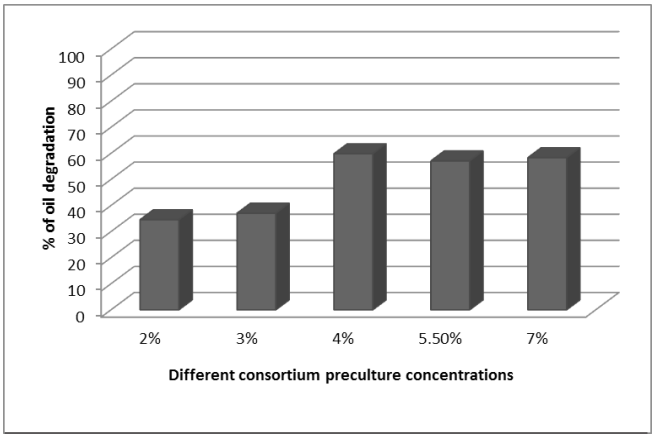

Fig. 1. % of oil degradation using different concentrations of consortium. The ratio of culture to distilled water: (2%; 1: 2.5 mL); (3%; 1.5: 2 mL), (4%; 2: 1.5 mL), (5.5%; 2.75: 0.75 mL), (7%; 3.5 mL culture) in 50 mL medium.

The results of enumeration of viable cells immobilized on rice straw showed that, when the carrier material was incubated for 3 h, the number of immobilized viable cells reached 6 X 109 CFU/ gm. An increase in the number of viable cells at longer incubation periods was insignificantly affected oil removal as represented in Table 2. This result ensures that the optimum incubation period necessary for the development of a well-established formulation was 3 h. Similar result was represented by Dengyong et al [23], where the carrier material was incubated with the F9 culture for various periods of time, and the viable cells immobilized on the carrier material were counted. His results showed that when the carrier material was incubated for 4 h, the number of immobilized viable cells reached 5 X 10^9 CFU/g. No significant increase in the number of viable cells was observed at longer incubation periods.

Assessment of immobilized bacterial consortium efficiency

The results showed that with increasing the concentration of crude oil the removal percentage decreased which could be rendered to the slow rate of biodegradation or the inhibitional effect of higher crude oil concentrations on the testing bacteria. The immobilized bacterial consortium on rice straw is more efficient than that immobilized in the foam. The percentages of biological oil removal using bacterial consortium immobilized on rice straw and foam at 1% oil concentration reach 61.6% and 15.4%, respectively and the percentage decreased to 29.8% and 12.5%, respectively at 4% oil concentration.

The combination of both techniques biological/ physical oil removal showed that the oil removal reaches a maximum percentage 97.14% and 98.2% using rice straw and foam respectively at 1% oil concentration and the percentage decreased slowly to reach 92.3% and 89.37% when using rice straw and foam respectively at 4% oil concentration. These results due to the synergistic effect of two techniques together as shown in Table 3. We conclude that the combination of two techniques in case of rice straw as carrier is better than foam as carrier due to higher efficiency of biological degradation by bacterial consortium when immobilized on rice straw than foam**.**

Biologically means: degradation of oil by bacteria immobilized on carrier without the effect of oil adsorption on the surface of carrier. Biologically& physically means: degradation of oil by bacteria immobilized on carrier in addition the effect of oil adsorption on the surface of carrier.

Tracking of crude oil degradation

Monitoring of crude oil removal rate biologically along 12 days incubation time is the best way to measure bioremediation efficiency of immobilized bacterial consortium. The results represent that by increasing time the biodegradation rate of crude oil increased and reach 61, 68.49, 71.3% after 6, 9, 12 days respectively as shown in Fig. 2. No significant increase in degradation was observed after 9 days. Similar observation was reported by Dengyong et al [23], who indicated that the majority of the diesel oil was degraded within the first 7 days using immobilized F9 cells on chitosan. Also in other study they were found that the rate of biodegradation of total petroleum hydrocarbon (TPH) was high during the first 7 days of incubation reaching to 54% and then decreased thereafter reaching to 78% TPH removal within 24 days of incubation period by using a consortium of degrading bacteria [26].

TABLE 2. % of oil degradation and viable cell counts at different incubation time of bacterial consortium with rice straw.

Different incubation time of culture with carrier by hour (h)	% of oil degradation	CFU/g dry formulation
1	49.04	5.8 X10^9
2	49.2	5.9 X10^9
3	53.37	6 X10^9
4	54.13	6.08 X10^9
5	54.27	6.16 X10^9

TABLE 3. % of oil consumption using immobilized consortium at different oil concentrations.

Different oil Concentrations	% of oil removal using rice straw		% of oil removal using foam	
	Biologically	Biologically & physically	Biologically	Biologically & physically
1%	61.6	97.14	15.47	98.2
2%	46.67	95.59	15	91.95
3%	37.1	93.6	14.25	91.15
4%	29.8	92.3	12.59	89.37

Biologically means: degradation of oil by bacteria immobilized on carrier without the effect of oil adsorption on the surface of carrier. Biologically& physically means: degradation of oil by bacteria immobilized on carrier in addition the effect of oil adsorption on the surface of carrier.

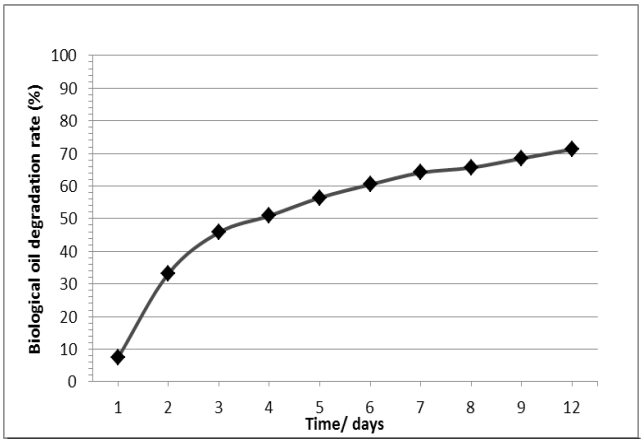

Fig. 2. Crude oil degradation rate using immobilized bacterial consortium.

Microscopic examination by Scanning Electron Microscope (SEM)

The morphology of the rice straw was examined under scanning electron microscope (Fig. 3 A). The results revealed that the rice straw is a porous carrier. The pores of the carrier material provided suitable and protective niches for bacteria. The bacterial cells were attached to the walls of the pores (Fig. 3 B) the pores may protect immobilized bacterial cells from antagonistic environmental factors. Figure (3 C) exposes that the bacterial diameter approximately (1.9- 1 µm) and (5.5- 0.8µm) for bacterial consortium (strains sp48 and M12). Figure (3 D) shows the attachment of crude oil on the surface of rice straw.

Optimization of oil removal by immobilized bacterial consortium in bioreactor

The assessments of oil removal using bacterial consortium immobilized on rice straw in a bioreactor at different times were represented in Fig. 4, which shows that the % of oil consumption increased by increasing the time in the two cases biologically and biologically/ physically which reach 63% and 90% respectively after 12 days. This indicates that the % of consumption increased considerably in case of biologically/ physically than biologically alone.

The behaviors depicted in Fig. 5 represent that the % of oil degradation biologically increased to 56.2% with increasing the rice straw weight to 1.6 gm after 6 days of incubation. As well by increasing the rice weight the % of oil consumption biologically/ physically increased and reaches to 87.2% when using 1.6 gm. From this result the rice weight 1.6 gm was selected for the further work which gives a comparable result instead of increasing the time.

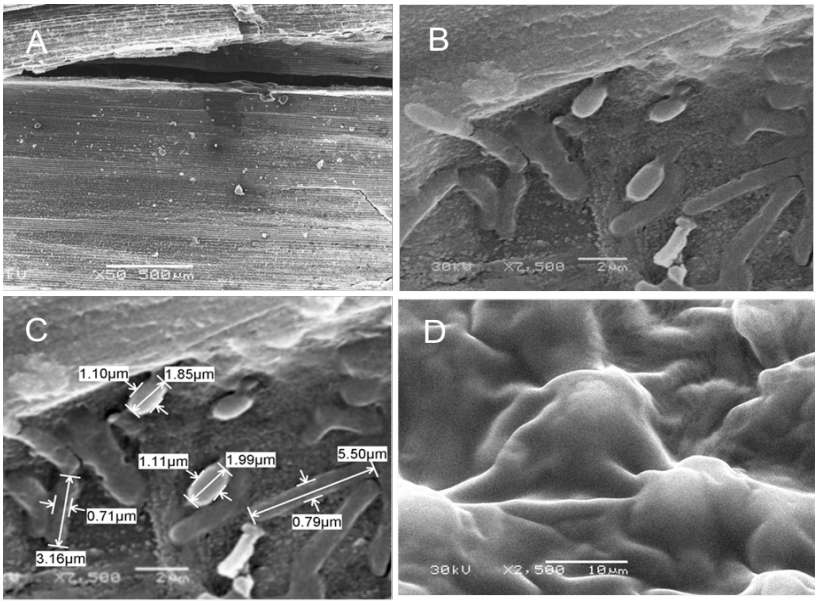

Fig. 3. Scanning Electron Micrograph (SEM), A: Structure of rice straw particles at X50,500; B, C: Bacterial consortium immobilized on rice straw surface at X2,500, D: Attachment of crude oil on the surface of rice straw as a carrier material at X2,500.

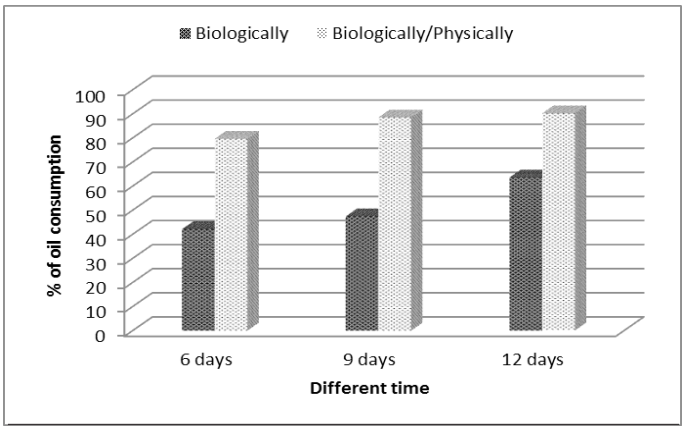

Fig. 4. Crude oil consumption % biologically and biologically/ physically at different time of incubation.

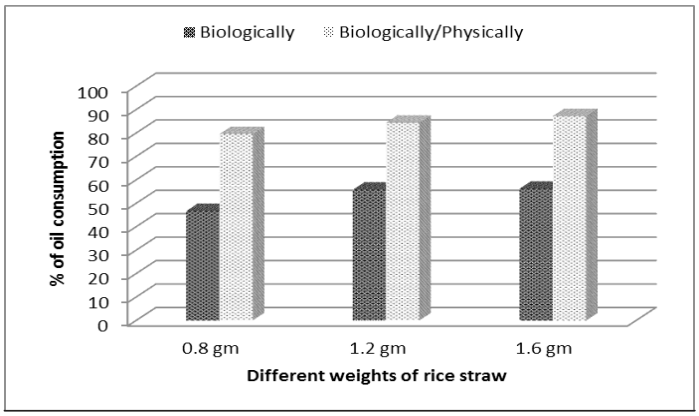

Fig. 5. Crude oil consumption % biologically and biologically/ physically at different weights of rice straw.

Efficiency of immobilized bacterial consortium under continuous addition of oil in bioreactor

The experimental data revealed that the % of oil consumption biologically/ physically decreased with continuous adding of 1% crude oil from 87.3% to 83.28% and 81.27% after three times of addition and the incubation time interval was 3 days. This indicates the great ability of immobilized bacteria to remove oil, so it can be applied in nature and give a good result.

The same result was represented by Qingxin et al [34] which showed that the immobilized cells of strain *Bacillus* sp. M-12 could be reused for many cycles. The immobilized M-12 by (polyvinyl alcohol) PVA could be used for more than seven cycles to treat waste water using a semi-continuous operation system. In each cycle, when the COD removal reached about or more than 70%, the waste water in the flask was removed and the immobilized cells retained. New waste water was then added into the flask and the COD of the waste water reduced from 2600 to 240 mg/L. Similarly Nichakorn et al [35] reported that the effectiveness of chitosan-immobilized cells (*Sphingobium* sp. P2) was investigated in a semi-continuous treatment system, where the stock oil/water emulsion of PTTV120 lubricant was added daily to the synthetic wastewater to maintain the initial lubricant concentration of 200 mg/L. The concentration of TPH in the control treatment increased daily and equaled 996± 8 mg/L at the end of study, this value corresponded to the total amount of added lubricant. In contrast, the lubricant was significantly removed from the systems with chitosan-immobilized cells, killed-immobilized cells and free cells, where the amounts of residual TPH were 214±4, 450±2 and 605±4 mg/L at the end of study, respectively. The efficiency of lubricant degradation was maximum at chitosan-immobilized cells.

Conclusions

Crude oil biodegradation by immobilized bacterial consortium is considered a valid process and has a high positive impact in oil environmental contaminants removal. In this study the rice straw represents the best carrier for bacterial immobilization. By studying different immobilization conditions as bacterial concentrations, incubation time with carrier, and % of oil concentration. It was found that 4% bacterial preculture, 3h incubation time, and 1% oil concentration are the finest conditions where oil removal reached to 61.6% and 97.14% biologically and biologically/ physically, respectively. By applying the same conditions in a small scale bioreactor the crude oil degradation by bacterial consortium reached to 56.2% and oil consumption biologically/ physically reached to 87.2% while three times addition of 1% oil concentration the removal reached to 81.27%. All these approaches proof that the immobilization process more applicable and environmentally viable mitigation technology.

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References

1. Gesinde A.F., Agbo E.B., Agho M.O. and Dike E.F.C., Bioremediation of some Nigerian and Arabian crude oils by fungal isolates, *International Journal of Pure and Applied Sciences,* **2** (3), 37-44 (2008).

2. Law A.T. and Button D.K., Multiple-carbon-source limited growth kinetics of a marine cory form bacterium, *Journal of Bacteriology,* **129** (1), 115-123 (1977).

3. Del'Arco J.P. and de França F.P., Biodegradation of crude oil in sandy sediment, *International Biodeterioration & Biodegradation,* **44**, 87-92 (1999).

4. Xu R., Lau N.L.A., Ng K.L. and Obbard J.P., Application of a slow-release fertilizer for oil bioremediation in beach sediment, *Journal of Environmental Quality*, **33**, 1210-1216 (2004).

5. Madigan M.T., Martinko, J.M. and Parker, J., *Brock Biology of Microorganisms*, ninth ed. Prentice-Hall International Ltd., London (2000).

6. Hii Y.S., Law Ah.T., Shazili N.A.M., Abdul-Rashid M.K. and Lee C.W., Biodegradation of Tapis blended crude oil in marine sediment by a consortium of symbiotic bacteria, *International Biodeterioration & Biodegradation,* **63**, 142-150 (2009).

7. Ito H., Hosokawa R., Morikawa M. and Okuyama H., A turbine oil-degrading bacterial consortium from soils of oil fields and its characteristics, *International Biodeterioration & Biodegradation,* **61**, 223-232 (2008).

8. Li X., Li P., Lin X., Zhang C., Li Q. and Gong Z., Biodegradation of aged polycyclic aromatic

hydrocarbons (PAHs) by microbial consortia in soil and slurry phases, *Journal of Hazardous Materials*, **150** (1), 21-26 (2008).

9. Radwan S.S., Al-Hasan R.H., Ali N., Salamah S. and Khanafer M., Oil-consuming microbial consortia floating in the Arabian Gulf, *International Biodeterioration & Biodegradation,* **56**, 28-33 (2005).

10. William P.S., *Review of Oil Spill Responses on Moderately-sized Spills in US Waters from 1993-2000.* Regional Citizens; Advisory Council, Elise De Cola (2001).

11. Ro K. S., Breitenbeck G. A. and Ghalambor A., Composting Technology for Practical and Safe Remediation or Oil Spill Residuals. Louisiana Oil Spill Coordinator's Office/Office of governor. Louisiana Applied Oil Spill Research and Development Program, Baton Rouge, Louisiana. *Technical Report Series*, 97-009 (1998).

12. Gonzalez E.C., Avelizapa L.I.R., Camarillo R.C. and Avelizapa N.G.R., Effect of keratinous waste addition on improvement of crude oil hydrocarbon removal by a hydrocarbon-degrading and keratinolytic mixed culture, *International Biodeterioration & Biodegradation,* **63**, 1018-1022 (2009).

13. Quek E., Ting Y.P. and Tan H.M., *Rhodococcus* sp. F92 immobilized on polyurethane foam shows ability to degrade various petroleum products, *Bioresource Technology,* **97,** 32-38 (2006).

14. Van V.J.A., Van O.L.S. and Van E.J.D., Fate and activity of microorganisms introduced into soil, *Microbiology and Molecular Biology Reviews,* **61**, 121 – 135 (1997).

15. Mohsen R.M., Abdel-Mohsen F.F., Deghiedy N.M. and Abu-Ayana Y.M., Review on the Manufacture of Particleboard from Agro-Wastes Using Different Adhesives, *Egyptian Journal of Chemistry,* **57**, 165-176 (2014).

16. Leenen E.J.T.M., Dos Santos V.A.P., Grolle K.C.F., Tramper J. and Wijffels R., Characterisitics of and selection criteria for support materials for cell immobilization in wastewater treatment, *Water Research,* **30**, 2985-2996 (1996).

17. Gentili A.R., Cubitto M.A., Ferrero M. and Rodriguez M.S., Bioremediation of crude oil polluted seawater by a hydrocarbon degrading bacterial strain immobilized on chitin and chitosan flakes, *International Biodeterioration & Biodegradation,* **57**, 222-228 (2006).

18. Wilson N.G. and Bradley G., Enhanced degradation of petroleum (slovene diesel) in an aqueous system by immobilized Pseudomonas fluorescens, *Journal of Applied Microbiology,* **80**, 99-104 (1996).

19. Obuekwe C.O. and Al-Muttawa E.M., Self-immobilized bacterial cultures with potential for application as ready-to-use seeds for petroleum bioremediation, *Biotechnology Letters,* **23**, 1025 -1032 (2001).

20. Oh Y.S., Maeng J. and Kim S.J., Use of microorganism immobilized polyurethane foams to absorb and degrade oil on water surface, *Applied Microbiology and Biotechnology,* **54**, 418-423 (2000).

21. Law A.T. and Teo K.S., Oil biodegradation in the Straits of Malacca: Phenanthrene degradation by AR-3, *Journal of Marine Biotechnology,* **5**, 162-167 (1997).

22. Giorgio T. and Jan H. C. A., Tucker model based approach for analysis of complex oil biodegradation data, *Journal of Chromatography A*, **1216**, 7865-7872 (2009).

23. Dengyong H., Xianrong S., Qun L., Ying H., Qingrong W. and Qiong L., Enhancement of the diesel oil degradation ability of a marine bacterial strain by immobilization on a novel compound carrier material, *Marine Pollution Bulletin*, **67,** 146-151 (2013).

24. Mang L., Zhongzhi Z., Shanshan S., Qinfang W. and Weizhang Z., Enhanced degradation of bioremediation residues in petroleum-contaminated soil using a two-liquid-phase bioslurry reactor, *Chemosphere*, **77**, 161-168 (2009).

25. Nawaz M.S., Billedeau S.M. and Cerniglia C.E., Influence of selected physical parameters on the biodegradation of acrylamide by immobilized cells of *Rhodococcus* sp., *Biodegradation*, **9**, 381-387 (1998).

26. Amer R., El-Gendy N. Sh., Taha T., Farag S. and Abdel Fattah Y., Biodegardation of crude oil and its kinetics using indigenous bacterial consortium isolated from contaminated area in Egyptian Mediterranean ecosystem, *Jokull Journal*, **64** (4), 42-58 (2014).

27. Lorda G.S. and Balatti A.P., Designing media I. Production of high cell concentrations of Rhizobium and Bradyrhizobium. In *Legume Inoculants. Selection and Characterization of Strains Production, Use and Management,* eds Balatti, A.P. & Freire, J.R.J. pp. 78±93. Editorial Kingra, Buenos Aires (1996).

28. Wang J. and Qian Y., Microbial degradation of 4-chlorophenol by microorganisms entrapped in carregeenan-chitosan gels, *Chemosphere*, **38**, 3109-3117 (1999).

29. Yamaguchi T., Ishida M. and Suzuki T., An immobilized cell system in polyurethane foam for the lipophilic micro-alga Protothecazopfii, *Process Biochemistry,* **34**, 167-171 (1999).

30. Na K., Lee Y., Lee W., Huh Y., Lee J., Lee J., Kubo M. and Chung S., Characterization of PCB-degrading bacteria immobilized in polyurethane foam, *Journal of Bioscience and Bioengineering,* **90**, 368-373 (2000).

31. Diaz M.P., Boyd K.G., Grigson S.J.W. and Burgess J.G., Biodegradation of crude oil across a wide range of salinities by an extremely halotolerant bacterial consortium MPD-M, immobilized onto polypropylene fibers, *Biotechnology and Bioengineering,* **79**, 145-153 (2002).

32. Farag S., Soliman N.A. and Abdel-Fattah Y.R., Optimization of Immobilization Conditions for Petroleum Oil Biodegradation by Candida tropicalis AQ1 using Wood Chips and Wax, *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, **7**(5), 200-210 (2016).

33. Arafa A., Farag S., Mahdaly M.A. and Badr M.M., Biodegradation of Petroleum Industry Oily Sludge by Bacterial Consortium and Its Application in Land Farming, *Pakistan Journal of Biotechnology,* **13** (1), 1-11 (2016).

34. Qingxin L., Congbao K. and Changkai Z., Waste water produced from an oil field and continuous treatment with an oil-degrading bacterium, *Process Biochemistry,* **40**, 873- 877 (2005).

35. Nichakorn K., Sitti T., Onruthai P., Sorawit P., Thawach C., Chalermchai R. and Ekawan L., Airlift bioreactor containing chitosan-immobilized Sphingobium sp. P2 for treatment of lubricants in wastewater, *Journal of Hazardous Materials*, **213**, 466-473 (2012).

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تحسين التكسير الحيوي للنفط الخام بواسطة تجمع بكتيري محمل بأستخدام مفاعل حيوى صغير بنظامى التشغيل ثابت المحتوى والمستمر

سهى فرج[1], نادية سليمان[2], ياسر عبد الفتاح[2]
[1]قسم التكنولوجيا الحيوية البيئية - معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية - مدينة الأبحاث العلمية والتطبيقات التكنولوجية - الإسكندرية - مصر.
[2]قسم تطوير الصناعات- معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية - مدينة الأبحاث العلمية والتطبيقات التكنولوجية- الإسكندرية - مصر.

انسكابات النفط إلى جانب التخلص من منتجات النفط وألقائها مباشرة في البيئة هي من المصادر الرئيسيه لتلوث الطبيعة مما يسهم في التلوث البيئي والمشاكل الصحية. أولاً لحل هذه المشكلة ، تم تحميل مجموع بكتيرى قادر على تكسير النفط على حوامل مختلفة (المجموع البكتيرى مكون من بكتيريا Pseudomonas sp. sp48 and Bacillus cereus M12 معزوله من عينات تربة من منطقة بحرى وسيدى كرير بالتتابع) ، وتم اختيار قش الأرز كأفضل مادة لتحميل البكتريا القادرة على تكسير النفط. وقد تم أجراء تحسين لخواص المجموع البكتيري فى أزالة النفط عن طريق اختبار كميات مختلفة من البكتريا (2 ، 3 ، 4 ، 5.5 ، 7٪) للتحضين مع قش الأرز، وتجربة وقت حضانة مختلف مع قش الأرز (5-1 ساعة) ، وعد الخلايا المحملة وأختبار تركيزات النفط المختلفة (1 ، 2 ، 3 ، 4٪). وقد وجد أن تكسير النفط الخام (تركيز 1٪) يصل إلى 61.6٪ بسبب العمل البيولوجي فقط بينما يرتفع إلى 97.14٪ بواسطة الاندماج الثنائي بين التأثير البيولوجي والفيزيائي للتخلص من النفط بعد 6 أيام من التحضين عند تطبيق الظروف المثلى وهى (4٪ مجموع بكتيري وفترة حضانة 3 ساعات مع قش الأرز). وأخيراً أجريت دراسة تطبيقية باستخدام الظروف المثالية في مفاعل حيوي صغير ثابت المحتوى وآخر مستمر لمعالجة المياه الملوثة بالنفط في نظام مفتوح. وقد أوضحت النتائج أن تكسير النفط بالطرق البيولوجية فقط أوالطرق البيولوجية والفيزيائية يصل الى 56.2% و 87.2% بالتتابع. بينما الأضافة المستمره للنفط بتركيز 1% لثلاث مرات فأن أزالة النفط تصل الى 81.27%. وهذه التقنية من التحميل تعد طريقة واعدة لحل مشكلة التلوث بالنفط بسبب التأثير المشترك لكلاً من الطرق الفيزيائية والبيولوجية.