



RESEARCH ARTICLE

STUDIES ON ALGINATE-BASED MACRO-ENCAPSULATION (IMMOBILIZATION) CAPSULES FOR CRUDE OIL DEGRADATION UNDER MICROBIAL CONSORTIUM CONDITIONS

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Abstract

Oil hydrocarbons are the most well-known natural poisons on the planet and oil slicks represent an incredible danger to earthly and marine environments. Oil contamination may emerge either coincidentally or operationally at whatever point oil is created, shipped, put away, and handled or utilized adrift or ashore. Oil slicks are a significant danger to nature as they seriously harm the encompassing biological systems. In this investigation, to improve the endurance and maintenance of the bioremediation specialists in the polluted locales, bacterial cells were immobilized. Immobilized cells are generally tried for oil debasement and degradative protein creation. There are numerous kinds of help and immobilization strategies that can be chosen dependent on such an application. We have contemplated the capability of immobilized microbial cells to corrupt oil hydrocarbons in oil. It also enhanced degradation with immobilized cells compared to a mixture of encapsulated bacteria for the treatment of oil and enzyme production. Encapsulated microbial consortium showed maximum (98%) followed by and ES10, HDUL4 (95%) of oil degradation. It was demonstrated that immobilized cell to be effective and is better, faster, and can be occurred for a longer period.

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Introduction:-

Oil hydrocarbons are one of the regular instances of these synthetics, which enter the earth now and again and in enormous volumes through various pathways [1]. Oil pollution has become a worldwide issue in industrialized and creating nations. It is one of the most hazardous contamination factors known today. It can make a danger to the earth. It is very dreaded by environmentalists and it's exceptionally difficult to control on the off chance that it spills out [2, 3]. There are many techniques for rewarding oil-contaminated locales, for example, mechanical and compound strategies, yet these strategies, by and large, are costly and have constrained adequacy. Then again, bioremediation is the promising innovation for the decrease of these oil toxin regions since it is cost-effective and will result in to finish mineralization. Bioremediation is a procedure that debases ecological contamination by microorganisms [4, 5]. Over the most recent couple of years, the utilization of biotechnological forms that involving microorganisms with the target of taking care of natural contamination issues is quickly growing. The analysts have demonstrated that natural technique is flexible, high soundness, wide applications in different zones, practical and productive for the remediation of petroleum [6]. One of the key points for bioremediation is keeping up high biomass of bacterial populaces. To improve the endurance and maintenance of the bioremediation specialists in the debased destinations, bacterial cells must be immobilized. Immobilized cells have been broadly utilized in the

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creation of helpful synthetic compounds, treatment of wastewaters, and bioremediation of contamination reason for its more extended working lifetime and upgraded dependability and endurance of the cells [7, 8]. The utilization of immobilized cells has been examined as an elective innovation for natural applications. These biocatalysts can offer the chance of a more extensive and increasingly prudent misuse in industry, squander treatment, medication, and improvement of bioprocess and observing gadgets like the biosensor [9]. Immobilization is a characteristic wonder existing in the globe. Radwan et al. [10] have given proof that the immobilization guideline is now found in nature, as microalgal tests gathered along the Gulf coast were secured by biofilms of oil using microscopic organisms that help debase hydrocarbons found in seawater. Exemplification is another irreversible procedure like ensnarement. This strategy can be accomplished by encompassing the organic parts inside different types of round semi-porous layers with a specific controlled penetrability [11].

Bioremediation of raw petroleum utilizing immobilized cells is seldom examined. The entirety of the techniques for immobilization, for example, Adsorption, Covalent Binding, Entrapment, and Encapsulation were gone after for bioremediation of unrefined petroleum. The high immobilization productivity of the cells onto the immobilization material and the high proclivity between the hydrophobic immobilization material and the substrates caused great corruption. Expanding accessibility of the substrates for the phones and superior communication between the substrates and the immobilized cells synergistically brought about building up the corruption rate [12]. Omar and Rehm [13] exhibited that *Candida parapsilosis* and *Penicillium* frequent when immobilized on granular mud in sections, adequately corrupted n-alkanes. They saw that residuals of C12 to C18 alkanes in immobilized bacterial cells framework are 13.4 to 32.3% while in free bacterial cells framework is 85.9 and 98.9%. Davis and Westlake [14] announced that the immobilization of cells onto dormant surfaces expanded accessible surface territory to encourage the development of biomass and improve the corruption rate. Obuekwe and M. Al-Muttawa [15], and *Arthrobacter* sp. what's more, a Gram-negative bacillus disengaged from Kuwait oil lakes, and then these microscopic organisms brooded with sawdust, Styrofoam or wheat grain, as transporters, under low supplement conditions, stable exopolysaccharide intervened immobilized societies were shaped. The creators tried the capacity to endure and corrupt hydrocarbons for about a month and a half at 45°C. Suspensions of free cells corrupted less raw petroleum than newly immobilized cells. Immobilization hydrocarbons degrader's microscopic organisms can tidy up oil painting and can be encouraging oil biodegradation in contaminated conditions. In some of the studies detailed that immobilized cells contrasted and free-living microorganisms progressively successful, have a longer period of usability, lower cost, and higher raw petroleum corrupting movement in different zones.

Methodology:-

The inoculum was prepared in a 10-ml Erlenmeyer flask containing 9,8 ml of medium and 0, 2 ml of pre-culture of selected isolates along with consortium. The flask was incubated overnight at 30°C, on a rotating shaker at 120 rpm. This culture was centrifuged for 10min at 8 720 x g at 4°C. The cells were then washed thoroughly with 10ml 0.8% of NaCl solution and resuspended in 1ml 1% peptone solution [16].

Macro encapsulation process

The encapsulation matrix is a mix of sodium alginate and starch and sterilized separately as a dry powder in the autoclave at 121°C for 20 min before dispersion in distilled water. Sodium alginate was first dissolved in water for 30 minutes, followed by the addition of modified and standard starch. Then, the cells were added into 30 ml of encapsulating matrix solution and mixed homogeneously. The mixture was introduced in a syringe and placed on the encapsulation device and extruded drop by drop through the needle (1.55 mm) by acting the syringe pump at the rate of 120 ml/h. The whole experiment is done under aseptic conditions in a laminar airflow hood. The drops fell directly in a 1.5% CaCl₂ solution for reticulation. After 30 minutes, the minimum time required for total reticulation, the microcapsules (about 5-6mm diameter) are washed 3 times with sterile tape water before drying. The cell concentration was determined during each step of the encapsulation process from the bacteria culture, centrifugation, mixing of the culture with the polymer, transfer from beaker to the encapsulating device, capsules production and drying [17].

Biodegradation of Crude Oil

Biodegradation Assay:

The individual and mixed bacterial consortiums from the overnight culture at the log phase of growth were transferred to 250mL conical flasks, each containing 100mL of sterile mineral salts medium with 1% (v/v) crude oil. The experiment was carried out in duplicate and uninoculated flasks constituted the controls, accounting for abiotic

losses. All flasks were incubated at 30°C for determining intervals of time (1 to 7 days). Residual concentrations of crude oil were determined gravimetrically analysis [18].

Gravimetric Analysis:

The whole content of each flask was taken at the end of each incubation period to assess residual concentrations of crude oil. The extraction was carried out by chloroform (3 sample: 1 chloroform). Sample with chloroform was placed in a separating funnel with continuous shaking, after which the contents were allowed to settle; two layers were formed: watery layer and chloroform layer containing the residual hydrocarbons. The last layer was decanted and air-dried. After chloroform evaporation, the residual oil was quantified gravimetrically (the consumed oil was calculated by subtracting the residual hydrocarbons from the original weight of hydrocarbons).

Extraction of degradation enzymes

In this method described by Rahman et al. (2002) with slight changes modifications selected macro to encapsulate cultures were transferred to 500 ml of Erlenmeyer flask, containing 100 ml of MSM with 1% (v/v) filter-sterilized crude oil as carbon resource. An un-inoculated flask was used as a control to monitor the abiotic loss of the crude oil substrate. The flasks were incubated at 37 °C for 7 days at 200 rpm. All the experiments were performed in triplicate. For determination of enzyme activity, cells were harvested every day by centrifugation at 10000 rpm at 4°C for 10 min and then used for enzyme assays.

Alkane Hydroxylase Activity

In this method, the collected macro encapsulated cultures were used for testing of alkane hydroxylase activity. 1 ml of testing solution contained 20 mM Tris-Hydrochloride and 0.15% CHAPS buffer (pH 7.4), 0.1 mM of Nicotinamide adenine dinucleotide (NADH), 10µL of hexadecane mixture (1% hexadecane diluted with 80% DMSO) and 50 µL of crude oil. The reaction was started by adding of 10µL of hexadecane mixture. The activity of the alkane hydroxylase was expressed as 1mM of NADH oxidized per minute. Absorbance was measured at 340 nm using a UV-Vis spectrophotometer.

Alcohol Dehydrogenase Activity

In this method, the collected macro encapsulated cultures were used for the assay. 1 ml of reaction solution contained 1 M of Tris- Hydrochloride buffer (pH 8.8), 4 mM of NAD⁺, 100 µL of ethanol (99% pure), and 50 µL of crude oil. The activity of the enzyme alcohol dehydrogenase was recorded as 1 mM of NADH formed per minute. Absorbance was measured at 340 nm using a UV-Vis spectrophotometer.

Results and Discussion:-

The total cell number did not change significantly during the first step of centrifugation and washing. It increased during the second step (mixing of the matrix with bacteria), due to bacteria multiplication that can be explained by the presence in the medium of high concentration of carbon source (starch, alginate). A slight decrease was observed during the transfer step due to losses of a small quantity of matrix/bacteria mixture in the syringe and the preparation beaker. The same observation was made during bead production and this could be explained by the fact that few bacteria were lost in the CaCl₂ gelling solution (0.2%) and the washing solution (0.07%). Finally, the viable cell number decreased during drying and this decrease is normal because some bacteria could not survive the dehydration stress, especially those at the top surface of the capsules.

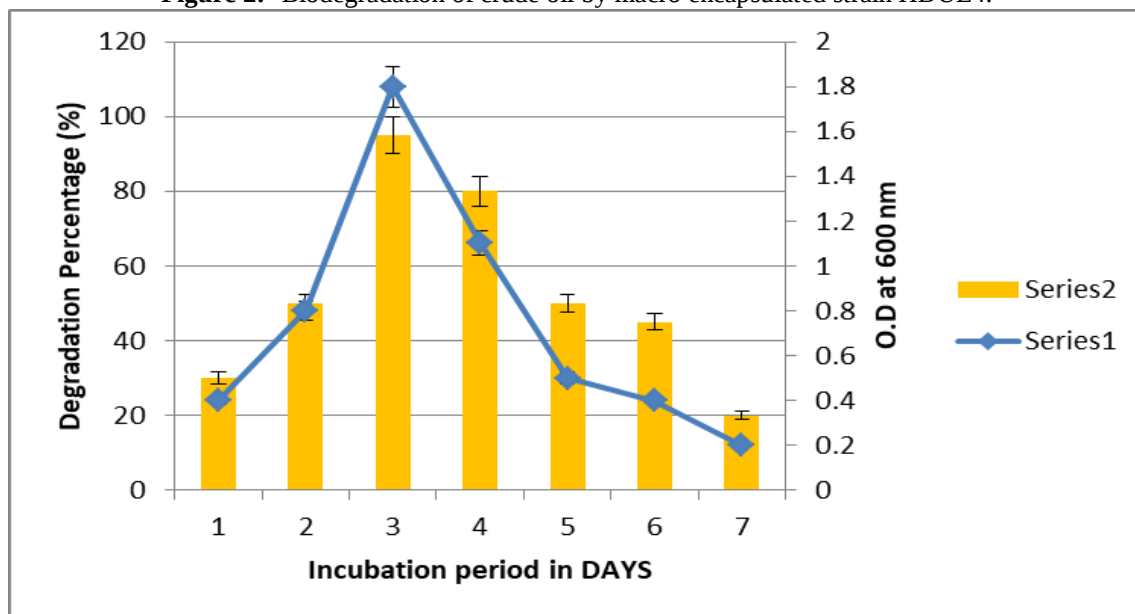
Fig 1:- Alginate based Macro encapsulation (Immobilization) capsules.



Crude oil degradation studies by different Macro encapsulated based strains

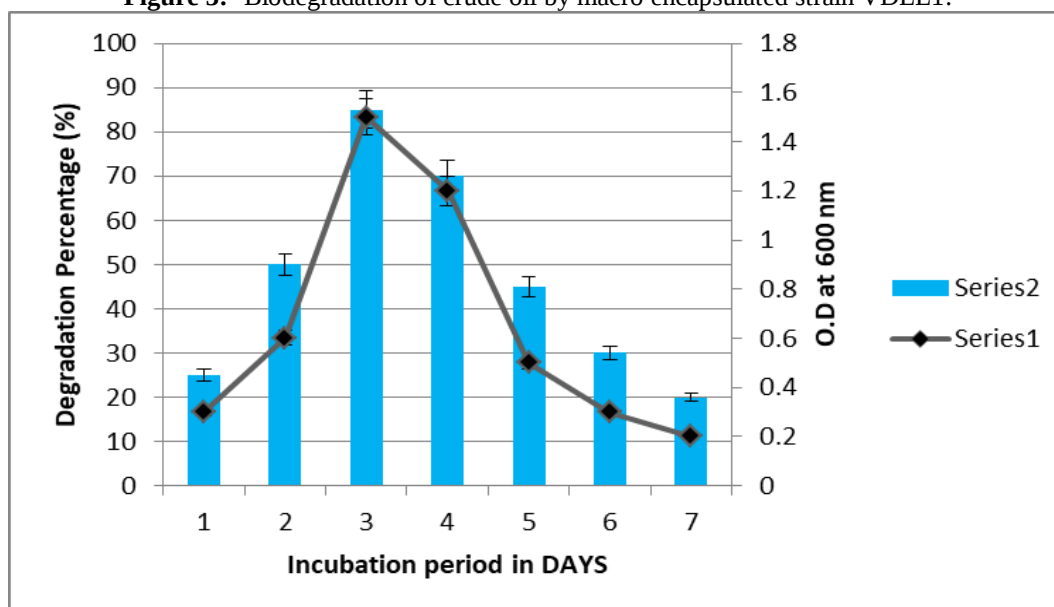
HDUL4 strain encapsulated strains have degraded crude oil up to 95% in seven days. The crude oil degradation was less on 7th day (20%). HDUL4 strain degraded crude oil in a range of 20-95% with the one week (Fig 2). Liang *et al.* [19] compared the amount of degradation of crude oil in contaminated soil with free-living bacterial cultures and activated carbon bio carrier. Results revealed that immobilization in enacted carbon bio carriers expanded the biodegradation of unrefined petroleum, bacterial populace, and complete microbial movement because of progress the oxygen, supplement mass exchange, and water holding capacity of the soil.

Figure 2:- Biodegradation of crude oil by macro encapsulated strain HDUL4.



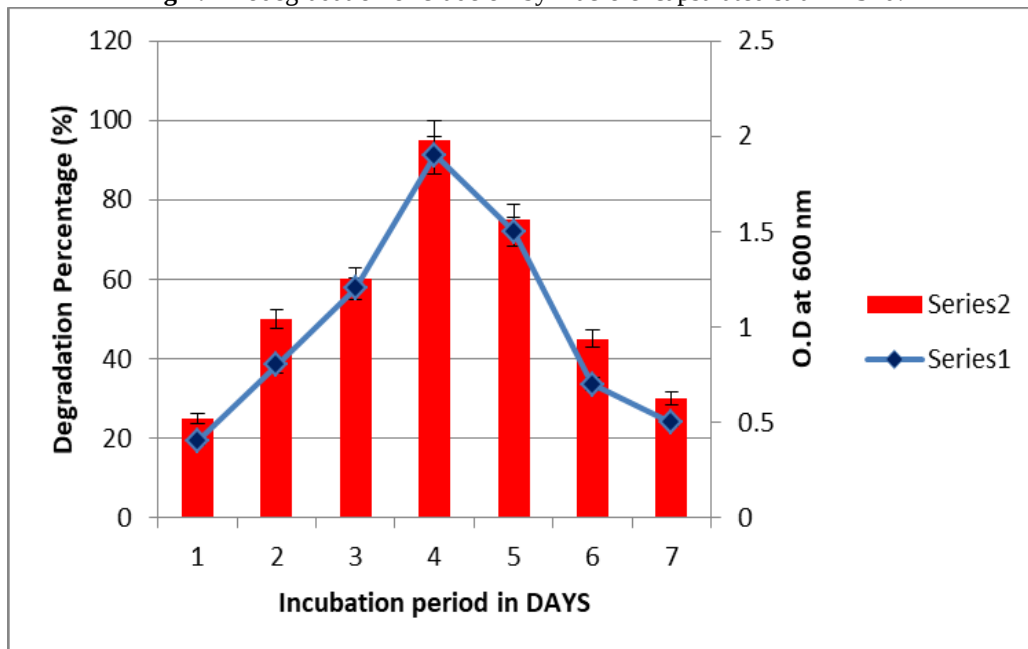
VDLL1 strain has degraded crude oil up to 85% in seven days. The crude oil degradation was less on 7th day (20%). VDLL1 strain degraded crude oil in a range of 20-85% with the one week (Fig 3). The oil-degrading capacity of the immobilized bacterial consortium in cocopeat, rice body powder, and sodium alginate cases was looked at by Nunal *et al.* [20].

Figure 3:- Biodegradation of crude oil by macro encapsulated strain VDLL1.



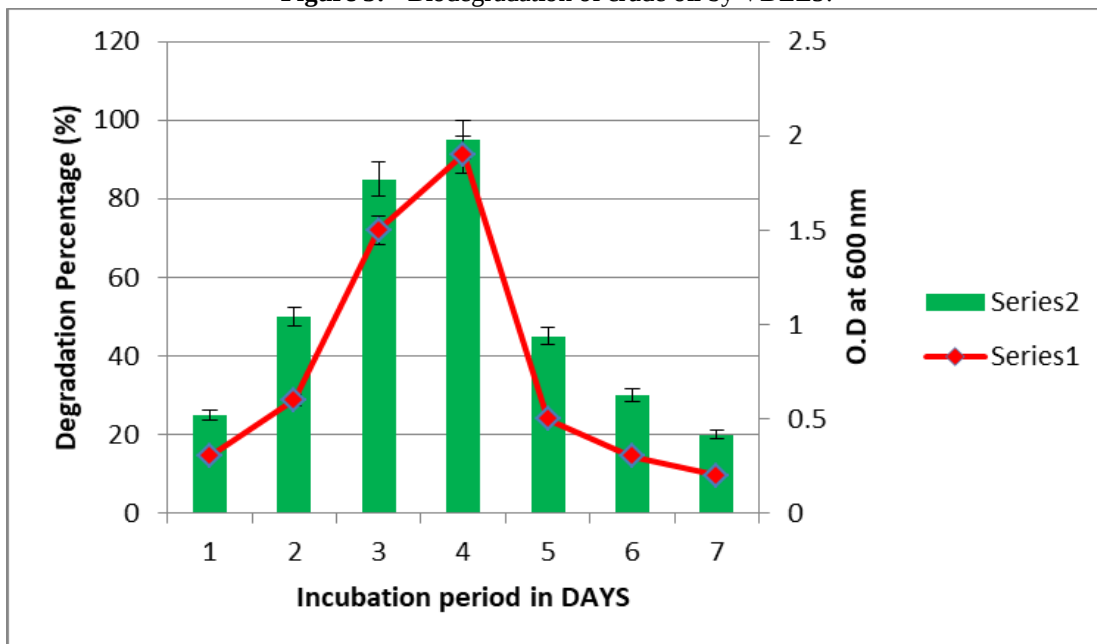
ES10 strain has degraded crude oil up to 95% in seven days. The crude oil degradation was less on 7th day (30%). ES10 strain degraded crude oil in a range of 30-90% with the one week (Fig 4). Xu and Lu [21] exhibited that oil expulsion in unrefined petroleum defiled soil was expanded by the use of hydrocarbon-degrading microbes immobilized on nut structure powder as bio carrier.

Fig 4:- Biodegradation of crude oil by macro encapsulated strain ES10.



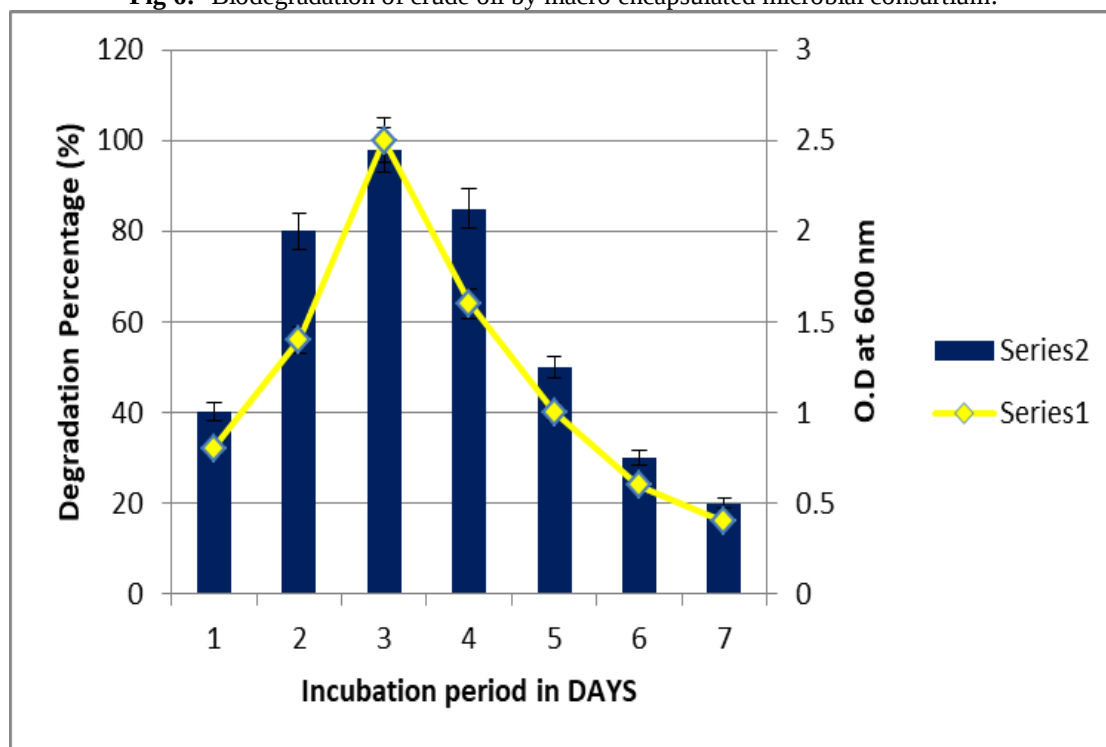
VDLL5 strain has degraded crude oil up to 95% in seven days of study. The crude oil degradation was less on 7th day (20%). VDLL5 strain degraded crude oil in a range of 20-95% with the one week (Fig 5). Diaz et al. [22] utilized immobilized the bacterial consortium MPD-M on polypropylene strands for biodegradation of raw petroleum in water with salinities differing from 0 to 180 g L⁻¹.

Figure 5:- Biodegradation of crude oil by VDLL5.



Macro encapsulated microbial consortium has degraded crude oil up to 98% in seven days of study. The crude oil degradation was less on 7th day (20%). VDLL5 strain degraded crude oil in a range of 20-98% with the one week (Fig 5). Wiesel et al. [23] observed that a blended bacterial culture immobilized on granular mud displayed great development, and exhibited proportionate debasement capability of polyaromatic hydrocarbons (PAHs) contrasted with uninhibitedly suspended cells in their model soil framework.

Fig 6:- Biodegradation of crude oil by macro encapsulated microbial consortium.



Enzyme activity of degradative enzymes produced by different encapsulated microbial strains

HDUL4 strain has produced alkane hydroxylase and alcohol dehydrogenase enzymes were studied in one week. In seven days HDUL4 showed the highest enzyme production on the 3rd day for both enzymes. HDUL4 produced 160 $\mu\text{mol/min/mg/protein}$ alkane hydroxylase and 120 $\mu\text{mol/min/mg/protein}$ alcohol dehydrogenase on 3rd day whereas HDUL4 showed very less for both enzyme productions on 7th day. The enzyme activity was in a range between 10 $\mu\text{mol/min/mg/protein}$ to 120 $\mu\text{mol/min/mg/protein}$ for alcohol dehydrogenase and Alkane hydroxylase range was between 12 $\mu\text{mol/min/mg/protein}$ to 160 $\mu\text{mol/min/mg/protein}$. VDLL1 strain has produced alkane hydroxylase and alcohol dehydrogenase enzymes were studied in one week. In seven days VDLL1 showed the highest enzyme production on the 3rd day for both enzymes. VDLL1 produced 150 $\mu\text{mol/min/mg/protein}$ alkane hydroxylase and 95 $\mu\text{mol/min/mg/protein}$ alcohol dehydrogenase on 3rd day whereas VDLL1 showed very less for both enzyme productions on 7th day. The enzyme activity was in a range between 10 $\mu\text{mol/min/mg/protein}$ to 95 $\mu\text{mol/min/mg/protein}$ for alcohol dehydrogenase and Alkane hydroxylase range was between 24 $\mu\text{mol/min/mg/protein}$ to 150 $\mu\text{mol/min/mg/protein}$. ES10 strain has produced alkane hydroxylase and alcohol dehydrogenase enzymes were studied in one week. In seven days ES10 showed the highest enzyme production on the 3rd day for both enzymes. ES10 produced 280 $\mu\text{mol/min/mg/protein}$ alkane hydroxylase and 120 $\mu\text{mol/min/mg/protein}$ alcohol dehydrogenase on the 3rd day whereas ES10 showed very less for both enzyme productions on 7th day. The enzyme activity was in a range between 35 $\mu\text{mol/min/mg/protein}$ to 120 $\mu\text{mol/min/mg/protein}$ for alcohol dehydrogenase and Alkane hydroxylase range was between 40 $\mu\text{mol/min/mg/protein}$ to 280 $\mu\text{mol/min/mg/protein}$.

VDLL5 strain has produced alkane hydroxylase and alcohol dehydrogenase enzymes were studied in one week. In seven days VDLL5 showed the highest enzyme production on the 3rd day for both enzymes. VDLL5 produced 250 $\mu\text{mol/min/mg/protein}$ alkane hydroxylase and 90 $\mu\text{mol/min/mg/protein}$ alcohol dehydrogenase on 3rd day whereas VDLL5 showed very less for both enzyme productions on 7th day. The enzyme activity was in a range between 15

$\mu\text{mol/min/mg/protein}$ to $90 \mu\text{mol/min/mg/protein}$ for alcohol dehydrogenase and Alkane hydroxylase range was between $28 \mu\text{mol/min/mg/protein}$ to $250 \mu\text{mol/min/mg/protein}$ (Table 1).

Table 1:- Degradative enzymes activity produced by different strains and under consortium conditions.

Isolates	The incubation period (days)	Alkane hydroxylase ($\mu\text{mol/min/mg/protein}$)	Alcohol dehydrogenase ($\mu\text{mol/min/mg/protein}$)
HDUL4	1	40	30
	2	90	65
	3	160	120
	4	120	80
	5	50	30
	6	20	20
	7	12	10
VDLL1	1	55	45
	2	90	60
	3	150	95
	4	105	60
	5	70	40
	6	50	20
	7	24	10
ES10	1	85	45
	2	120	80
	3	280	120
	4	200	80
	5	140	65
	6	60	50
	7	40	35
VDLL5	1	60	45
	2	120	60
	3	250	90
	4	180	60
	5	80	45
	6	50	20
	7	28	15
Consortium (Mixture)	1	125	95
	2	240	180
	3	480	220
	4	320	160
	5	240	90
	6	120	65
	7	80	55

Macro encapsulated microbial consortium produced alkane hydroxylase and alcohol dehydrogenase enzymes were studied in one week. In seven days consortium showed the highest enzyme production on the 3rd day for both enzymes. Consortium produced $480 \mu\text{mol/min/mg/protein}$ alkane hydroxylase and $220 \mu\text{mol/min/mg/protein}$

alcohol dehydrogenase on 3rd day whereas consortium showed very less for both enzyme productions on 7th day. The enzyme activity was in a range between 55 $\mu\text{mol/min/mg/protein}$ to 220 $\mu\text{mol/min/mg/protein}$ for alcohol dehydrogenase and Alkane hydroxylase range was between 80 $\mu\text{mol/min/mg/protein}$ to 480 $\mu\text{mol/min/mg/protein}$ (Table 1).

Conclusion:-

In this study, Macro encapsulated *Pseudomonas* ES10 and VDLL5, *Stenotrophomonas* HDUL4, and VDLL1 strains isolated from dairy and bulk drugs were used to study crude oil degradation and degradative enzyme productions along with encapsulated microbial consortium. From the results, it was concluded that the strains isolated from dairy and bulk drug industries in this study have degradation activity of crude oil, heavy metals, and phenol. Encapsulated microbial consortium showed maximum (98%) followed by and ES10, HDUL4 (95%) of oil degradation. Consortium produced 480 $\mu\text{mol/min/mg/protein}$ alkane hydroxylase and 220 $\mu\text{mol/min/mg/protein}$ alcohol dehydrogenase on 3rd day whereas consortium showed very less for both enzyme productions on 7th day. Among the strains, the ES10 strain showed the highest activities and can be further developed for industrial purposes. ES10 strain has produced alkane hydroxylase and alcohol dehydrogenase enzymes were studied in one week. The enzyme activity was in a range between 35 $\mu\text{mol/min/mg/protein}$ to 120 $\mu\text{mol/min/mg/protein}$ for alcohol dehydrogenase and Alkane hydroxylase range was between 40 $\mu\text{mol/min/mg/protein}$ to 280 $\mu\text{mol/min/mg/protein}$.

References:-

1. Hassanshahian M, Emtiazi G, Caruso G, Cappello S. Bioremediation (bioaugmentation/biostimulation) trials of oil polluted seawater: a mesocosm simulation study. *Mar Environ Res* 2014; 95: 28-38. a
2. Tebyanian H, Hassanshahian M, Kariminik A. Hexadecane-degradation by *Teskumurella* and *Stenotrophomonas* strains isolated from hydrocarbon contaminated soils. *Jundishapur J Microbiol* 2013; 26(7): 82-91.
3. Hassanshahian M, Ahmadinejad M, Tebyanian H, Kariminik A. Isolation and characterization of alkane degrading bacteria from petroleum reservoir waste water in Iran (Kerman and Tehran provenances). *Mar Pollut Bull* 2013; 73(1): 300-5.
4. Hassanshahian M, Zeynalipour MS, Musa FH. Isolation and characterization of crude oil degrading bacteria from the Persian Gulf (Khorramshahr provenance). *Mar Pollut Bull* 2014; 82(1-2): 39-44. b
5. Hassanshahian M, Emtiazi G. Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation. *Int Biodeterior Biodegradation* 2008; 62: 170-8.
6. Ghanavati H, Emtiazi G, Hassanshahian M. Synergism effects of phenol-degrading yeast and ammonia-oxidizing bacteria for nitrification in coke wastewater of Esfahan Steel Company. *Waste Manag Res* 2008; 26(2): 203-8.
7. Cassidy MB, Lee H, Trevors JT. Immobilized microbial cells: a review. *J Ind Microbiol Biotechnol* 1996; 16: 79-101.
8. Scott CH. Immobilized cells: a review of recent literature. *Enzyme Microb Technol* 1987; 9: 66-79.
9. Margaritis A, Merchant FJ. Advances in ethanol production using immobilized cell systems. *Crit Rev Biotechnol* 1984; 2: 339-93.
10. Radwan SS, Al-Hasan RH, Salamah S, et al. Bioremediation of oily sea water by bacteria immobilized in biofilms coating microalgae. *Int Biodet Biodeg* 2002; 50: 55-9.
11. Bickerstaff GF Jr. Immobilization of enzymes and cells *Methods in Biotechnology*. New Jersey: Humana Press 1997.
12. Wilson NG, Bradley G. Enhanced degradation of petroleum (slovene diesel) in an aqueous system by immobilized *pseudomonas fluorescens*. *J Appl Microbiol* 1996; 80: 99-104.
13. Omar SH, Rehm HJ. Degradation of n-alkanes by *Candida parapsilosis* and *Penicillium frequentans* immobilized on granular clay and aquifer sand. *Appl Microbiol Biotechnol* 1988; 28(1): 103-8.
14. Davies JS, Westlake DW. Crude oil utilization by fungi. *Can J Microbiol* 1979; 25(2): 146-56.
15. Obuekwe CO, Al-Muttawa M. Self-immobilized bacterial cultures with potential for application as ready-to-use seeds for petroleum bioremediation. *Biotechnol Lett* 2001; 23: 1025-32.
16. Martin AM, Ed. *Bioconversion of waste materials to industrial products*. New York: Springer 1998.
17. Zhang YQ, Tao ML, Shen WD, et al. Immobilization of L-asparaginase on the microparticles of the natural silk sericin protein and its characters. *Biomaterials* 2004; 25(17): 3751-9.

18. Rahman RN, Ghaza FM, Salleh AB, Basri M. Biodegradation of hydrocarbon contamination by immobilized bacterial cells. *J Microbiol* 2006; 44(3): 354-9.
19. Liang Y, Zhang X, Dai D, et al. Porous biocarrier enhanced biodegradation of crude oil contaminated soil. *Int Biodeterior Biodegradation* 2009; 63: 80-7.
20. Nunal SN, Santander DE, Leon SM, et al. Bioremediation of oil-contaminated seawater and sediment by an oil-degrading bacterial Consortium. *Biocontrol Sci* 2014; 19(1): 11-22.
21. Xu Y, Lu M. Bioremediation of crude oil-contaminated soil: comparison of different biostimulation and bioaugmentation treatments. *J Hazard Mater* 2010; 183(1-3): 395-401.
22. Díaz MP, Boyd KG, Grigson SJ, Burgess JG. Biodegradation of crude oil across a wide range of salinities by an extremely halotolerant bacterial consortium MPD-M, immobilized onto polypropylene fibers. *Biotechnol Bioeng* 2002; 79(2): 145-53.
23. Wiesel I, Wubker SM, Rehm HJ. Degradation of polycyclic aromatic hydrocarbons by an immobilized mixed bacterial culture. *Appl Microbiol Biotechnol* 1993; 39: 110-6.