Isolation of nitrifying bacteria from oil contaminated soil to study their biodegradability and enhancing crude oil degradation with the use of selected bulking agents

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Abstract : Oil exploration activities have consequential environmental impact on air, water and land. The present study deals with crude oil degrading potential of nitrifying bacteria isolated from oil contaminated soil near Valsad District. Total 21 isolates were obtained using Winogradsky medium and further 14 oil degrading isolates were obtained using Mineral basal medium with crude oil as carbon and energy source. 5 isolates OCSAz1, OCS1, GDSAz1, OCS-C and OCS-D was used to study their oil degrading activity. Inoculum size of OCSAz1was 1.0×10^7cfu/ml and OCS-1 was 1.25×10^6 cfu/ml. Temperature, pH, optical density, total viable cells by SPC and residual oil were measured gravimetrically for these isolates. Nitrifying bacteria successfully utilize crude oil as carbon and energy source and also able to utilize engine oil, toluene, phenol and kerosene. From these isolates, OCSAz1 showed highest activity with 45% degradation rate with total cell count of 4.68×10^8cfu/ml after 15 days of degradation. Followed by this, OCS1 showed 40% degradation rate with total viable cells of 3.35×10^8cfu/ml. OCSAz1 and OCS-1 gave their activity at temperature of 27 C and pH value of 4.0 and 5.5, respectively. Result of BATH test showed adherence ability of OCSAz1 with 45 % and OCS1 with 20 %. The degradation rate was further enhanced by using bulking agents such as Rice husk, Sugarcane bagasse, vermicompost and NPK. Rice husk treated medium gave best result with 67% of degradation with total viable cells of 2.1×10^8 cfu/ml within 96 hours followed by sugarcane bagasse gave 60% degradation with total viable cells of 1.2×10^8 cfu/ml. The high crude oil utilization of isolate implies that nitrifying bacteria isolated from contaminated ecosystem are excellent crude oil degraders and can be used for bioremediation purpose.

KEYWORDS: - Biodegradation, Nitrifying bacteria, Crude oil, Winogradsky, Bioremediation.

I. INTRODUCTION

Oil exploration activities have consequential environmental impact on air, water and land. About 40% of the world's energy supply comes from crude oil. With the advent of the industrial revolution and the ever increasing economic growth thereafter, there has been a concomitant increase of problems of accidental oil spills throughout the world. In addition to oil tanker accidents, another important source of oil in the sea in ballast water. Some petroleum products have been found to exert carcinogenic and neurotoxic effects. Though several mechanical and chemical methods have been implemented for the degradation of these products, the cost and rate of contamination is higher, there for bioremediation gives better solution. So, Conventional methods employed in the removal of spilled oil have deleterious side effects to the environment. So, the recent focus is on bioremediation process. Bioremediation is defined as the process in which microorganisms are stimulated to rapidly degrade hazardous organic contaminants to environmentally safe level. The microbes involved in a particular bioremediation process may obtain both energy and **carbon source** through the metabolism of target organic contaminants.

Oil spills can have a **major impact on the temporary animal and fish loss of habitat.** Heavy oils may affect several organism functions like respiration, feeding, and thermo-regulation. At the same time, the entire ecosystem can change temporarily because of the chemical components and elements of the spilled oil that are toxic to the environment. The effects of oil spills on humans may be direct and indirect, depending on the type of contact with the oil spill. The main oil spill effects include a variety of diseases, negative economic impact, pollution with crude oil or petroleum products and the aesthetic issues that affect the residents of the affected areas in multiple ways. **Biodegradation** is the dominant and ultimate natural mechanism by which petroleum pollutants are eradicated from an environment. Jones *et al.*, (1983) reported biodegradation of alkyl aromatics which is petroleum-derived aromatic hydrocarbon in marine sediments. Due to a wide range of molecular structures present in crude oil in soil, freshwater and marine environments, many scientists prescribed the use of mixed microorganism populations, capable of rapidly degrading crude oil in the biotransformation of these complex hazardous environmental contaminants (Bartha and Bossert, 1984; Cooney, 1984 Atlas, 1985; Floodgate, 1984). Nitrifying bacteria are gram-negative, chemoautotrophic, aerobic bacteria that oxidize ammonia to nitrate in the soil in a process known as nitrification (Willey *et al.*, 2011). The conversion of ammonia to nitrate is carried out by two groups of nitrifying bacteria; the ammonia-oxidizing bacteria and the nitrite-oxidizing bacteria and occurs in a two-step process that depend on the activities of at least two different genera. Considering the important role played by nitrifying bacteria in soil fertility in our ecosystem and in waste water treatment plant, the assessment of the response of nitrifying bacteria to pollution stress exerted by crude and refined petroleum fraction needs to be investigated. It is found that organisms isolated from oil contaminated soil have much more degradation rate than compared to normal soil because they are adapted to that situation. This adaptation is because of Plasmids. So, certain plasmid plays important role in the adaptation of natural microbial populations to oil and other hydrocarbons. Therefore, nitrifying bacteria isolated from contaminated ecosystem has more beneficial effect to soil and used for bioremediation. Bioremediation had been independently identified as potential emerging technology within weeks of the spill. Fertilizer addition would enhance the rates of oil

degradation. The focus of this study was on the changes in oil composition due to microbial degradation. Several agricultural wastes are used as bulking agents such as Rice husk, Sugarcane bagasse, NPK and Vermicompost to enhance degradation of oil by microorganisms and used for bioremediation of oil.

II. MATERIALS AND METHODS

1. Sample collection and enrichment

The oil contaminated soil samples were collected from 3 different areas Hingraj road, Garrage near Lillapore and garden soil of Hingraj, Valsad region. Samples were collected in a clean bottle by a clean spatula. The soil sample was collected from a depth of 1-15 cm from the top of the soil. For the isolation of Nitrifying bacteria, 5 g of freshly collected soil sample were inoculated on the same day of the collection into sterile Phosphate buffer and shaken at 100 rpm for 2 hours at room temperature for the enrichment (Okpokwasii *et al.*, 2012).

2. Isolation

The enriched soil samples were diluted on Winogradsky Phase 1 and Winogradsky Phase 2 medium for the isolation of Nitrifying bacteria. All the plates were incubated at 28 ˚C for 48-72 hrs. After incubation, different types of colonies were observed and morphology and colony characteristics were noted down. Also Gram staining was performed and results were observed. Isolation of oil degrading organism was cultured on Mineral Basal Agar medium (MBA) supplemented with 1% v/v crude oil as carbon and energy source. All the plates were incubated at 28 ˚C for 24-48 hrs. After incubation, organism which are able to utilize crude oil as carbon and energy source were able to grow on medium and growth were observed and noted down. The pure isolated bacterial colonies were maintained on Nutrient agar slant and preserved at 4˚C for further studies. Biochemical tests such as Nitrate reduction test, Ammonia Oxidation test, Ammonia production test and Nitrate production test were preformed for the confirmation of nitrifying bacteria

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3. Checking Biodegradability of Isolates

In addition to growth on crude oil, the purified strains were also tested on other hydrocarbons such as Kerosene, Engine oil, Benzene and Toluene. Mineral basal medium containing 1 % v/v of the above product was inoculated and incubated for 72-96 hrs at room temperature. Cultures without increase in turbidity over initial optical density and non-inoculated control were scored as no growth while cultures with increased turbidity significantly greater than the control were scored as growth. Degradation rate were calculated by using value of optical density. The degradation rates of bacterial isolates were determined using crude oil supplemented medium. Organism was introduced into different flask of sterile Mineral basal medium supplemented with 2 ml of crude oil as carbon source. Flask was agitated at 180 rpm at RT for 20-25 days. Control of given procedure is also performed. During incubation, representative samples were withdrawn at interval of 2, 5, 8, 11 and 14 days using destructive approach. Temp. , pH, OD of the medium was measured. Also total viable cells are counted by spread plate count. Residual crude oil was determined gravimetrically using diethyl ether as extraction solvent. For each sample, 5 ml diethyl ether was vigorously shaken manually. The mixture was then separated using laboratory funnel and evaporated at room temperature to remove the solvent thus leaving residual crude oil.

Empty beaker was pre-weighted and residual oil containing beaker was than weighted and percentage biodegradation of crude oil was determined as follows:

$$\% \text{ degradation} = \frac{a - b \times 100}{a}$$

Where, a = weight of crude oil control

b = weight of crude oil remaining in each case.

4. BATH Test

The adherence ability of the bacterial isolate with highest activity with oil was measured using the BATH test as described by Rosenberg *et al.*,(1980). Ten milliliter of culture containing cfu/ml was added to 0.7 ml of crude oil in a 20 ml glass tube and mixed well. The optical density of the cells in the culture broth was determined before the addition of crude oil. After 2 hrs of static storage, the aqueous phase was carefully removed by a Pasture pipette and the optical density was measured again. The percentage of bacteria adhered to the oil was calculated using the following formula:

$$\text{Percentage of bacterial adherence} = (1-(OD_{\text{shaken with oil}}/OD_{\text{original}})) \times 100 = \%$$

5. Enhancement of biodegradability of isolates using bulking agents

An experiment was designed to study the effect of bioaugmentation in the presence of biostimulants like inorganic fertilizer (NPK) and bulking agents such as rice husk, sugarcane bagasse and vermicompost. Five different tubes containing 40 ml of Mineral basal medium was prepared. Each medium was inoculated with 0.4 ml of bacterial culture which has highest activity. The bulking agents were added in different tubes such as NPK (1%), Rice husk (3%), Sugarcane bagasse (3%) and vermicompost (3%). Tube without addition of bulking agents was taken as control as it corresponded with natural conditions. All the tubes were kept at room temperature for 4 days (Kumari *et al.*, 2016). Samples from each tube were collected at interval of 24 hours and all the parameters such as residual oil (gravimetrically), microbial biomass, pH of medium and temperature were analyzed and degradation rate were calculated.

III. RESULTS AND DISCUSSION

A. Isolation of oil degrading nitrifying bacteria

Total 21 isolates were screened on this selective medium in which 3 isolates obtained on Nitrogen free mineral basal medium, 7 isolates obtained on Winogradsky phase 1 medium and 11 isolates were obtained on Winogradsky phase 2 medium.

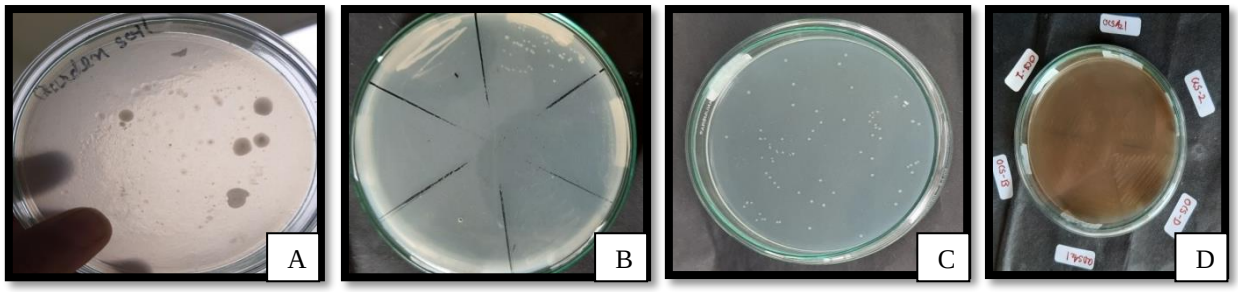


Figure 1: Isolates on A) Nitrogen free mineral basal medium B) and C) Winogradsky phase 1 medium D) Winogradsky phase 2 medium

From above 21 isolates, further 14 oil degrading nitrifying bacteria were isolated on Mineral basal medium containing 1% v/v crude oil as carbon and energy source.

Table 1: Isolation of oil degrading bacteria

Sr. No	Sample	Isolates obtained
1	Garden soil	GDSAz1, GDS-3, GDS-A, GDS-B, GDS-C
2	Garrage soil	GSAz1, GS2
3	Oil contaminated soil	OCSAz1, OCS-1, OCS-2, OCS-A, OCS-B, OCS-C, OCS-D

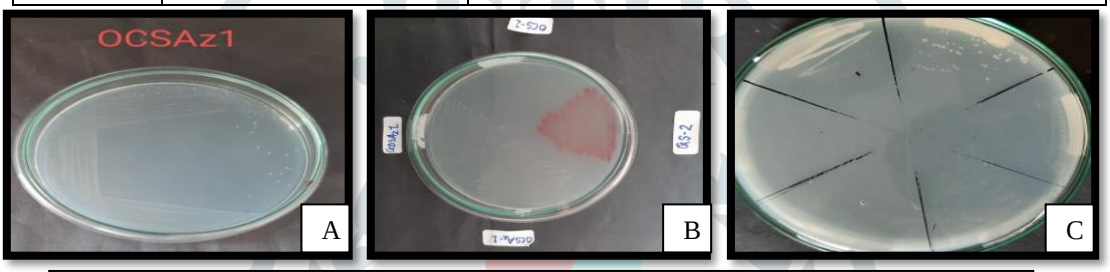


Figure 2: Isolates on A, B, C) Mineral basal medium supplemented with crude oil

B. The result of gram staining showed that all the isolates are gram negative either short or long rods and biochemical test confirms the present of oil degrading nitrifying bacteria. The level of hydrocarbons utilized by the organisms is presented in Table 2.

Table 2: Degradation of other isolates

Strain code	Toluene	Kerosene	Engine Oil	Benzene
OCSAz1	4%	1%	11%	3%
GSAz1	8%	0%	10%	8%
GDSAz1	4%	0%	13%	5%
OCS-1	5%	0%	10%	4%
OCS-2	11%	3%	10%	7%
GS-2	8%	7%	12%	7%
GDS-3	8%	1%	10%	9%
OCS-A	8%	0%	14%	10%
OCS-B	5%	0%	11%	12%
OCS-C	10%	5%	11%	10%
OCS-D	4%	0%	7%	10%
GDS-A	1%	0%	6%	4%
GDS-B	5%	0%	10%	8%
GDS-C	5%	1%	8%	9%

Growth was measured by increase of OD at 620 nm of the culture.

++ Heavy growth: OD 620 nm >

+ Moderate growth: OD 620 nm >

-No growth: OD 620 nm >

The result indicated that the organisms could utilize crude oil as well as other hydrocarbons as energy and carbon source.

C. Results of degradation rate before and after the induction with selected bulking agents

Table 3: OD of medium at 620 nm (**Before** induction)

Strain Code	Day-0	Day-2	Day-5	Day-8	Day-11	Day-14
Control	0.0	0.0	0.0	0.0	0.0	0.0
OCSAz1	0.03	0.05	0.08	0.13	0.20	0.28
GDSAz1	0.01	0.04	0.02	0.10	0.05	0.09
OCS-1	0.01	0.05	0.07	0.12	0.18	0.18
OCS-C	0.01	0.04	0.05	0.11	0.16	0.18
OCS-D	0.02	0.03	0.03	0.14	0.15	0.19

Table 4: OD of medium at 620 nm (**After** induction)

Strain OCSAz1	24 hrs	48 hrs	72 hrs	96 hrs
Control	0.0	0.0	0.0	0.0
Rice husk	0.40	0.543	0.64	1.37
Sugarcane bagasse	0.23	0.39	0.482	0.97
NPK	0.11	0.33	0.61	1.22
Vermicompost	0.10	0.10	0.38	0.84

Table 5: Residual oil of medium (**Before** induction)

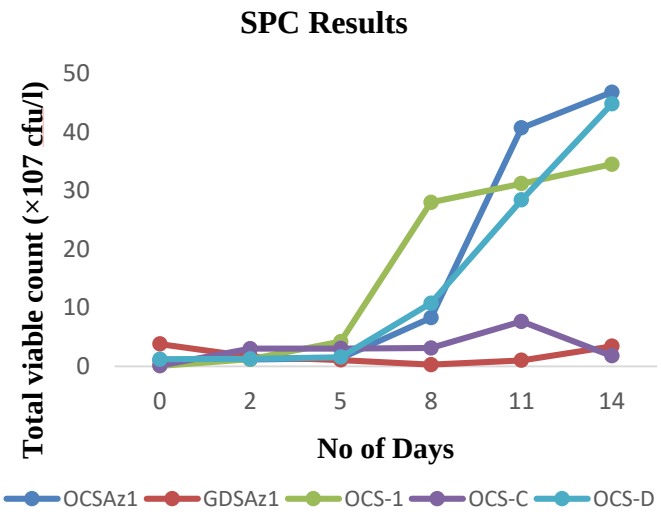
Strain Code	Day-0	Day-2	Day-5	Day-8	Day-11	Day-14
Control	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.49 ml
OCSAz1	0.5 ml	0.48 ml	0.46 ml	0.40 ml	0.32 ml	0.28 ml
GDSAz1	0.5 ml	0.5 ml	0.5 ml	0.49 ml	0.49 ml	0.47 ml
OCS-1	0.5 ml	0.46 ml	0.40 ml	0.39 ml	0.35 ml	0.30 ml
OCS-C	0.5 ml	0.45 ml	0.44 ml	0.43 ml	0.40 ml	0.38 ml
OCS-D	0.5 ml	0.48 ml	0.45 ml	0.40 ml	0.40 ml	0.37 ml

Table 6: Residual oil of medium (**After** induction)

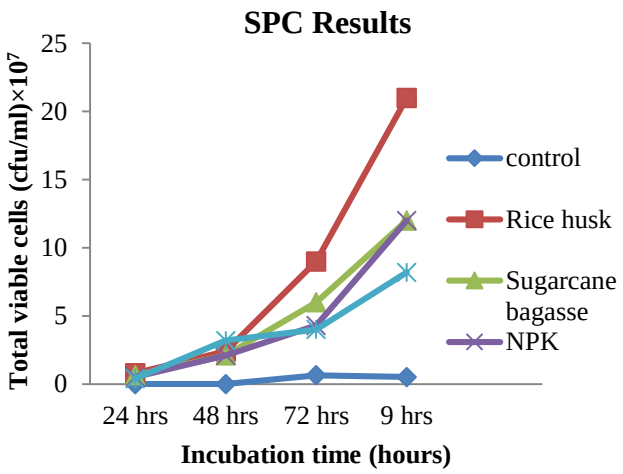
Strain OCSAz1	0 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Control	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml
Rice husk	0.4 ml	0.28 ml	0.24 ml	0.192 ml	0.132 ml
Sugarcane bagasse	0.4 ml	0.32 ml	0.24 ml	0.21 ml	0.16 ml
NPK	0.4 ml	0.32 ml	0.29 ml	0.24 ml	0.195 ml
Vermicom post	0.4 ml	0.36 ml	0.30 ml	0.30 ml	0.28 ml

As the incubation time increased, turbidity of medium increased and therefore optical density of medium increased which showed that organisms are able to grow on medium and utilize crude oil.

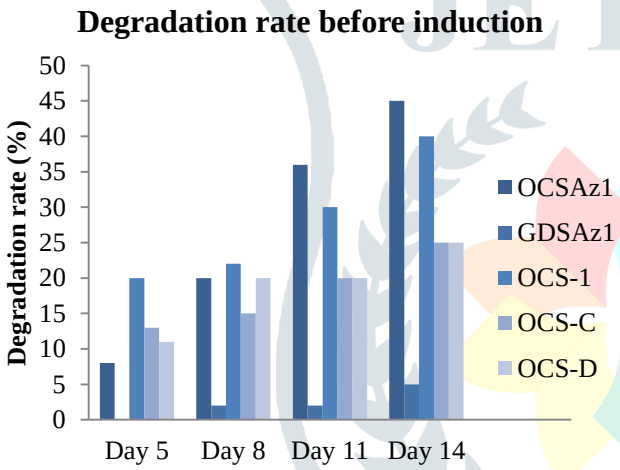
After treating medium with selected bulking agents, it was found that growth rate of organism OCSAz1 was increased by increase in incubation period. It shows that OCSAz1 was able to utilize crude oil as carbon source and bulking agents helped that to grow faster in medium and therefore increase degradation of crude oil. Rice husk gave best growth of OCSAz1 with 2.1×108 cfu/ml followed by Sugarcane bagasse treated medium contain 1.2×108 cfu/ml after 96 hours of incubation.



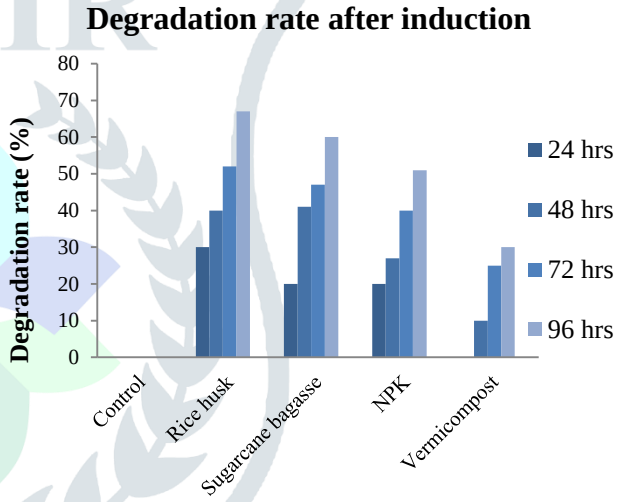
Graph 1: Total viable cells **before** treating medium with bulking agents



Graph 2: Total viable cells **after** treating medium with bulking agents



Graph 3: Degradation rate of isolates **before** induction

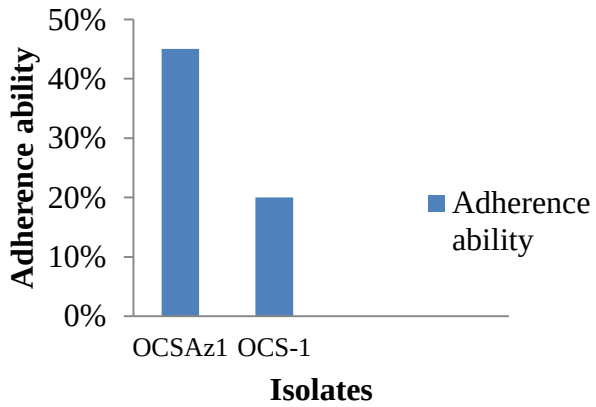


Graph 4: Degradation rate of isolates **after** induction

D. Result of BATH Test

Table 7: Result of Optical density for BATH Assay

Sr. No.	Bacterial isolates	Initial OD at 620 nm	OD of aqueous phase	Adherence ability (%)
1	Control	0.0	0.0	0%
2	OCSAz1	0.09	0.05	45%
3	OCS-1	0.05	0.04	20%



Graph 5: Graph of BATH Assay

As shown in the above given graph the OCSAz1 organism obtained from oil contaminated soil has got the highest percent of bacterial adherence to hydrocarbon that is 45% followed by OCS-1 has 20% activity.

After this experiment, degradation rate of strain OCSAz1 increased from **45% to 68%** within only 4 days. So, selected bulking agents are used for the induction of oil degradation by providing nutrients. OCSAz1 gave highest activity while strain isolated from garden soil (GDSAz1) gave lowest activity of degradation.

IV. CONCLUSION

Oil degrading nitrifying bacteria were isolated for bioremediation purpose and also demonstrated the application of bulking agents such as Rice husk, Sugarcane bagasse, vermicompost and NPK. Organisms grown on MBA plates were potential crude oil degraders as they were utilizing the crude oil which was added as a substrate. According to my study, I concluded that organism isolated from oil contaminated soil coded as **OCSAz1** is an efficient strain with high potential of crude oil degradation. This study revealed that nitrifying bacteria isolated from contaminated ecosystem have ability to proliferate and degrade hydrocarbons. It was also concluded that organism isolated from oil contaminated ecosystem have much more degradation rate than compared to normal soil because they are adapted to particular situation and this adaptation is because of plamids. Selected bulking agents helped organism to grow and proliferate in the medium and enhance the degradation of crude oil. Therefore, the degradative capabilities of the evaluated organisms could be explored in bioremediation campaigns for contaminated soils. It is necessary to clean up the oil contaminated beaches and other environment. This strain has capacity to degrade oil compound and used for bioremediation purpose and control pollution.

V. ACKNOWLEDGMENT

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