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ORIGINAL ARTICLE **OPEN ACCESS**

Screening and isolation of bacterial species from oil sludge contaminated soil to study its oil sludge degradation activity

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ABSTRACT

The oil refining, manufacturing units and petrochemical industries generates solid waste and waste water which are environmental concern and need proper treatment prior to their disposal. Huge amount of oil waste have been produce worldwide. Oil refinery wastes is the mixture of water soluble and insoluble substance having high carbohydrate and lipid content. The main of the study to evaluate the efficiency of low-cost waste degradation technology for reducing hydrocarbon content of oil sludge waste, so need detail study and degradation of oil sludge is essential. There are several environmental and economic benefits arising from the application of new waste management process. In present work, oil sludge waste degradation was studied by using minimal medium. Checking of the oil sludge degradation by bacterium M1 was studied at room temperature. It shows that almost all degradation of oil sludge is within 2 days. After the analysis and study it conclude that the isolated strain M1belongs from the Bacillus subtilis subsp.

Keywords: *Oil sludge, Minimal medium, Hydrocarbon, Waste degradation.*
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INTRODUCTION

Industries like oil refinery, petroleum, automobile etc. generates huge amount of oily sludge as a waste byproduct. The oil sludge comprises chemically complex mixtures of variety of hydrocarbons, sediments and water which is hazardous to all living beings on planet[1].Composition of oil sludge is complex in nature and completely depends on the sources. Among the different components in oil sludge waste, most of having high affinity towards the particulate matter and components of soil. The different ongoing processes in industries like crude oil refining, cleaning in place (CIP) of oil storage vessels and waste treatment processes generates large quantities of oil sludge. Oil sludge resides in fine pores that's why it is difficult to eliminate[2]. Oil is considered as one of the principle sources of energy for human being and also it proved as severe environmental pollutant [3]. Petroleum hydrocarbons can be divided into four classes: saturates, aromatics, asphaltenes (Phenols, fatty acids, Ketones, esters and porphyrins) and resins (pyridines, quinolines, carbazoles, sulfoxides and arnides). Hydrocarbons differ in their susceptibility to microbial attack and rank in the following order of decreasing susceptibility: n- alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes. It has very low aqueous miscibility and high hydrophobicity. Large amount of oil containing sludge has generated in oil refineries from process of waster oil separation[4]. Without any prior treatment of oily sludge, if simply dumping or burning process carried out, then it will seriously affect on ecosystems and all living life on planet [5]. As per the reports by United States Environmental Protection Agency, oily sludge listed as priority pollutant due to their mutagenic, carcinogenic and toxic potential[6].After considering the toxicity and polluting ability of oil hydrocarbons, there is urgent need of the development of well-studied, effective and ecofriendly methodology for treatment. The study and reports by various environmental regulatory agencies form different countries has motivated the companies for development of ecofriendly technologies, highlighting the bioremediation [7].The physicochemical treatments for oily sludge includes thermal desorption, incineration, cement kiln, solvent extraction, Coker, and land filling etc. Microorganisms especially bacteria, has ability to synthesize different enzymes during their metabolic activity and are useful for bioremediation process. It is has been effective in reduction of organic load from effluent and is a cheaper and environment friendly process of degradation[8].Bioremediation in oil refinery waste can be stated as the use of efficient microorganisms to reduce or remove the environmental pollutants from soil and water, aiming the degradation of hydrocarbons into CO_2 and H_2O [9].Optimization study at different environmental parameters like pH, temperature and nutrient supplements like carbon source, nitrogen source etc. was proved to be effective in degradation study of

oil sludge. The temperature plays an important role in petroleum hydrocarbons biodegradation, by their direct effect on physiology and chemistry of the pollutants as well as on diversity of the micro flora. Ambient temperature of an environment affects both the property of silled oil and the activity of microorganisms. The aim of present research work is to isolate indigenous bacterial species from oil sludge contaminated soil and to study their oil degradation activity.

MATERIALS AND METHODS

Sampling

The oil sludge samples were collected from A. R. Engineering Pvt. Ltd. located in Satara district, Maharashtra, India. The samples were kept in sterile clean plastic bottles and immediately transferred to laboratory for further analysis. The soil samples were collected from the oil contaminated site of industrial premises in sterile plastic bags.

Isolation and screening of oil sludge degrading bacteria

The oil sludge degrading bacteria were isolated from oil sludge contaminated soil through the enrichment method. Enrichment was performed in 250ml Erlenmeyer flask with 50ml of sterile minimal medium inoculated with 1 gm of oil contaminated soil sample and 1ml oil sludge as a carbon source was added. The flask was kept in incubator for 7 days at 37°C. After proper incubation period, the loop full enriched cultures were streaked on sterile minimal agar plates separately. Plates were incubated at 37°C for 24 hrs. Morphologically different colonies were selected and studied for colony characteristics. The single morphologically different isolate was grown on minimal medium named as isolate M1.

Composition of Minimal agar

Sr. No.	Ingredients	Quantity	Sr. No.	Ingredients	Quantity
1	K_2HPo_4	0.7gm	5	Ammonium sulfate	0.1gm
2	KH_2Po_4	0.2gm	6	pH	7.2$\pm$ 2
3	Sodium citrate	0.05gm	7	Agar- Agar	3gm
4	$MgSo_4$	0.01gm	8	Distilled water	100ml

Morphological and biochemical characterization of oil degrading bacteria

The isolated bacterial strain was identified by morphologically for gram nature and motility. Further, it was identified as per standard keys described in Bergey's manual of determinative bacteriology [10]. Following are the biochemical tests were performed for identification.

1. **Amylase activity**– The loopful of pure culture of selected bacterial isolate was spot inoculated on sterile starch agar plate, after proper incubation period (at 37°C for 24 hours), plate was observed for zone of hydrolysis of starch surrounding the colony. Experiment was done in triplicates [11].
2. **Gelatinase activity**- The loopful of pure culture of selected bacterial isolate was spot inoculated on sterile gelatin agar plate, after proper incubation period (at 37°C for 24 hours), plate was observed for zone of hydrolysis of gelatin surrounding the colony. Experiment was done in triplicates[12].
3. **Caseinase activity**– The loopful of pure culture of selected bacterial isolate was spot inoculated on sterile skimmed milk agar plate, after proper incubation period (at 37°C for 24 hours), plate was observed for zone of hydrolysis of casein surrounding the colony. Experiment was done in triplicates [13].
4. **Catalase activity**-Catalase activity was detected by the liberation of free O_2 when pure culture of selected bacterial isolate was dipped in substrate H_2O_2 solution[14].
5. **Oxidase activity**- The oxidase activity was detected on sterile filter paper strip dipped with oxidase test reagent. The presence of dark purple coloration represents a positive test[15].
6. **Sugar utilization test**- The selected bacterial culture was tested for glucose, sucrose, fructose and lactose utilization test separately, using peptone water base medium using phenol red indicator. After incubation, the tubes were checked for change in color of the medium.
7. **Indole test**- The test culture was tested for indole production in sterile 1 % tryptone broth. After incubation, xylene and kovacs reagent was used for detection of indole production.
8. **Methyl red test-** Sterile glucose phosphate broth tubes were inoculated with selected test culture. After incubation period, methyl red indicator was used as reagent in test tubes to check the results.
9. **VogesProskauer test**-Sterile glucose phosphate broth tubes were inoculated with selected test culture. After incubation period, Barrit's A -40% KOH, Barrit's B- α naphthol in ethanol was used as reagent in test tubes to check the results.
10. **Citrate utilization test**- The test culture was tested for citrate utilization in sterile Kosser's citrate broth. After incubation, turbidity was checked to interpret results.

Biodegradation study of oil sludge using efficient bacterium

The ability of selected bacterium culture was tested for biodegradation of oil sludge. In experimental setup, 0.5 ml of selected bacterium culture was inoculated in 100 ml minimal medium containing predetermined quantity (5 ml)of oil sludge and kept for incubation at 37℃ fortwo days [16].The present study aiming to determine biodegradation of organic contaminants. During experiment, control set was used consisting of minimal medium with oil sludge and in blank set, minimal medium was considered.

Extraction:

Extraction procedure was performed for the IR spectrometric and HPLC analysis. The minimal medium containing flask (50ml)was inoculated with 0.5 ml of selected bacterial culture, containing 5 ml oil sludge sample and kept at 37℃ for 48 hrs. Control flask containing minimal medium and oil sludge was kept at 37℃ for 48 hrs.After incubation medium as well as oil sludge sample were centrifuge at 7000rpm for 10 minutes, separately. Supernatant was collected and pellet was discarded. Supernatant and petroleum ether mixed in 1:1 concentration, shakes it well after every 20 min for 2-3 times and kept for settling pellet and separated out petroleum ether layer. Pellet was discarded and supernatant (petroleum ether layer) was kept for evaporation at room temperature. Extract was collected named as M extract and oil sludge extract in sterile vial and used for further testing.

Analysis

UV-Vis Spectrophotometric analysis:

The test sample (2ml) was filtered using Whatman filter paper no. 1, centrifuged at 7,000 rpm for 10 minutes. The supernatant was observed for U.V. spectrophotometric pattern and compared with control set.

IR Spectrometric analysis:

For the determination of functional groups, IR spectrometric analysis of M extract and oil sludge extract were performed using F.T.I.R. (CFC/D.R. No. 07) instrument. IR spectrometric analysis was carried out in Common Facility Center of Yashavantrao Chavan Institute of Science, Satara.

RESULT AND DISCUSSION

Screening of oil sludge degrading bacteria

Naturally, microorganisms showing the catabolic activity, and found remarkable presence at crude oil polluted sites especially soil [17]. Crude oil contaminated soil badly effects on environment especially marine life, human health, desert plants, birds, crops etc. Microbes has ability to catabolize crude oil by synthesizing different enzymes during their metabolism and are useful for bioremediation process. This research study was carried out to screen the microorganism (bacteria) from crude oil contaminated soil, where indigenous bacteria use crude oil as a sole carbon and energy source and thus it can be used as a potential candidate for the biodegradation of crude oil contamination by breaking down of hydrocarbons into CO_2, H_2O and humus.

In our study, one isolated bacterium was taken through the enrichment technique. It was selected on the basis of their early growth on oil sludge containing minimal agar media. Morphological and colony characteristics of isolate are presented in (Table 1). As per observation of study, the selected bacterium is Gram positive, non-motile short rod.

Table 1: Morphological and colony characteristics of isolate

Isolate	Size	Shape	Color	Margin	Elevation	Consistency	Opacity	Gram nature	Motility
M1	0.1mm	Circular	White	Entire	Convex	Moist	Opaque	Gram positive short rod	Non motile

The morphologically screened isolate was further identified using its biochemical characterization as per standard keys described in Bergey's manual of determinative bacteriology [9]. The isolate was tested for enzymatic activities such as amylase, gelatinase, caseinase, catalase and oxidase. As per results, isolate was found positive for amylase, catalase and oxidase activity. Results are shown in table 2. Further, identification of isolate was carried out by biochemical tests, such as sugar utilization test and IMViC test.In sugar utilization test, selected bacterial culture was tested for glucose, sucrose, fructose and lactose utilization test. As per results, isolate was able to utilize sugars such as glucose, sucrose and fructose (Table 3). In IMViC test, isolate showed positive results for indole production, Methyl red test and citrate utilization test (Table 4).

Table 2: Detection of enzymatic activity by selected bacterial isolate

Sr. No.	Name of isolate	Amylase	Gelatinase	Caseinase	Catalase	Oxidase
1	M1	++	--	--	++	++

Table 3: Detection of sugar utilization by selected bacterial isolate

Sr. No.	Name of isolate	Glucose	Sucrose	Fructose	Lactose
1	M1	++	++	++	--

Table 4: IMViC test results by selected bacterial isolate

Sr. No.	Name of isolate	Indole	Methyl Red	Citrate Utilization	Voges Proskauer
1	M1	++	++	++	--

Biodegradation study of oil sludge using selected bacterium

Degradation of oil sludge in minimal medium

The experiment was performed to check degradation capacity of selected isolate, with 0.5 ml of selected bacterium culture added in 100 ml minimal medium containing predetermined quantity (5 ml)of oil sludge and kept for incubation at 37°C for two days. The present study aiming to determine biodegradation of organic contaminants. During experiment, control set was used consisting of minimal medium with oil sludge. As per results, it showed that, the total degradation of oil sludge was observed within 48 hrs (Figure 1).

Figure 1: Degradation of oil sludge

Above figure depicts, minimal medium with bacterial isolate at first day was blackish in color whereas after the degradation of oil sludge, medium turns to the transparent medium within 48hrs. This was another indicator of determining the oil sludge degradation rate via color observation.

UV Vis Spectrophotometric analysis

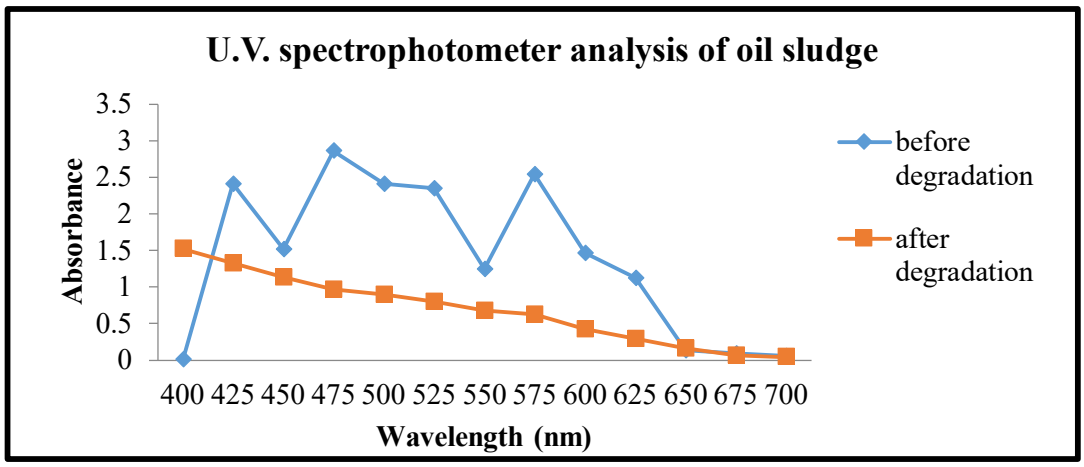

Figure 2: First day U.V. spectrophotometer analysis of oil sludge

In this U.V. spectrophotometric analysis study, biodegradation of oil sludge was studied by using selected isolate. The absorbance of test sample on first and second day shows the degradation of oil sludge. As per observations, it was concluded that the selected isolate is potent for degradation of hydrocarbons.

IR spectrometric analysis

IR spectrometric analysis of oil sludge extract was compared with other three IR spectrometric analysis. They showed the different pattern of analysis in which complex compounds are converted into simple compound. Extract of the medium not harmful to environment and ecosystem.

IR spectrometric analysis of oil sludge extract

In IR spectrometric analysis of oil sludge extract (control) carboxylic acid, aldehydic, aromatic, nitroalkanes, carbon fluoride, carbon bromide etc. compounds are detected (Figure 3). These all compounds are harmful to ecosystem. They are non-disposable.

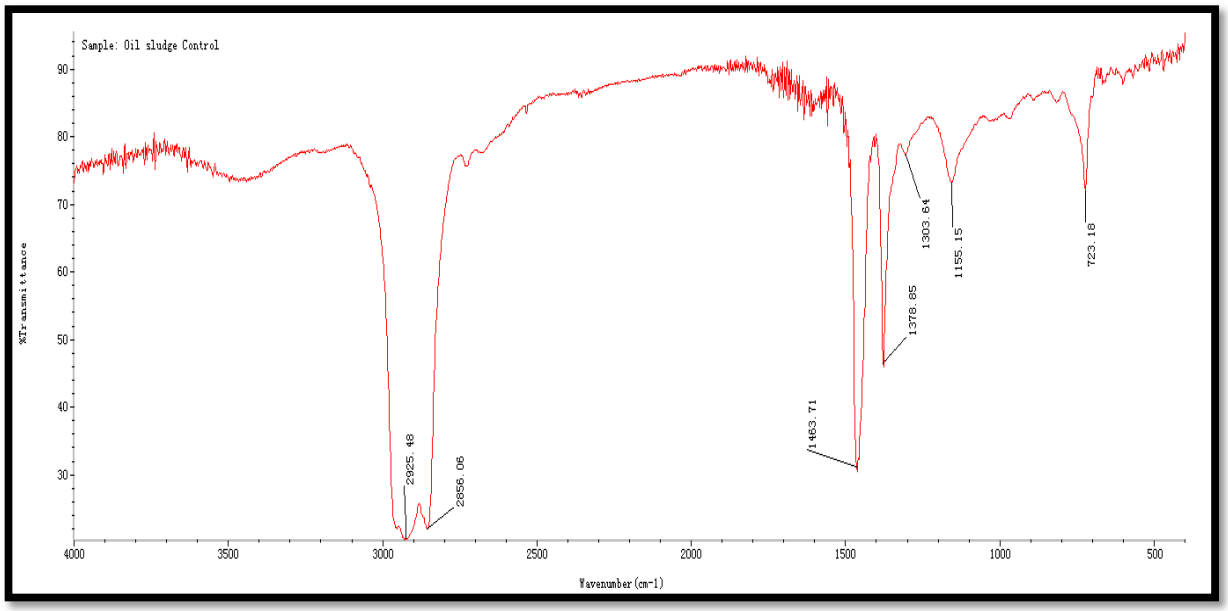

Figure 3: IR spectrometric analysis of oil sludge extract

IR spectrometric analysis of minimal extract

In IR spectrometric analysis of minimal extract ester, alkane, methane compounds are detected. This are harmless compounds as compare to compounds detected in oil sludge extract (Figure 4).

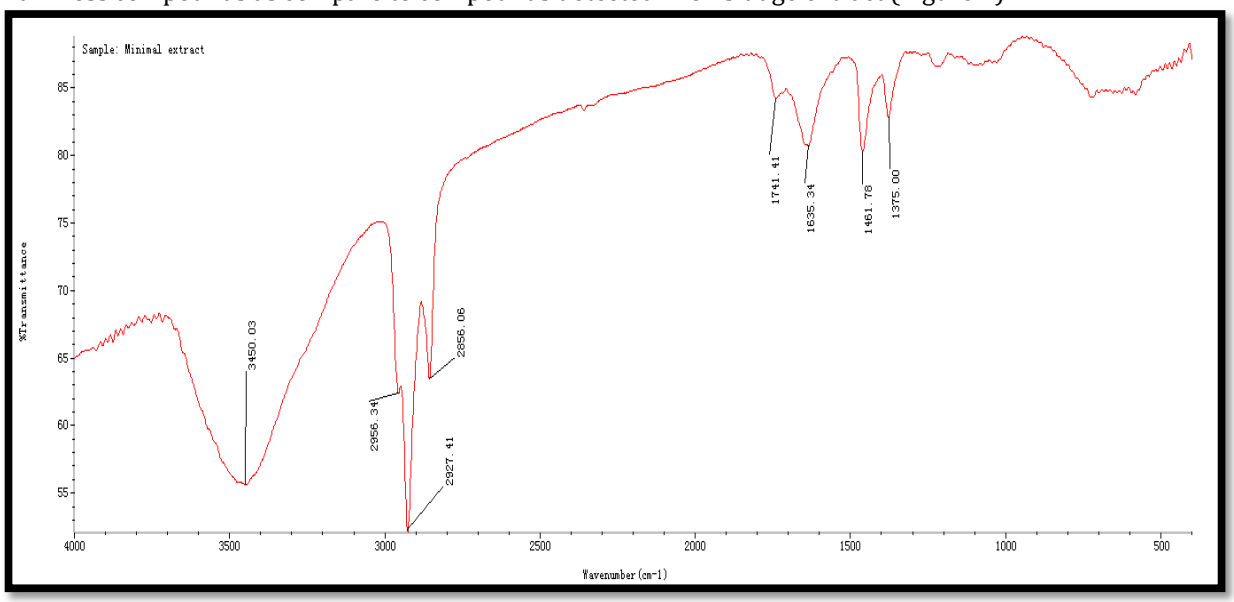

Figure 4: IR spectrometric analysis of minimal extract

Following is the detailed account of active group study (Table 5).Active group detection after biotransformation was done by IR. The remarkable changes was observed in biotransformation.

Table 5: Active group detection after biotransformation via IR

Oil sludge		Minimal	
Wavelength(cm^{-1})	**Compound Name**	**Wavelength(cm^{-1})**	**Compound Name**
2925.48	Carboxylic acid OH stretch	2956.34	Carboxylic acid OH stretch
2856.06	-C-H Aldehyde	2927.41	=C-H stretch
1463.71	C=C Aromatic	2856.06	-C-H Aldehyde
1378.85	NO_2 stretch	1741.41	C=O ester
1303.64	NO_2 stretch	1635.34	C=C Alkane
1155.15	C-F	1461.78	CH_3 bend
723.18	C-Br	1375.00	NO_2 stretch

CONCLUSION

Petroleum refineries generates large amount of oil sludge as waste by product. Simply dumping this waste in environment affects to living organism and disturbs the ecosystem. Hence, we try to apply green technology for oil sludge hydrocarbon degradation. The isolated bacterium shows high degradation rate of hydrocarbons. After the analysis and study it conclude that the isolated strain M1belongs from the *Bacillus subtilis subsp.* Degradation rate also concluded using color changes of blackish oil sludge containing medium to transparent medium. The different types of analysis like UV-Vis and IR spectrophotometry show the remarkable degradation of the oil sludge. This method for hydrocarbon degradation is clean, eco-friendly, time saving and low cost effective.

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