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Isolation and Characterization of Microbial Strains from Refinery Effluent to Screen their Bioremediation Potential

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Abstract

In the present world of industrial revolution, spills of petroleum products and oil are one of the major sources of the contamination in the ecosystem and cause serious health hazards for humans and livestock. Therefore, the removal of these pollutants has become necessity of the time. Applications of new techniques such as bioremediation and biodegradation, over conventional methods are much more promising for safe and sustainable environment. Aim-The aim of this study is to isolate and characterize microbial strains from effluent of Mathura Refinery – Indian Oil Corporation Limited, the microscopic and biochemical studies and their potential of bioremediation. Bacteria were isolated from Mathura refinery IOCL, Based on the biochemical studies six morphologically distinct bacterial strains with promising bioremediation role were identified. To check the degradation of crude oil sample by bacterial isolates Bushnallhass media and separation funnel method were performed. Six bacteria were isolates from Mathura refinery effluentsand all six bacteria have ability to degrade the crude oil in different range. Maximum potential to degrade the crude oil were identified in Pseudomonas. This study might be important in an important step towards bioremediation techniques

Keywords: *Biodegradation, Bioremediation, Petroleum products, Safe and sustainable environment.*

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INTRODUCTION

Water pollution is one of the serious ecological threats among various types of pollutions. Various anthropogenic activities, such as heavy industrialization including crude oil refinery, chemical, textiles, nuclear industries etc., have led to the contamination of ground water in both urban and rural area leading to serious health hazard1.

Release of crude oil or petroleum products in the ecosystem severely affects the biotic communities. To overcome such ecological extermination several physical and chemical methods are conventionally used whichleads to the conversion of comparatively less toxic forms instead of the complete degradation and removal[2]. However, the modified form of petrochemical still causes cellular toxicity leading to various deformities. The hydrocarbons released in the ecosystem can be metabolized by many communities of bacteria inbiodegradable nontoxic products[3-4]. The advantage of bacteria mediated remediation of ecosystem (i.e. bioremediation) over conventional methods is that the bioremediation provides a complete solution of toxic substances rather than converting their forms in a cost effective manner.

Use of microbial strainsfor treatment of polycyclic aromatic hydrocarbons from contaminated locations has already been reported[1,6,9].

Numerous residential microflora naturally do have inherent properties for degrading these pollutants from soil and aquatic ecosystems. The purpose of present work is isolation and characterization of bacterial strains from oil refinery effluent and their identification through colony morphology, microscopic and biochemical studies[6-7].

Toxic pollutants cause harm to living and non-living things but before devising solutions to cure them, we need to understand that their sources, leaching process, chemical conservation and their mode of deposition[8].

MATERIALS AND METHODS
Sample collection

Industrial effluent was obtained, in sterile sample collection screw cap bottles of 100 ml volume aseptically, from the effluents of oil petroleum refinery, Mathura (IOCL Mathura Refinery Mathura, Uttar Pradesh, India). The collected sample was then immediately bought to the laboratory for further processing.

Isolation of pure bacterial strains
Initial isolation

For initial isolation of bacterial strains serial dilution in 0.8% NaCl solution of collected industrial effluents was performed. 5ml NaCl solution was transferred in five test tubes labeled as 10^{-1} to 10^{-5}. Followed by addition of 50⁷l inoculum (oil refinery effluent) in the first tube and serial diluted. From 10^{-1}, 10^{-3} and 10^{-5} tubes 10⁷l sample was spread on solidified Nutrient Agar plates and incubated overnight at 37°C.

Primary bacterial screening

After overnight incubation colonies found on Nutrient medium plates, were selected for secondary screening of pure culture. The single morphological bacterial colony was transferred separately to another Nutrient Agar Medium (NAM) plates by the means of inoculation loop and were incubated overnight at 37°C. The isolated bacterial strains were grown-up on MacConkey Agar media with pH 7.2 at 37°C. Finally pure and axenic cells were transferred aseptically to NAM slants and test tubes containing broth media for further investigation in incubator at 37°C[3].

Confirmatory test for oil degradation microbes by using bushnellhaas media

For detecting oil degrading ability of axenic strains, method introduced by Bushnell and Haas, (1941), has been used with Bushnell-Haas media. Bushnell Haas Agar was used for microbiological examination of crude oil it uses for the study of microbial utilization of hydrocarbon. This media contains Composition (g/l) Magnesium Sulphate 0.2, Calcium Chloride 0.02, Monopotassium Phosphate 1.0, Dipotassium Phosphate 1.0, Ammonium Nitrae 1.0, Ferric Chloride 0.05, Agar 20.0, Final pH (at 25°C) 7.0±0.2.

After preparation of Bushnell Haas agar plate the isolated bacterial cultures were inoculated into the medium[3] and one control plate and six bacteria isolates with Bushnell Hass Agra with the inoculum of six bacterial isolates and 15 ml of crude oil sample in each plates were kept inthe incubation at 37°C for 7 days. Finally positive results were observed by measuring the quantity

of oil degraded by the each bacterial strain after 7 days.

Inoculation of pure culture isolates into oil containing nutrient broth

  In this stage the oil degradation capacity is confirmed by each bacterium. 20 ml of crude oil sample were taken in six conical flask of nutrient broth. Place the conical flask in autoclave at 15lb on 120°C for 30 minutes. Inoculate the pure culture isolates of bacteria with the help of inoculation loop under Laminar in each flask. Put the flasks for incubation at 37°C in incubator for 30 days,

with the interval of 3-5 days put the conical flasks in the shaker also to provide the proper agitation of bacteria.
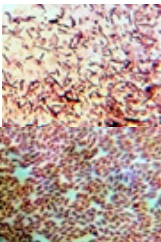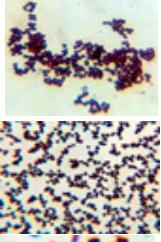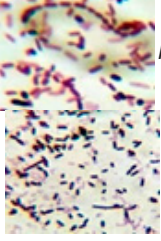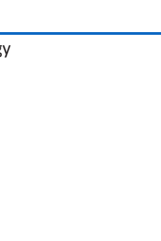
Separation funnel method for measurements of oil degradation analysis

  After the incubation of 30 days degraded oil amount were measured with the help of separation funnel method by putting the nutrient broth with oil and bacteria isolates in separation funnel for 35 minutes. Amount of oil measured in measuring cylinder with the help of micropipette for each sample and analysis shows that all six

Table 1. The bacterial class identification and suspension turbidity used for card inoculation

S.No.	Bacteria classes	Identification	McFarland Turbidity
1	GN	Gram negative fermenting and non-fermenting bacilli	0.50-0.63
2	GP	Gram positive cocci and non-spore forming bacilli	0.50-0.63
3	YST	Yeast and yeast like organisms	1.80-2.20
4	BCL	Gram- positive spore- forming bacilli	1.80-2.20

Table 2. Morphological characteristics of the bacterial isolates cultured on Nutrient agar medium

S.No	Colony Color	Microscopic View	Genus	Gram Staining	Colony Size	Colony Form
1	Yellow color colony		*Bacillus*	GramPositive	Small	Circular
2	Pink color colony		*Algoriphagus*	GramNegative	Medium	Irregular
3	White color colony		*Bacillus sp.*	GramNegative	Small	Irregular
4	Milky white colony		*Acinetobacter*	GramNegative	Large	Irregular
5	Green color colony		*Pseudomonas sp*	GramNegative	Small	Circular
6	Cloudy white colony		*Bacillus sp*	GramNegative	Medium	Irregular

bacteria have ability to degrade the amount of crude oil sample after measuring the quantity of crude oil.

Biochemical analysis

For biochemical analysis, we used VITEK2 which is an automated system for microbial growth analysis and provide highly accurate data. It performs 64 individual tests in 64 different well like various metabolic activities (alkalinization, acidification, enzymes hydrolysis and growth) in the presence of inhibitory substance.

To identify the different classes of organism, four reagents card were used; turbidity was also measured with the help of turbidity meter- DensiChek.

RESULTS
Isolation of bacteria

A total of six bacteria from refinery effluents were screened on Nutrient agar media. By understanding the biochemical and morphological analysis six strains are *Bacillus sp1*,*Algoriphagus*, *Bacillus sp2*, *Acinetobacter, Pseudomonas, Bacillus sp3*.

Identification and characterization of oil degrading bacterial isolates

On the basis of colony characterization, morphology, species, growth, stain test, colony size, colony form (Table 2) and biochemical analysis (Table 3) six different bacteria were identified. From the analysis one bacteria out of six were gram positive remaining five were gram negative; Three out of six bacteria were different shades of white in color.

Screening of bacteria on crude oil

Crude oil has different hydrocarbons it also includes olefins paraffin and aromatic compounds[10,11]. Crude oil is the great substrate for screening of hydrocarbon degradation capabilities in microorganism because it has the variation in hydrocarbons and its composition[3,4].

The ability of oil degradation was determine by the growth of bacteria on Bushnell Haas Agar media and add the same amount of oil in all six petriplates and keeps it for up to 7 days at 30°C. The effect of variation in quantity of oil after seven days defined each bacterium has the ability of oil degradation at different level [3].Separation
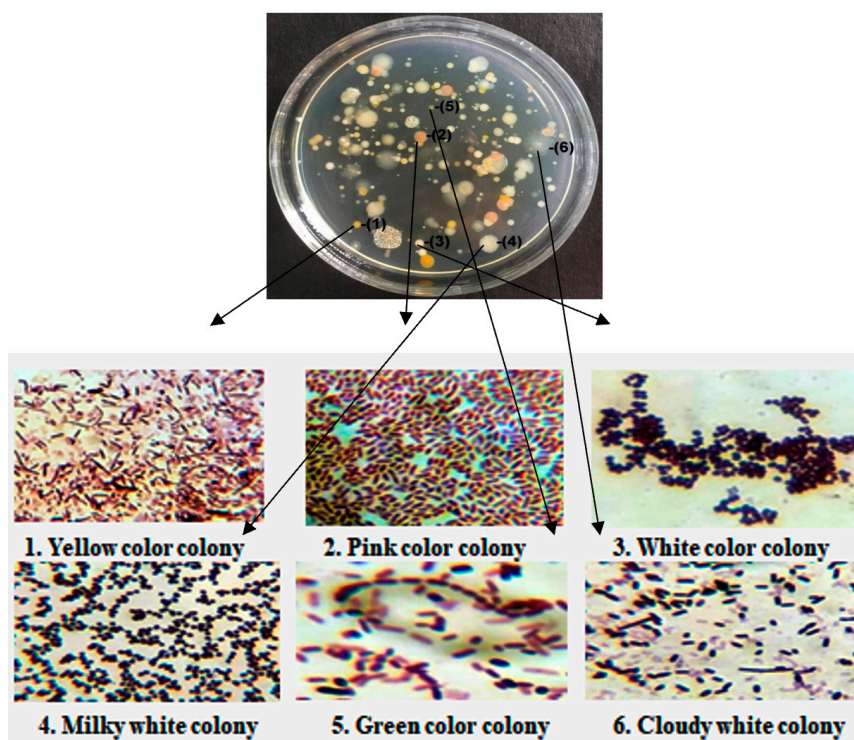

Fig. 1. Representing bacterial colony and microscopic view

Table 3. Biochemical Test Results

GRAM NEGATIVE

Well	Test	Mnemonic	Algoriphagus	Bacillus sp.	Pseudomonas sp.	Bacillus sp.
2	Ala-Phe-Pro ARYLAMIDASE	APPA	-	-	(+)	-
3	ADONITOL	ADO	-	-	-	-
4	L-Pyrrolidonyl- ARYLAMIDASE	PyrA	-	(+)	(+)	-
5	L-ARABITOL	IARL	-	-	-	-
7	D-CELLOBIOSE	dCEL	-	-	-	(+)
9	BETA- GALACTOSIDASE	BGAL	-	-	-	-
10	H2S PRODUCTION	H2S	-	(+)	-	-
11	BETA-N-ACETYL GLUCOSAMINIDASE	BNAG	-	(+)	-	-
12	GlutamylArylamidasepNA	AGLTp	-	-	-	-
13	D-GLUCOSE	dGLU	+	(+)	-	(+)
14	GAMMA-GLUTAMYL- TRANSFERASE	GGT	-	-	(+)	-
15	FERMENTATION/GLUCOSE	OFF	-	-	-	-
17	BETA GLUCOSIDASE	BGLU	+	(+)	-	(+)
18	D-MALTOSE	dMAL	-	(+)	-	-
19	D- MANNITOL	dMAN	-	-	-	(+)
20	D-MANNOSE	dMNE	-	-	-	(+)
21	BETA- XYLOSIDASE	BXYL	-	-	-	-
22	BETA-ALANINE arylamidasepNA	BALap	-	-	-	-
23	L-Proline ARYLAMIDASE	ProA	+	-	(+)	-
26	LIPASE	LIP	+	-	(+)	-
27	PALATINOSE	PLE	-	-	-	-
29	Tyrosine ARYLAMIDASE	TyrA	+	-	(+)	-
31	UREASE	URE	-	(+)	-	-
32	D-SORBITOL	dSOR	-	-	-	(+)
33	SACCHAROSE/SUCROSE	SAC	-	-	-	(+)

GRAM POSITIVE

Well	Test	Mnemonic	Bacillus
2	D-AMYGDALIN	AMY	-
4	PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C	PIPLC	-
5	D-XLOSE	dXYL	-
8	ARGININE DIHYDROLASE 1	ADH1	-
9	BETA- GALACTOSIDASE	BGAL	+
11	ALPHA-GLUCOSIDASE	AGLU	(+)
13	Ala-Phe-Pro ARYLAMIDASE	APPA	+
14	CYCLODEXTRIN	CDEX	-
15	L-Asppartate ARYLAMIDASE	AspA	+
16	BETA GALACTOPYRANOSIDASE	BGAR	+
17	ALPHA-MANNOSIDASE	AMAN	-
19	PHOSPHATASE	PHOS	+
20	Leucine ARYLAMIDASE	LeuA	+
23	L-Proline-ARYLAMIDASE	ProA	+
24	BETA-GLUCURONIDASE	BGURr	+
25	ALPHA-GALACTOSIDASE	AGAL	+
26	L-Pyrrolidonyl-ARYLAMIDASE	PyrA	+
27	BETA-GLUCURONIDASE	BGUR	-
28	Alanine ARYLAMIDASE	AlaA	-
29	Tyrosine ARYLAMIDASE	TyrA	+
30	D-GALACTOSE	dSOR	-
31	UREASE	URE	-
32	POLYMIXIN B RESISTANCE	POLYB	-
37	D-GALACTOSE	dGAL	-
38	D-RIBOSE	dRIB	-

Cont... Table 3.

GRAM NEGATIVE

Well	Test	Mnemonic	Algoriphagus	Bacillus sp.	Pseudomonas sp	Bacillus sp
34	D-TAGATOSE	dTAG	-	-	-	(+)
35	D-TREHALOSE	dTRE	-	(+)	-	(+)
36	CITRATE(SODIUM)	CIT	+	-	(+)	-
37	MALONATE	MNT	-	-	-	-
39	5-KETO-D-GLUCONATE	5KG	-	-	-	-
40	L- LACTATE Alkalinization	ILATk	-	(+)	(+)	-
41	ALPHA- GLUCOSIDASE	AGLU	-	(+)	-	-
42	SUCCINATE alkalinization	SUCT	+	-	(+)	-
43	BETA-N-ACETYL-GALACTOSAMINIDASE	NAGA	-	-	-	-
44	ALPHA-GALACTOSIDASE	AGAL	-	-	-	-
45	PHOSPHATASE	PHOS	+	(+)	-	-
46	Glycine ARYLAMIDASE	GlyA	-	-	(+)	-
47	ORNITHINE DECARBOXYLASE	ODC	-	-	-	-
48	LYSINE DECARBOXYLASE	LDC	-	(+)	-	-
53	L-HISTIDINE Assimilation	IHISa	-	-	-	-
56	COUMARATE	CMT	+	(+)	-	-
57	BETA-GLUCORONIDASE	BGUR	-	-	-	-
58	O/129 RESISTANCE (comp.vibrio)	O129R	-	(+)	-	-
59	Glu-Gly-Arg-ARYLAMIDASE	GGAA	-	-	-	-
61	L-MALATE Assimilation	IMLTa	+	-	(+)	-
62	ELLMAN	ELLM	-	-	(+)	-
64	L-LACTATE assimilation	IIATA	-	-	(+)	-

GRAM POSITIVE

Well	Test	Mnemonic	Bacillus
39	L-LACTATE alkalinization	ILATk	-
42	LACTOSE	LAC	-
44	N-ACETYL-D-GLUCOSAMINE	NAG	-
45	D-MALTOSE	dMAL	-
46	BACITRACIN RESISTANCE	BACI	-
47	NOVOBIOCIN RESISTANCE	NOVO	-
50	GROWTH IN 6.5% NaCl	NC6.5	-
52	D-MANNITOL	dMAN	-
53	D-MANNOSE	dMNE	-
54	METHYL-B-D-GLUCOPYRANOSIDE	MBdG	-
56	PULLULAN	PUL	-
57	D-RAFFINOSE	dRAF	-
58	O/129 RESISTANCE (comp.vibrio.)	O129R	-
59	SALICIN	SAL	-
60	SACCHAROSE/SUCROSE	SAC	-
62	D-TREHALOSE	dTRE	-
63	ARGININE DIHYDROLASE	ADH2s	-
64	OPTOCHIN RESISTANCE	OPTO	-

funnel method also shows that the amount that remains after analysis was less than the amount that added at the initial, after considering the amount remains in control in both the analysis. This ability determines that all 6 isolates obtained from the sampling site have metabolic active and ability to utilize the hydrocarbon as their energy source

Identification of potent bacterial strain for oil degradation

After Bushnell Hass media and separation funnel analysis, the confirmatory test for oil degradation was found maximum in *Pseudomonas*[12] by consuming the maximum oil amount in both the analysis. Other than *Pseudomonas, bacillus sp1, Algoriphagus, Bacillus sp2, Acinetobacter* and *Bacillus sp3* also have potential of crude oil degradation.

DISCUSSION

Nowadays, activities related to the petrochemical industry are one of the serious environmental concerns in terms of releases of hydrocarbon contaminates in the ecosystem[13]. These are the organic toxic pollutants often associated with neurotoxicand carcinogenic activities in humans. Conventional methods for disposal of these pollutants have their limits in terms of effectiveness and cost. Oil degradation bacteria are present in extensive range in environment and well known for their ability of degradation of hydrocarbons present in oil[14]. Microorganism benefits in the removal of contaminant because of the presence of some special proteins and enzymes.

Application of nonpathogenic microbial stains for solving this problem is a promising tool for treatment of contaminated sites[9]. These microorganisms utilize complex toxic hydrocarbon contaminants into their metabolic cycles and transform them into simple non toxic forms with complete mineralization. Various physical and chemical factors are responsible for influencing the phenomena. Numerous residential microfloraas naturally possess inherent capabilities of degrading these toxic pollutants from soil and water ecosystems.

Hydrocarbon degrading potential of pseudomonas has recently been delineated and attributed to presence of naphthalene dioxygenase operon in its genome which is involved in the degradation of Benzene, Toluene, Ethylbenzene, Xylene[15]. Similarly biodegradation of patroleum hydrocarbon has also been reported by Bacillus *sp.*[16] a metagenomic study has also reported the enrichment of Acinetobacter *sp*. in a marine environment after an oil spill event[17]. All these recent works corroborate with our findings.

CONCLUSION

The successful bioremediation techniques and approaches practiced using microbes for petroleum hydrocarbon degradation[3]. The road ahead for implementation of these organisms for the improvement of the environment and ultimately, public health is indeed long and worthwhile.

The aim of this study was to proposes the ecofriendly approach for the problem of discharging waste in the environment. We further endorse our approach for identifying native bacterial strains from refinery effluent, for consumption of used engine oil.

Six bacterial strains were isolated, purified and identified on the basis of colony characteristic, microscopic studied and biochemical analysis. In which oil degradation capacity was found in *Pseudomonas bacillus sp1, Algoriphagus, Bacillus sp2, Acinetobacter* and *Bacillus sp3*. Oil degrading microorganisms are wide spread in nature. They are well known for their ability to degrade variety of hydrocarbons present in the crude oil and harbor catabolic enzymatic activity to utilize organic contaminants as sole carbon and energy source converting them into less harmful substances[12]. Use of indigenous microorganisms aids in the removal of organic contaminants due to the specific enzyme systems synthesized by microorganisms.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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