ISSN: 2320 - 4230

Journal of Drug Discovery and Therapeutics 1 (11) 2013, 01-17

RESEARCH ARTICLE

ISOLATION OF OIL DEGRADING BACTERIA FROM OIL CONTAMINATED SOIL AND EXPRESSION OF OIL DEGRADING GENES IN NON OIL DEGRADING BACTERIA

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Received 10 November 2013; Revised 14 November 2013; Accepted 15 November 2013

ABSTRACT

The present study is carried out by isolation and characterization of oil degrading microbes from oil contaminated sites. In this work the *Bacillus megaterium* , *Pseudomonas fluorescenes* and *S.aureus* were isolated according to Bergey's manual and further used for oil degradation to check the production of biosurfactant. The Shake flask method, Agar well diffusion method and Spread plate method were used for oil degradation. Further the optimization was carried out for best carbon, nitrogen sources and also for pH and temperature. And in last the plasmid was isolated from production media and after restriction digestion , ligation the transformation was done to transform oil degrading genes in to non oil degrading microbes.

Key words: Bioremediation, Shake flask method, Agar well diffusion method, Spread plate method and optimization.

INTRODUCTION:

Bioremediation is the use of micro-organism metabolism to remove pollutants. Bioremediation is the branch of biotechnology which deals with the methods of solving the environmental problems. It also plays vital role in cleaning the environment from pollutants and contaminants by using the microorganisms and fungi. Bacteria are the most important microbes in this process because they break the dead materials into organic matter and nutrients. To investigate the countermeasure to remediate soils contaminated with oils, bioremediation provide an effective and efficient strategy to speed up the clean-up processes [1] .Industrial wastewaters containing petroleum hydrocarbon is highly toxic and posed a great danger especially to refinery nearby communities. Therefore, there is need for effective treatment before discharge. Natural process employing microorganisms is considered to be very effective and environmentally friendly method of decontamination [2]. For the process of bioremediation, it is necessary that microorganisms should be healthy and active so that they can perform their duty efficiently. It is not necessary that all the microorganisms detoxify the same contaminants and toxins but for different toxins there are different microorganisms because there are different habitats for different organisms [3] [4]. Bioremediation can take place in two conditions that are aerobic and anaerobic conditions. There are three types of bioremediation and all are used to remove toxic substances and contaminants from the environment whether they are rivers or crude oils. The present study is carried out by isolation of oil degrading microbes from the soil sample and their characterization. It also involved the expression of oil degrading genes in non oil degrading microbes [5] [6].

METHODOLOGY:

Sample collection:

Soil Sample (A) - Soil+ Mobil oil, from Pepper mill Colony, Nishatganj, Lucknow.

Soil Sample (B) – Soil+ Mobil oil, from Gomti nagar Petrol pump, Lucknow.

Soil Sample (C) - Soil+ Engine oil, from Unnao Petrol pump.

Serial dilution method:

Microorganisms are used in industries for enzyme production as well as in antibiotic production and serial dilution method is used to get reduced number of bacterial colonies in order to get pure colonies.

Characterization of bacterial culture:

Gram Staining:

Gram staining is a method of differentiating bacterial species into two large groups Gram-positive and Gram-

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negative. The name comes from its inventor, Hans Christian Gram[7].

Oil degrading activity:

1) Shake flask method:

Prepare NB (Nutrient Broth) and add half amount of oil (petrol, diesel, and engine). After autoclaving cool at room temperature then inoculate bacterial culture and incubate at 37°C in shaker for 7- 10 days. Day by day measurement was done and also the growth of culture was observed with the help of colorimeter taking OD at 620 nm.

2) Agar well diffusion method:

Prepare Nutrient Agar plates and spread 100 µl. of oil (diesel, petrol, engine oil) prepare wells and load 50 µl. of bacterial culture then incubate at 37°C for overnight and observe result, if cultures showing growth in the presence of oil it means culture have properties to degrade oil.

3) Spread plate method: Prepare Nutrient Agar plates and spread 100 µl. of oil (petrol, diesel, engine) and after it spread 50 µl. of bacterial culture over it and then incubate at 37°C for overnight and observe result, if cultures show growth in the presence of oil it means culture have properties to degrade oil.

Growth kinetic study: Growth Kinetics process is applied to determine the time period at which the culture shows its optimum activity (stationary phase). Growth of any microbes occurs in different stages which are indicating by growth curve. In Growth kinetic study there four stages occur.

Horikoshi alkaline media:

It is used for growth kinetic energy of a culutre. Including day by day OD at 600 nm. **Identification of culture through Bergey's manual** [7]

Catalase Test:

Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it function to catalyze the decomposition of hydrogen peroxide to water and oxygen. Catalase has the one of the highest turnover number of all enzyme. one catalase enzyme can convert 40 million molecules of hydrogen peroxide to water and oxygen each second.

MRVP Test:

MR and VP broth is used for the identification of microorganism. It contains peptone, buffers and dextrose or glucose. In MR test their methyl red is used which is work as a indicator. In MR (methyl red) test bacteria convert Dextrose /Glucose to pyruvate with the help of metabolic reaction. The color of broth changes yellow to red. In VP test bacteria convert Dextrose/Glucose to acetine in the presence of α- Napthol and alkaline and alkaline condition.

Mannitol Test:

In their acid formation is occurs. Phenol red is used as a indicator. In Mannitol test their beef extract, peptone, Mannitol, NaCl, Phenol red indicator etc and pH is 7.

OF Broth (Oxidation fermentation broth):

Oxidative/fermentation glucose test (O/F test) is a biological technique utilized in microbiology to determine the way a microorganism metabolizes a carbohydrate such as glucose. Dextrose can be used in the test. The power of breakdown of carbohydrates is possessed by a large number of bacteria, fungi and yeasts. This microbial activity is of great significance in the carbon cycle in nature and industrially.

Litmus milk test:

Milk is an excellent medium for the growth of microorganisms because it contains the milk protein casein, the milk sugar lactose, vitamins, minerals and water. Litmus, a pH indicator is incorporated in the medium for the detection of production of acid or alkali and oxidation-reduction activities. A variety of different chemical changes occur in milk, depending upon which milk ingredients are utilized by the bacteria and is dependent upon the types of enzymes that the organism is able to produce

Starch hydrolysis:

Bacillus culture is having properties to produces amylase enzyme which act on starch and hydrolyses starch solution. In the presence of iodine solution give blue color and if bacillus culture is producing metabolic than that culture will show clear zone of inhibition in the presence of iodine that shows positive result.

Nitrate Reduction:

Nitrate reduction test indicates that the reduction of nitrate is possible or not through culture in this test Napthol solution is used and also acid is used that will help Nitrate Reduction.

Oxidase test:

Oxidase test **Gordon Macleod Reagent** is used which shows purple color in the presence cytochrome Oxidase enzyme this reagent contain endophenol which react with cytochrome Oxidase enzyme give purple color, if the bacterial culture is not produce cytochrome Oxidase enzyme than no color is observe.

Growth Kinetic Study:

Prepare NB and autoclave it after autoclaving inoculate bacterial culutre after incubation of culutre incubates at

37^0c for overnight. After incubation next day take OD at 600nm through colorimeter.

Optimization:
Optimization techniques used to check growth activity of different microbes by carbon sources, nitrogen sources, different pH (4, 6, 7, 8,10) and different temperature (4^0C, 50^0C, 37^0C, at room temp.) Optimization is the technique in which provides suitable condition for the growth of culture.

Carbon sources:
1% Carbone sources was used for culture. The different carbon sources were taken like Sucrose, maltose, Mannitol, glucose, starch and beef extract. In their 5ml D/W and 1% carbon sources was taken[8].

Nitrogen sources:
1% Nitrogen sources was used for culture. The different nitrogen sources were taken like Urea, peptone, ammonium chloride, and CH_3COONa.

pH: 4, 6, 7, 8, and 10.
Temperature: 4^0C, 50^0C, 37^0C, and at room temp.
Protein isolation of bacterial culture:
Procedure:
Take 1 ml. of Bacterial Culture in a centrifuge tube. Centrifuge at 10,000 rpm for 10 min take supernatant as a crude sample (production of extracellular amylase).
After that the supernatant was taken into two palaces-

1) Bradford method to check protein content.
2) Enzyme assay to measure enzyme activity.

Bradford assay:
Principle:
C.B.B dye (Commassie Brilliant Blue Dye) reacts with protein sample and gives blue color solution where absorption shifted from 465 – 595 nm and optical density can be measured at 595nm.

Enzyme assay DNS method:
DNS(3, 5 dinitro salicylic acid) which reacts with reducing sugar and itself converted into 3 amino 5 nitro salicylic acid, to stop the reaction it will give orange color solution at λmax 540nm.[9]

Extraction of Lipid:
Take 0.5ml of supernatant and 0.5ml chloroform mixed gently by inversion for 1hours. Centrifuge at 10,000rpm for 10 min than transfer bottom layer to new tube, air dry properly than dissolve in TE buffer and check the activity.

Plasmid isolation: Plasmid isolation was done by alkaline denaturation method[10]

Restriction Digestion: Restriction digestion was done with the help of restriction enzyme which are also known as molecular scissors, they will cut DNA at specific site either blunt ends or sticky ends.

Ligation: Ligation indicates joining of phosphodiester bond in this case joining will take place between 5′ phosphate and 3 OH group.

Competent cell preparation: Competent cell are the cell which are ready to uptake foreign DNA and for transformation we need competent cell.

Transformation: Transfer of genetic material from one cell to another cell with the help of plasmid is known as transformation.

RESULTS:

Isolation of oil degrading microorganism from oil contaminated sites:
Microbes from soil were isolated by serial dilution method and mixed culture was obtained by spreading as shown in figure:

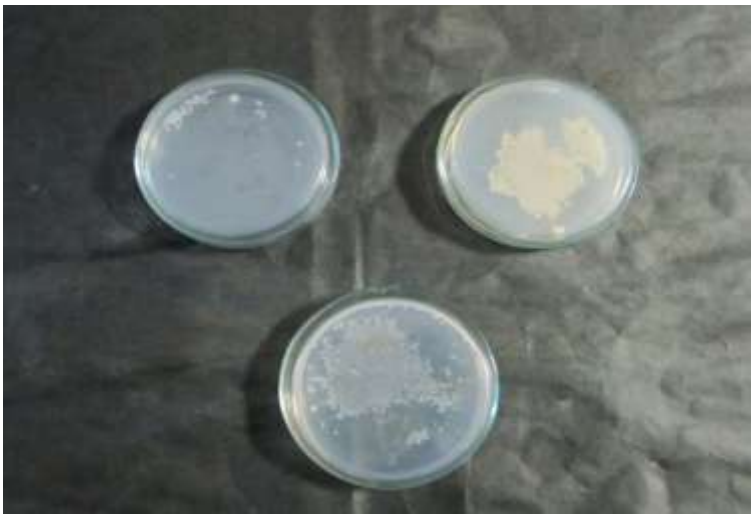


Figure 1: Mixed cultures in spreading

Table 1: Colony morphology of culture from sample C1, C2, C3 and C4

Colony Morphology.	Culture C1	Culture C2	Culture C3	Culture C4
Size	4 mm.	3.5 mm.	4.5 mm.	1.5 mm.
Shape	Circular	Irregular	Fusiform	Irregular
Color	Off white	Off white	White	White
Texture	Sticky	Smooth	Smooth	Rough
Margin	Entire	Lobate	Undulate	Lobate
Opacity	Opaque	Opaque	Translucent	Translucent
Elevation	Flat	Flat	Flat	Flat

Sub culturing: The cultures C1, C2, C3 and C4 obtain through primary screening were purified by quadrant streaking was shown in fig. 2:

Figure 2: Sub culturing through Quadrant Streaking

Gram's staining result:

After gram's staining slides were observed under microscope and some colonies (C2 and C4) get pink color and some get purple color (C1, C3 and C5). The colonies showing pink color are gram negative rods and gram negative cocci and colony which shows purple color are gram positive rods and gram positive cocci.

Table 2: Observation of cultures through Gram staining:

Cultures	Occurrence
C1	Gram positive rod
C2	Gram negative rod
C3	Gram positive rod
C4	Gram negative cocci
C5	Gram positive cocci

Method to check oil degrading activity:
A). Shake Flask Method results:

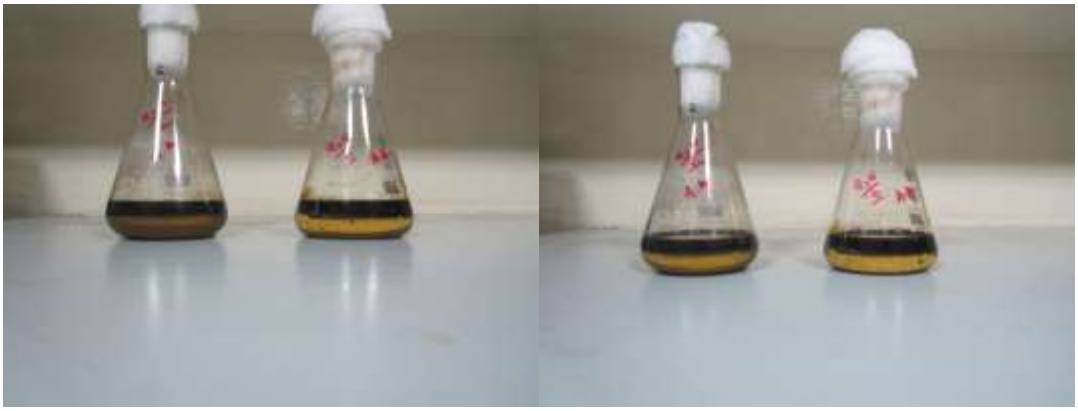


Figure 3: Oil degradation activity on first and second day by culture C2 (Mobil oil was used)

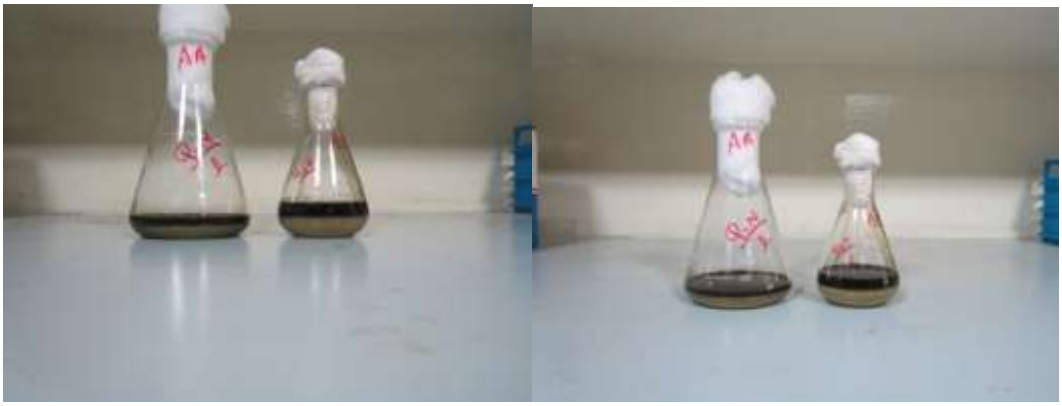


Figure 4: Oil degradation activity on third and fourth day by culture C2 (Mobil oil was used)

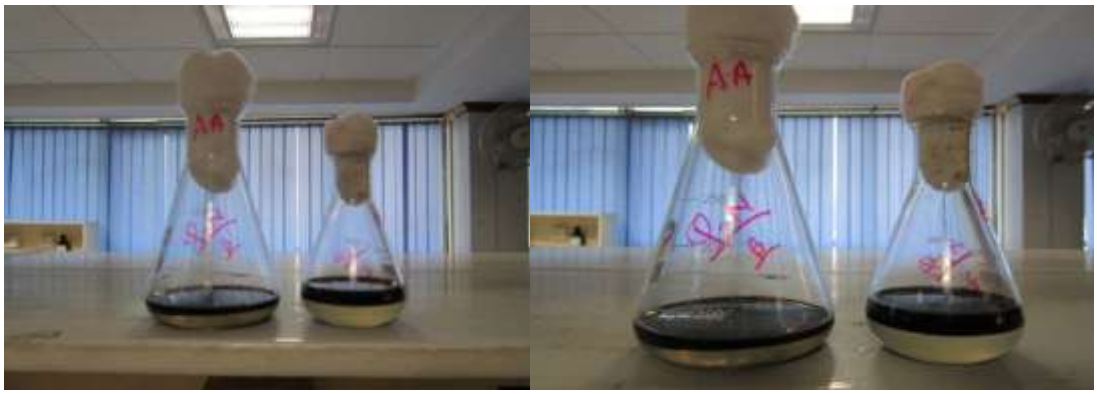
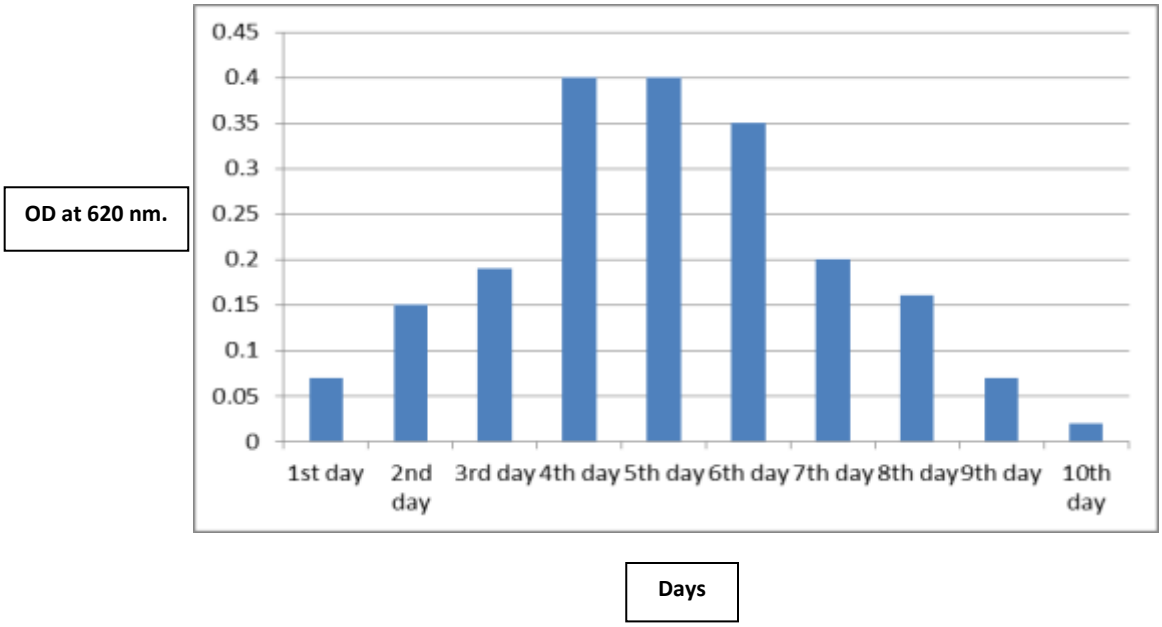


Figure 5: Oil degradation activity on fifth and 7th day by culture C2 (Mobil oil was used)

Figure 6: Day by day oil degradation activity on seventh day by culture C2 which degrade oil completely (Mobil oil was used)

Table 3: Optical Density day by day to check oil degradation activity by culture C2 in shake flask method:

Days	Optical Density
1st day	.07
2nd day	0.15
3rd day	0.19
4th day	0.40
5th day	0.40
6th day	0.35
7th day	0.20
8th day	0.16
9th day	0.07
10th day	0.02



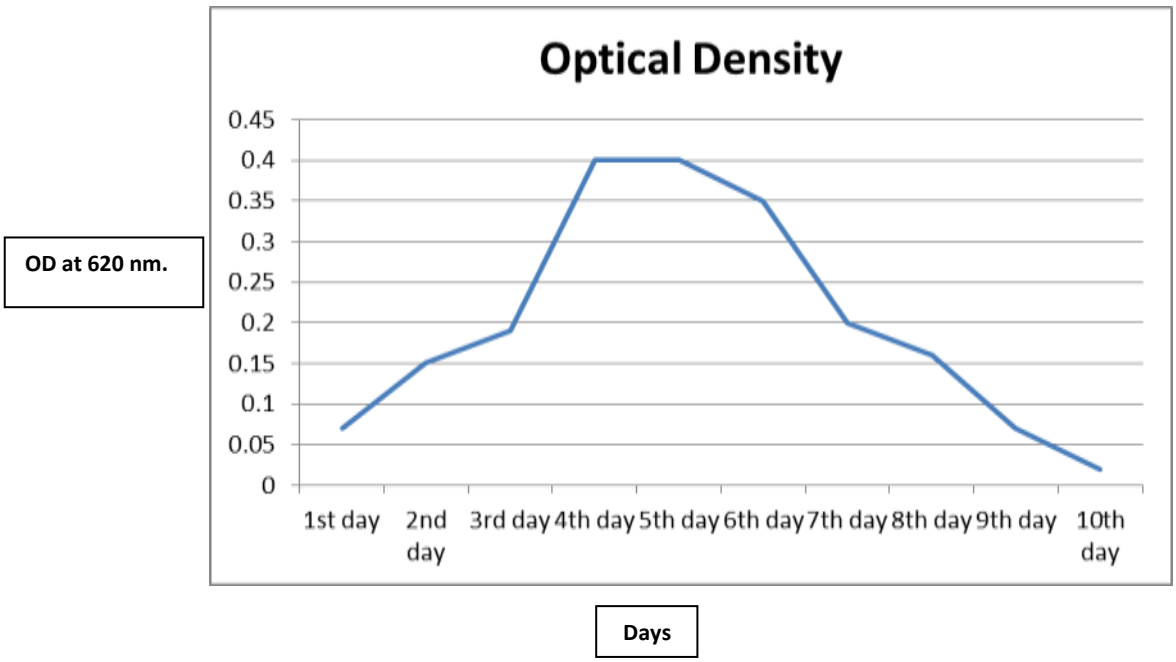


Figure 7: Graph between OD and days of Oil degradation activity by culture C2 which was showing log phase on 4th and 5th day

Figure 8: Oil degradation activity on first, second and third day by culture C1 and C3 (vegetable oil and petrol was used)

Figure 9: Oil degradation activity on sixth and seventh day through Shake flask method by culture C1 and C3 (vegetable oil and petrol was used)

Table 4: Optical Density day by day to check oil degradation activity by culture C1 and C3 in shake flask method:

Days	OD for culture C1	OD for culture C3
1st day	0.05	0.08
2nd day	0.15	0.18
3rd day	0.20	0.23
4th day	0.27	0.28
5th day	0.19	0.21
6th day	0.15	0.16
7th day	0.08	0.07

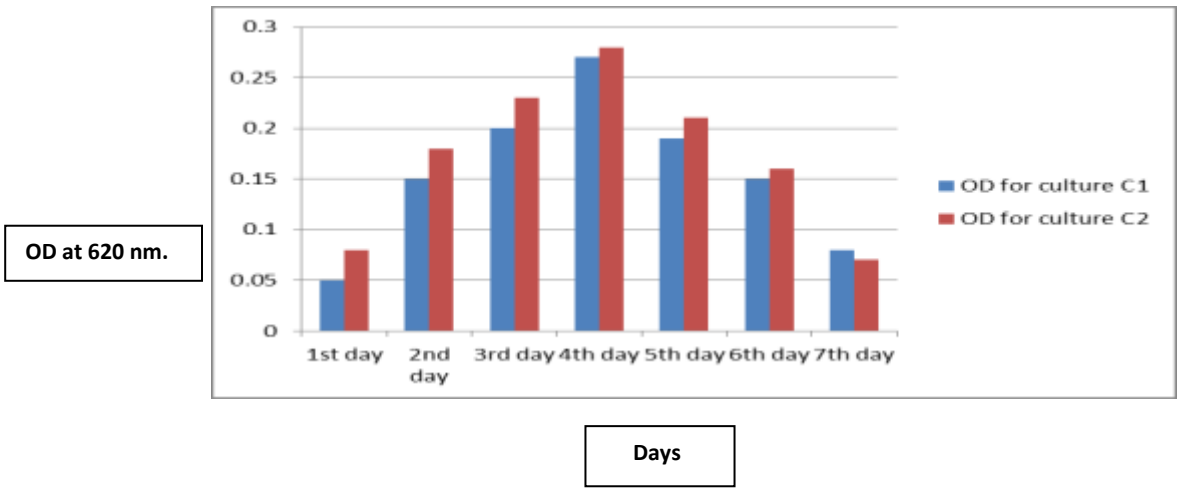


Figure 10: Graph between OD and days of Oil degradation activity by culture C1 and C3 which was log phase on 3rd and 5th day.

B). Spread Plate Method results:

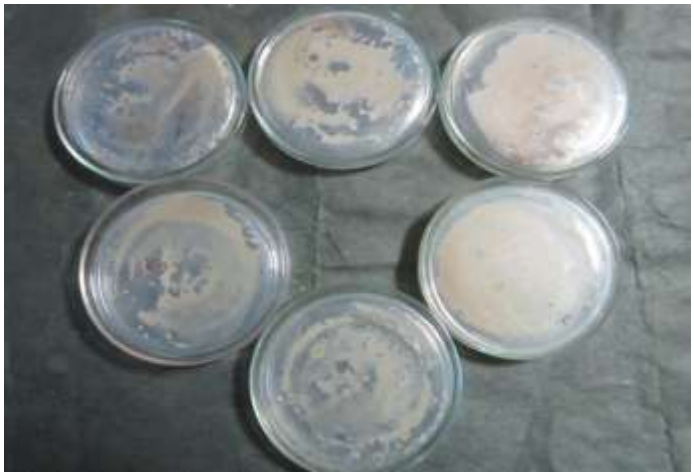


Figure 11: Oil degradation activity through Spread Plate Method

C). Agar well diffusion method:

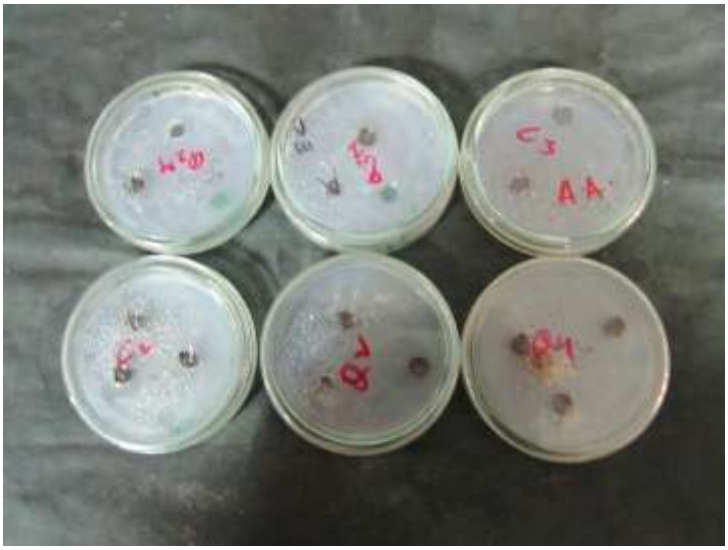


Figure 12: The figure shows the degradation of oil in Agar well diffusion method

Growth kinetics:

Horikoshi media:

Figure 13: This figure shows the growth of microorganisms in Horikoshi media (production media)

Table 5: OD to check the growth of microorganisms in Horikoshi media (Growth Kinetics)

Day	Optical Density (OD)	Phase
1st	0.60	Lag
2nd	0.92	Log
3rd	0.96	Stationary
4th	0.98	Stationary
5th	0.46	Death or Decline
6th	0.16	Death or Decline

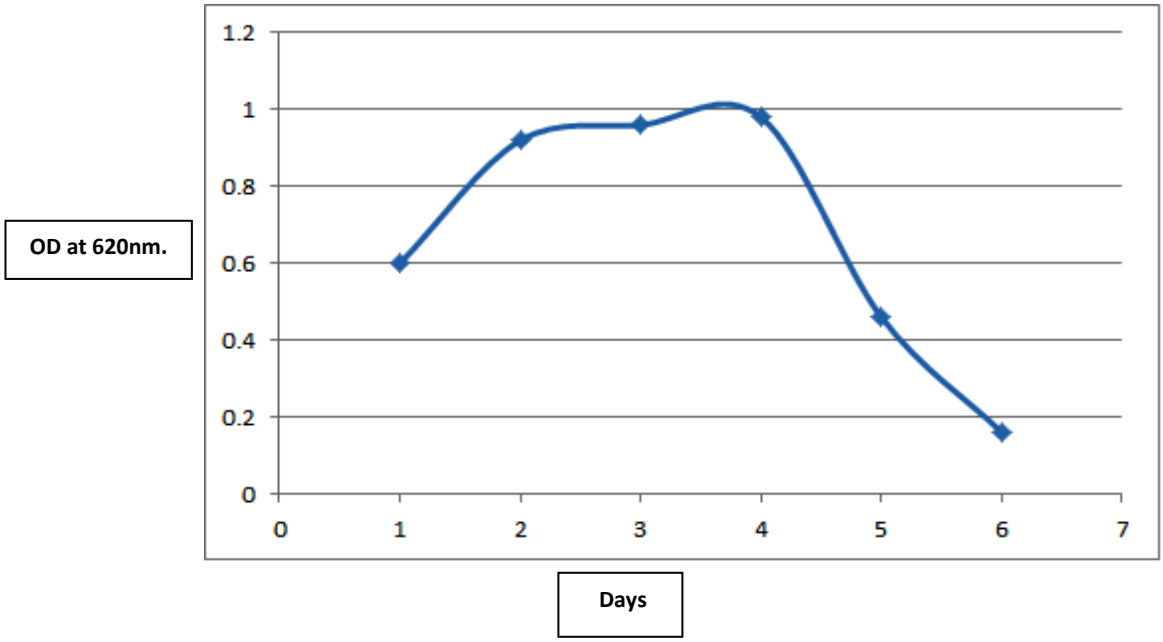


Figure 14: Growth curve between OD and number of days (Growth Kinetics) showing log phase by culture on 2nd day. Identification through Bergey's manual:

Table 6: Biochemical test for different cultures followed by Bergey's manual:

Biochemical Tests	*Bacillus cereus*	*Pseudomonas fluroscence*	*Staphylococcus aureus*
Endospore test	Positive	Negative	Negative
Catalase test	Positive	Positive	Positive
Oxidase fermentation test	Positive	Negative	Negative
Nitrate test	Positive	Positive	Positive
Starch hydrolysis	Positive	Negative	Negative
Oxidase test	Positive	Positive	Positive
MR test	Negative	Negative	Positive
VP test	±	Negative	±
Mannitol test	Negative	Negative	Positive
Litmus milk test	_____	Positive (alkaline)	_____

Optimization result (through Optical density):

Table 7: OD for 1% Carbon sources:

Sources	*Pseudomonas fluroscence*	*Bacillus megaterium*	*Bacillus cereus*	*Staphylococcus aureus*
Sucrose	0.03	0.02	0.05	0.00
Maltose	0.05	0.04	0.03	0.00
Mannitol	0.02	0.02	0.02	0.00
Glucose	0.06	0.05	0.05	0.00
Starch	0.15	0.24	0.11	0.08
Beef extract	0.20	0.29	0.19	0.39

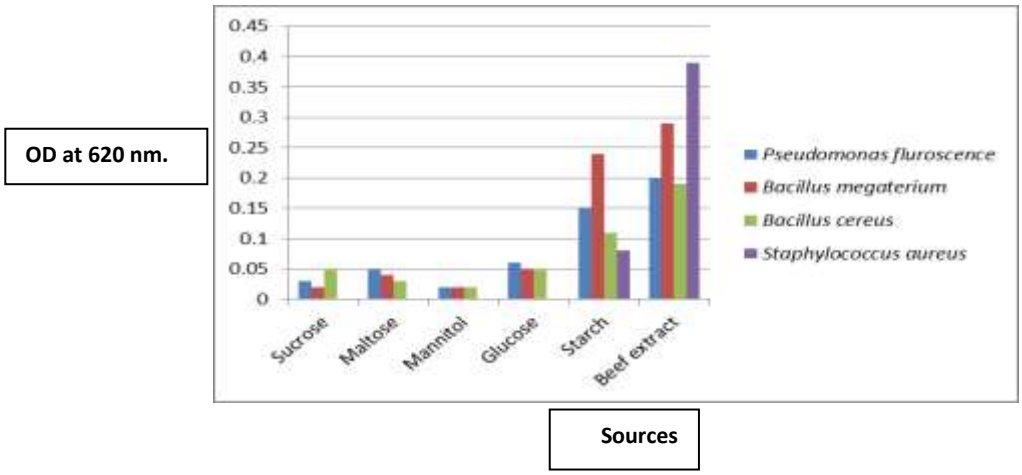


Figure 15: Graph between OD and sources showing best carbon source beef extract for all cultures

Table 8: OD for 1% Nitrogen sources:

Sources	*Pseudomonas fluroscence*	*Bacillus megaterium*	*Bacillus cereus*	*Staphylococcus aureus*
Urea	0.02	0.01	0.01	0.00
NH4Cl	0.01	0.02	0.02	0.00
Peptone	0.22	0.30	0.19	0.20
CH3COONa	0.04	0.03	0.02	0.00

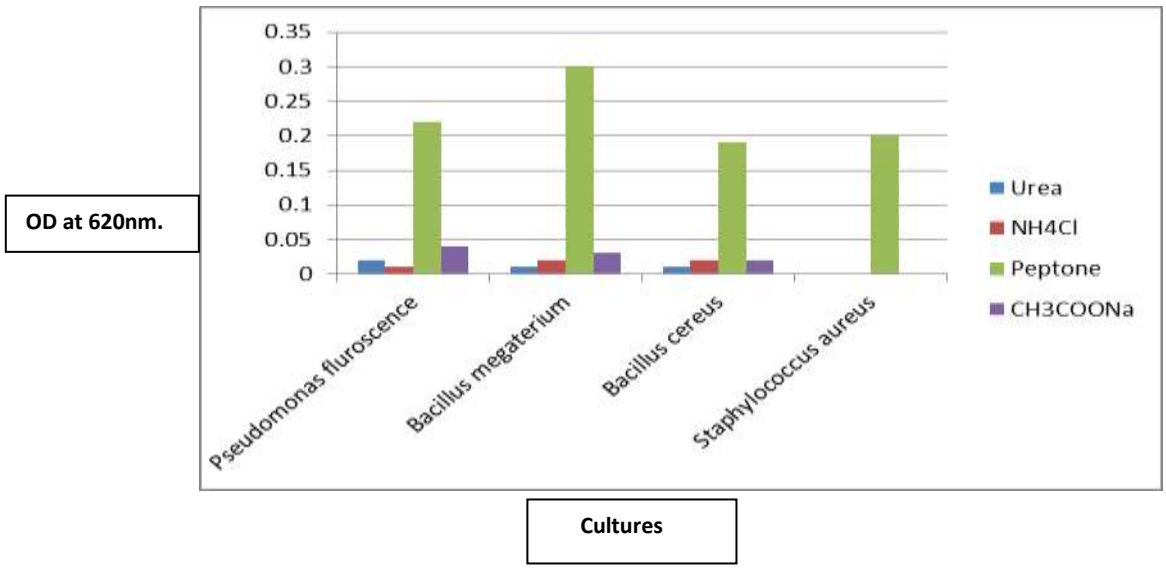


Figure 16: Graph between OD and Cultures showing best nitrogen source peptone for all cultures

Table 9: OD for different pH:

pH	*Pseudomonas fluroscence*	*Bacillus megaterium*	*Bacillus cereus*	*Staphylococcus aureus*
pH 4	0.03	0.01	0.01	0.08
pH6	0.12	0.08	0.08	0.18
pH7	0.13	0.10	0.14	0.24
pH8	0.10	0.13	0.15	0.26
pH10	0.01	0.02	0.01	0.15

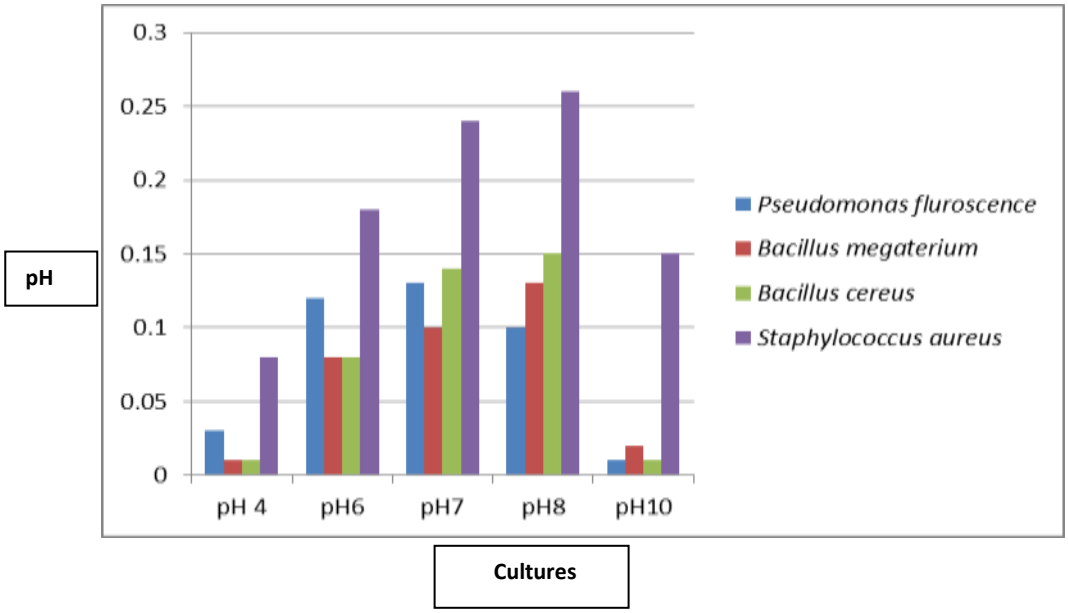


Figure 17: Graph between OD and cultures showing best pH from 6 to 8 for all cultures

Table 10: OD for different temperature:

Temperature	*Pseudomonas fluroscence*	*Bacillus megaterium*	*Bacillus cereus*	*Staphylococcus aureus*
4°C	0.00	0.00	0.00	0.19
50°C	0.31	0.25	0.26	0.31
37°C	0.48	0.62	0.64	0.42
Room Temp.	0.27	0.15	0.24	0.25

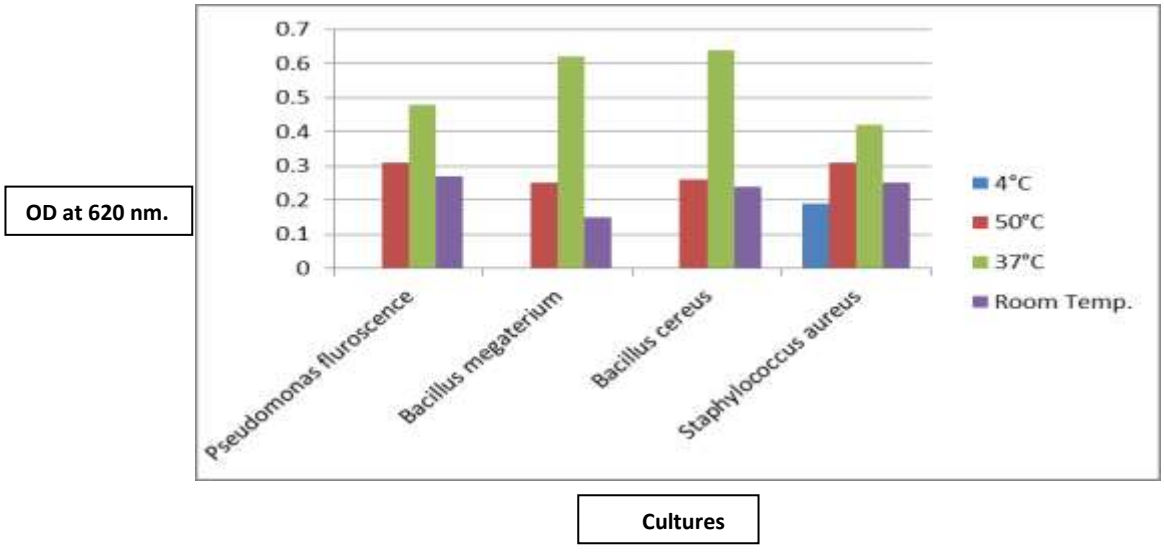


Figure 18: Graph between OD and cultures showing best temperature 37^{0}C for all cultures

Production on Optimized Media:

The best carbon, nitrogen, pH and temperature were used for production of Biosurfactant and these cultures were incubated at 37 ^{0} C for 48hrs in shaker incubator.

Table 19 – Best carbon, nitrogen, pH and temperature for appropriate culture

Cultures	Carbon source	Nitrogen source	pH	Temperature
Pseudomonas fluroscence	Beef extract	Peptone	7	37°C
Bacillus cereus	Beef extract	Peptone	8	37°C
Bacillus megaterium	Beef extract	Peptone	8	37°C
Staphylococcus aureus	Beef extract	Peptone	8	37°C

Figure 20: Oil degradation activity through cultures in present of best sources on first day

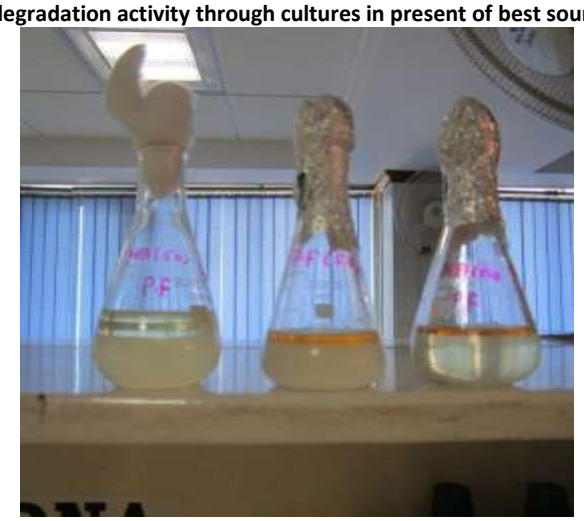


Figure 21: Oil degradation activity through cultures in present of best sources on third and fourth day

Figure 22: Oil degradation activity through cultures in present of best sources on sixth day

Table 12: Optical Density day by day to check oil degradation activity by cultures in shake flask method:

Days	*Pseudomonas fluroscence*	*Bacillus cereus*	*Bacillus megaterium*	*Staphylococcus aureus*
1st day	.04	.06	.07	.04
2nd day	0.11	0.14	0.12	0.10
3rd day	0.17	0.19	0.17	0.15
4th day	0.19	0.21	0.23	0.21
5th day	0.13	0.15	0.13	0.11
6th day	0.08	0.07	0.05	0.06

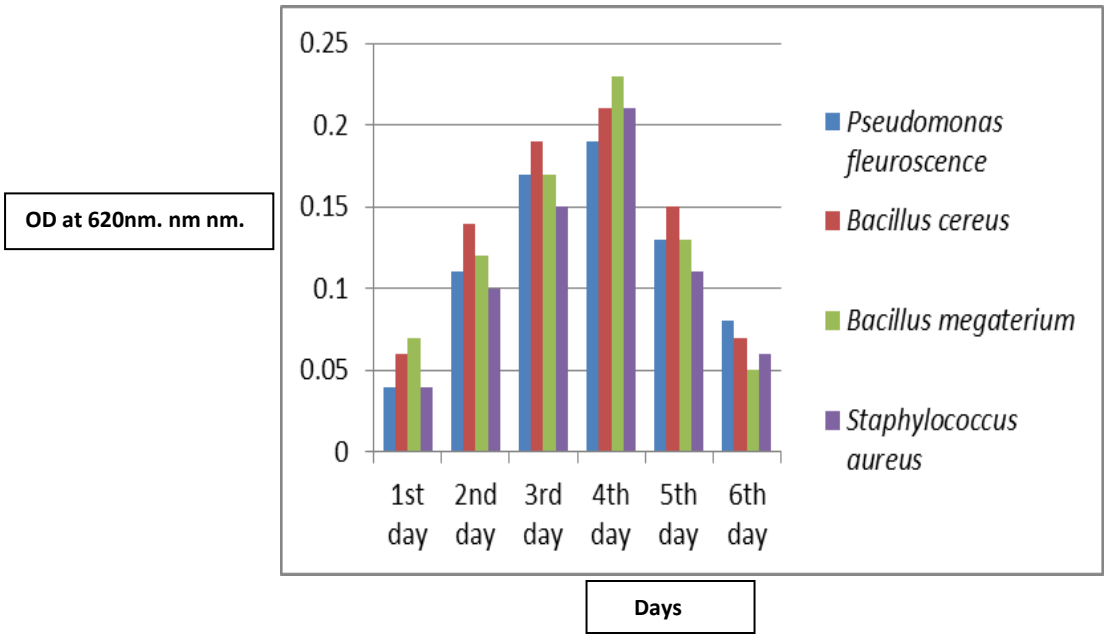


Figure 23: Graph between OD and days for cultures degrade oil in presence of best sources

Biosurfactant Isolation Results:

Bradford assay:

Table 13: Preparation of Standard Graph

Test Tube	Vol. of BSA (µl)	Vol. of D/W (µl)	Conc. Of BSA (mg/µl)	Vol. of Bradford	Incubation time	OD at 620nm.
1.	0	500	0	2.5 ml.	10 min. at room temp. at dark	00
2.	50	450	10	2.5 ml.		.07
3.	100	400	20	2.5 ml.		.16
4.	150	350	30	2.5 ml.		.26
5.	200	300	40	2.5 ml.		.32
6.	250	250	50	2.5 ml.		.36
7.	300	200	60	2.5 ml.		.43
8.	350	150	70	2.5 ml.		.43
9.	400	100	80	2.5 ml.		.46
10.	450	50	90	2.5 ml.		.47
11.	500	0	100	2.5 ml.		.55

OD for Supernatant- .11

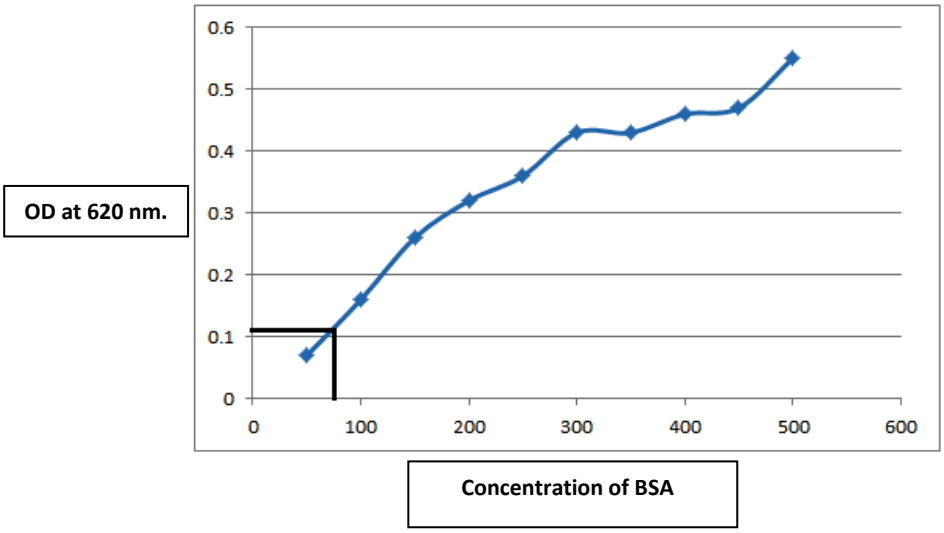


Figure 24: Standard graph between OD and Concentration of BSA

Enzyme assay DNS method:

Table 14: Preparation of Standard Graph

Test Tube	Vol. of standard maltose (µl)	Vol. of D/W (µl)	Conc. Of Maltose.	Vol. of DNS	Incubation time	Vol. of D/W	OD at 540nm.
1.	0	1000	0	1 ml.	In water bath at 100°C for 10 min.	5 ml.	0
2.	100	900	50	1 ml.		5 ml.	0.01
3.	200	800	100	1 ml.		5 ml.	0.02
4.	300	700	150	1 ml.		5 ml.	0.06
5.	400	600	200	1 ml.		5 ml.	0.07
6.	500	500	250	1 ml.		5 ml.	0.10
7.	600	400	300	1 ml.		5 ml.	0.15
8.	700	300	350	1 ml.		5 ml.	0.21
9.	800	200	400	1 ml.		5 ml.	0.19
10.	900	100	450	1 ml.		5 ml.	0.25
11.	**1000**	**0**	**500**	**1 ml.**		**5 ml.**	**0.28**

OD for Supernatant- 0.62

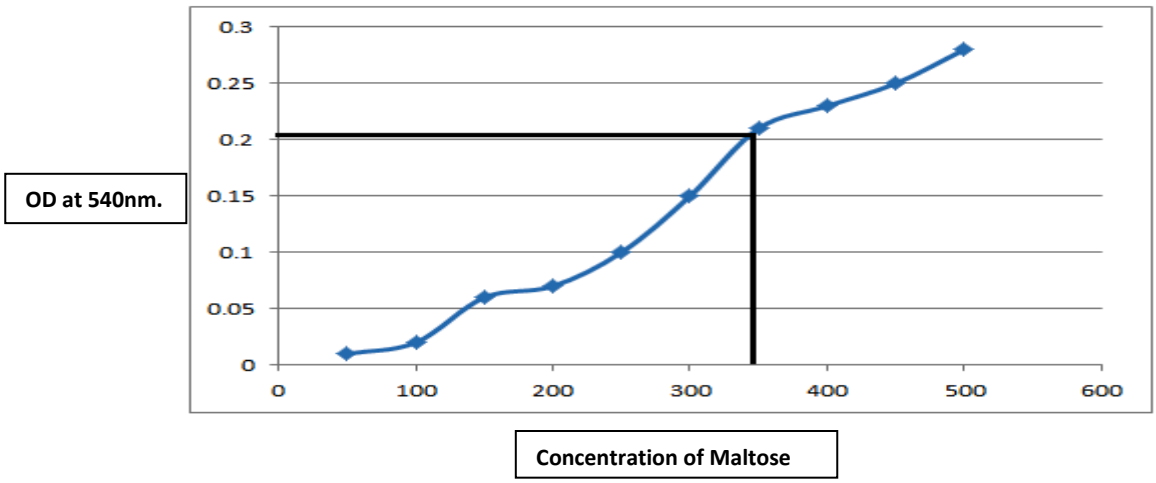


Figure 32: Standard graph between OD and Concentration of Maltose

Agarose Gel Electrophoresis result:

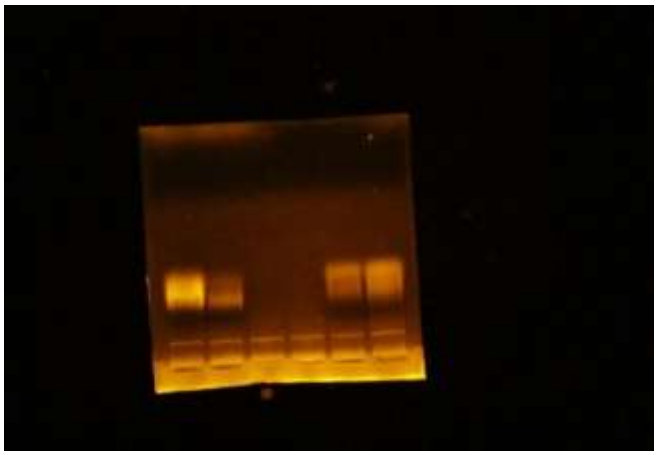


Figure 25: Shows the result of movement of plasmid bands from negative to positive poles

Transformation results:

Spreading:

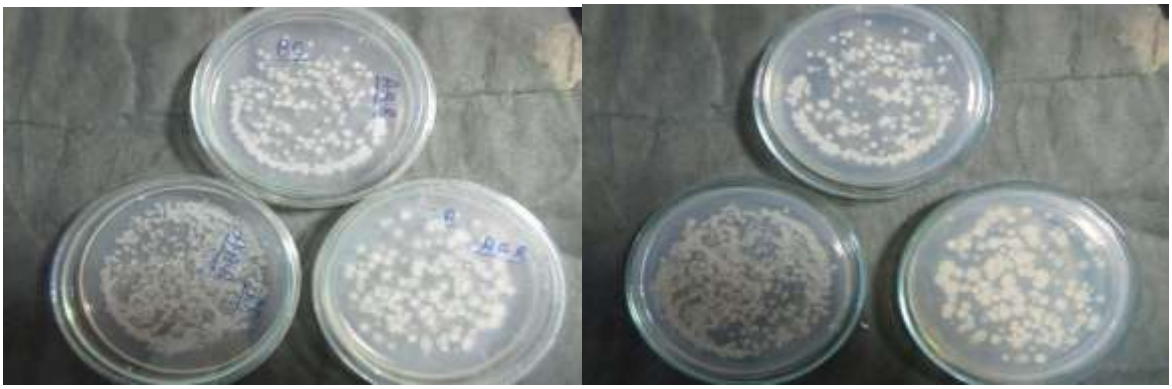


Figure 26: Spreading of transformed cultures

Oil degradation activity of transformed cell:

Figure 27: Oil degradation activity on first day

Figure 28: Oil degradation activity on fifth day through transformed plasmid of *Pseudomonas fluroscence* in *E. coli* which gives positive result

Figure 29: Oil degradation activity on fifth day through transformed plasmid of *Staphylococcus aureus* in *E. coli* which gives positive result

Figure 30: Oil degradation activity on fifth day through transformed plasmid of *Bacillus cereus* in *E. coli* which gives positive result

DISCUSSION:

Bioremediation is the use of micro-organism metabolism to remove pollutants. Bioremediation is the branch of biotechnology which deals with the methods of solving the environmental problems. It also plays vital role in cleaning the environment from pollutants and contaminants by using the microorganisms and fungi. Bacteria are the most important microbes in this process because they break the dead materials into organic matter and nutrients[11] [12].

Bioremediation looks at the whole system, the living soil communities, and aims to restore the maximum health, diversity, and life. bioremediation uses microbes to degrade hydrocarbons in soil. today bioremediation is considered as the most effective and pollution free method for removing crude oil pollution from contaminated sites, as this method makes use of microorganism.

Those bacteria which are degrades oil know as oil degrading microbes. In this present study there were three oil contaminated soil was taken and five cultures isolated and out of five cultures, only three cultures were identified and further used for experiment. The degradation of oil from culutre is check from three methods 1st is shake flask method 2nd is spread plate method and the last 3rd one is agar well diffusion method. There 3 cultures were isolated *Bacillus, Pseudomonas* and *Staphylococcus* they are showing positive results.[13] [14]

The best oil degradation result found in engine and mobil oil in the presence of *Pseudomonas fluroscence* culture. For checking the growth activity in bacterial culture. optimization technique was used and provide different carbon, nitrogen, suitable pH and optimum temperature as sources in the result we obtain beef extract and peptone were good carbon and nitrogen sources respectively and suitable pH was between 6 to 7 and optimum temperature was 37°C for their growth. After getting best carbon - nitrogen sources, pH and temperature for particular culture, shake flask method was again performed to check oil degradation activity of culture. Biosurfactant isolation was done and confirmation of these biosurfactant were done by Bradford assay (For protein) and Enzyme assay DNS method (for Proteins and Enzyme) and isolation of lipid was also performed by using chloroform ($CHCl_3$) and plasmids were isolated from all cultures and further restriction digestion and ligation were performed and *E.coli* cells were used as a host cell for competent cells preparation and Transformation was performed. Further the activity of non-oil degrading *E.coli* culture was checked compare to transformed *E.coli* cells which shows the positive result of oil degradation activity[15][16].

CONCLUSION AND FUTURE PROSPECTS:

The present study involved optimization production and characterization of oil degradation producing microbes isolated from oil contaminated sources and further gene

expression was done to convert non - oil degrading producing microbes into oil degrading microbes. According to results basis the samples were collected from automobile shop and the identified culture were *Bacillus cereus, Pseudomonas fluroscence and Staphylococcus aureus.* These cultures were tested for degradation of mobil oil, engine oil and petrol. The degradation was possible after ten days and further the optimization was done with the help best carbon sources, nitrogen sources, pH and temperature. The biosurfactant can be the form of protein, enzyme and lipids etc. that can isolated from each culture and activity was tested against oil. The expression of gene was conducted using plasmid isolation, restriction- digestion, ligation and transformation which transformed non- oil degrading producing microbes into oil degrading microbes.

The future prospect for the study can involve in- situ and ex- situ remediation as well as phytoremediation. The bioremediation is an eco-friendly process which cleans up the environment by using biological agents. The microbes which degrade the oil can be off various types and the type of biosurfactant can also be varied. So the biosurfactants can be isolated and there activity can be tested by using different types of substrate in the form of oil.

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