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OPTIMIZATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCING MICROBES ISOLATED FROM OIL CONTAMINATED SOIL AND EXPRESSION OF BIOSURFACTANT GENES IN *E.COLI*

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Abstract

The present study is carried out by optimization, isolation and characterization of biosurfactant producing microbes and also expression oil degrading genes from oil degrading microbes to non-oil degrading microbes. Bioremediation is a process in which the environment can cleanup through microbes. The various types of soil sample were collected from different regions of Unnao and Lucknow. The soil was contaminated with oil. Total 15 cultures were isolated through serial dilution method out of which 4 cultures were characterized through Bergey's manual. These cultures were tested for oil degradation by test tube method in small scale and large scale method. Four types of oils were used: petrol, diesel, kerosene and vegetable oil. The best results were obtained for all these cultures against petrol. The identified cultures were: *M.luteus, S.aureus, B.cereus, N.flavescens.* Further plasmid isolation was done by alkaline denaturation method and transformation process was completed by heat shock treatment. The host cells used as *E.coli* which was not oil degrading microbes and after transformation the oil degrading capacity was developed which shows positive results and also optimization was done to enhance the capacity of oil degrading microbes. The optimization parameters include carbon source (glucose, dextrose, maltose, sucrose, beef extract), nitrogen source (urea, peptone, KNO_3, NH_4Cl), metal ion (Zn, Pb, Ca, Mg) and pH (5,6,7,8).

INTRODUCTION

Bio surfactants are a structurally diverse group of surface-active substances produced by microorganisms. All bio surfactants are amphiphiles, they consist of two parts—a polar (hydrophilic) moiety and non polar (hydrophobic) group. A hydrophilic group consists of mono, oligoor polysaccharides, peptides or proteins and a hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohol. A characteristic feature of bio surfactants is a hydrophilic-lipophilic balance (HLB) which specifies the portion of hydrophilic and hydrophobic constituents in surface-active substances.Due to their amphiphilic structure, bio surfactants increase the surface area of hydrophobic water-insoluble substances, increase the water bioavailability of such substances and change the properties of the bacterial cell surface. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents. The bio surfactants accumulate at the interface between two immiscible fluids or between a fluid and a solid. By reducing surface (liquid-air) and interfacial (liquid-liquid) tension they reduce the repulsive forces between two dissimilar phases and allow these two phases to mix and interact more easily [1]. The unique properties of bio surfactants allow their use and possible replacement of chemically synthesized surfactants in a great number of industrial operations. A new experimental procedure and robust equipment have been developed for studying oil degradation at sea and the system has been proved under harsh environmental conditions [2].There are many advantages of bio surfactants as compared to their chemically synthesized counterparts. Some of those are:

- Biodegradability.
- Generally low toxicity.
- Biocompatability and digestibility, which allows their application in cosmetics, pharmaceuticals and as functional food additives.

Concerning disadvantages, one of the problems is related to large scale and cheap production of biosurfactants. Large quantities are particularly needed in petroleum and environmental applications, which, due to the bulk use, may be

expensive. To overcome this problem, processes should be coupled to utilization of waste substrates combating at the same time their polluting effect, which balances the overall costs [3].

Objective:

- Isolation of biosurfactant producing microbe from oil contaminated site.
- To check biosurfactant activity in small scale and large scale.
- Identification of biosurfactant producing microbe through Bergey's manual.
- Expression of biosurfactant genes in *E.coli*.
- Optimization of cultural conditions to enhance biosurfantant activity.

MATERIALS AND METHODS

Sample collection:

Soil samples were collected from various region-

- R and S- Gomtinagar, Lucknow.
- A- Nagar Palika Road, Unnao.
- B- Lucknow Kanpur Highway.
- C- Babuganj, Unnnao.

Serial dilution:

This method is used on the principle that when soil sample or water sample along with bacterial colonies taken, the result obtained in the form of reduce number of colonies.The microbes are having importance in the industries for enzyme as well as antibiotic production.

Dilution = volume of the sample/ total volume of the sample and the diluents.

Pure culture:

A pure culture is a culture is a culture containing a single colony of an organism. A pure culture is usually derived from mixed culture by transferring a small sample in to a new sterile growth medium in such a manner as to disperse the individual cell across the surface medium or by thinning the individual cells so that when multiply each will from a discrete colony.

Small scale activity test:

A cross check was performed at small scale in order tocheck the activity of various cultures for production ofbiosurfactant [4].

Growth kinetics study:

Growth is orderly increase in all major constituents of an organism, involving several structures, nucleic acid, protein and all other cell components from nutrient obtained from outside the cell. Growth kinetics process was used to determine the time period at which the culture showed optimum activity.

Large scale activity test:

The cultures which showed the best activity with specific substrate initially were further used for large scale method [5][6].

Characterization of bacterial culture:

Characterization of micro-organisms according to Bergey's manual:

Bio-chemical test:

Biochemical method was performed to differentiate between the unknown culture.

Catalase test, Endospore staining, Acid-Fast staining, Carbohydrate test, Mannitol test were performed according to Bergey'smanual[7].

Confirmatory test for *Micrococcus luteus*:

Oxidase test:

This test is used for the confirmation of desired bacteria. Cytochrome oxidase is an enzyme found in some bacteria that transfer electrons to oxygen. Presence of cytochrome oxidase can be detected through the use of an oxidase reagent (GordnMcleod reagent) which acts as an electron donor to cytochrome oxidase.If bacteria oxidize the reagent it turns dark purple due to formation of indophenol blue indicating positive reaction [8].

Confirmatory test for *Bacillus cereus*:

Starch hydrolysis:

Starch is a complex carbohydrate (Polysaccharide) composed of two constituents amylose, a straight chain polymer of 200-300 glucose units, and amylopectin a larger branched polymer with phosphate groups [9].

Confirmatory test for *Staphylococcus aureus*:

Mannitol salt agar media:

The differential medium allows the investigator to distinguish between different types of bacteria based on some observable trait in their pattern of growth on the medium. The essential feature of

this media is a very high salt concentration which mainly *Staphylococcus* can tolerate and inhibits the growth of most other bacteria. It also contains mannitol which acts as fermentable sugar and a pH indicator dye. Mannitol fermenting colonies produce acid which react with the indicator dye forming a colored halo around the colonies.

Confirmatory test for *Neisseria flavescens*:

Oxidase test:

It was performed similarly as mentioned above for *Micrococcus luteus.*

Nitrate reduction test:

Nitrate may be reduced to multiple compounds by two processes- anaerobic respiration and denitrification. In aerobic respiration the bacterium uses nitrate as it's terminal electron acceptor, reducing nitrate to a variety of compounds, while denitrification reduces nitrate solely to molecular nitrogen. The appearance of red color indicates positive result and no red color indicates negative result.

Plasmid isolation:

In addition to chromosomal DNA, a bacterium may also carry an additional circular piece of DNA called a plasmid. The plasmids were isolated from the respective cultures by using the alkaline lysis method. The entire process involved use of three buffers which are as follows:

- **P1 buffer:**Resuspension buffer.

It consists of-50 mMTrisHCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNAse A.

- **P2 buffer:**lysis buffer.

It consists of-200 mMNaOH, 1% SDS.

- **P3 buffer:** Neutralization buffer.

It consists of-3M Potassium Acetate (pH 5.5).

Agarose gel electrophoresis:

For the DNA gel electrophoresis generally TAE buffer is preferred as it provides faster electrophoretic migration of linear DNA and it is easier to recover DNA fragment from the gel for cloning or preparing probes.

Transformation:

Transfer of genetic material from one cell to another with the help of plasmid which works as vector.

Competent cells preparation:

Cells which are ready to uptake foreign DNA are called competent cells.

Activity test after transformation:

After the transformation of *E.coli*with the oil degrading genes, theactivity was further tested.

Production in production media:

The characterized cultures were produced in production media i.e. inselective media which favors the growth of particular microbe.

Optimization:

Optimization refers to limiting of sources. We chose various sources but only some specific are suitable for growth of bacteria.

- **Carbon sources:** The effect of carbon sources such as- maltose, glucose, starch, dextrose and lactose at a concentration of 1% was examined by replacing in the production media.
- **Nitrogen sources:** Various nitrogen sources like- beef extract, tryptone, glycine, yeast extract and peptone at a concentration 0.5% was examined by replacing in the production media.
- **Metal ions:** Various metal ions like- $CaCl_2$, $MgSO_4$, Na_2HPO_4, $Pb(NO_3)_2$, $CuSO_4$ at concentration of 0.2% was examined by replacing in the production media.
- **pH:** The effect of pH such as- 5, 6, 7, 8 was examined by replacing in production media.

Final optimization:

The final optimization aimed at focussing on best carbon source, nitrogen source, metal ion and pH to achieve the better cultural growth. We chose the best parameters from the results of initial optimization and then subjected the isolated culture to those conditions.

Results

A total 15 cultures of bacteria were isolated from 4 different areas of Lucknow and Unnao. Out of 15 isolates there were 4 cultures detected for their activity of producing biosurfactant and thus were helpful for bioremediation. The biosurfactant genes were further isolated and were expressed in non-biosurfactant

producing microbe. The cultures were also maintained for optimization of carbon sources, nitrogen sources, metal ions and pH.

Serial dilution:

The serial dilution method was performed in ordered to get pure and reduced number of bacterial colonies. Total 15 isolates were found and out of 15 cultures 4 cultures were used for further work.

Sub culturing:

The procedure of transfer of microorganisms from their parent growth source to a fresh one or from one medium to another.

Small scale activity test:

Growth kinetics study:

Large scale activity test:

Colony morphology:

A1: Nagar Palika Road, Unnao.

B2: Lucknow Kanpur Highway.

B3: Lucknow Kanpur Highway.

S1: GomtiNagar,Lucknow.

Biochemical tests:

The occurrence of bubbles on slide indicates the positive test for catalase test. The carbohydrate test uses phenol red as an indicator. If the color change occurs from red to yellow then it is considered as positive test.

The mannitol test uses phenol red as an indicator. If the color change occurs from red to yellow then it is considered as positive test.

The glucose test uses bromothymol blue as an indicator. If the blue changes then it is considered as positive test.

Confirmatory test:

Oxidase test for *M.luteus*:

The occurence of dark purple color indicates the presence of *M.luteus*.

Starch hydrolysis for *B.cereus*:

The yellow zone around growth of culture indicates that the starch is being hydrolysed by *B.cereus.*

Mannitol salt agar test for *S.aureus*:

The growth on mannitol salt agar media indicates the presence of *S.aureus*.

Coagulase test for *S.aureus*:

The occurrence of agglutination in test sample indicates the presence of *S.aureus.*

Oxidase test for *N.flavescens*:

The occurrence of dark purple color indicates the presence of *N.flavescens.*

Nitrate reduction test for *N.flavescens*:

The nitrate reduction test is considered positive when red or pink color appears. The above test was negative as there was no change in color. This indicates the presence of *N.flavescens.*

Plasmid isolation:

Activity test after transformation:

Production in production media:

Table 8 showed that for all the isolates stationary phases were obtained at 4^{th} day.

Optimization Parameters

Table 9 showed that best carbon source for *M.luteus* was Maltose, for *B.cereus*,Glucose , for *S.aureus* , Lactose and for *N.flavescenes* it was Dextrose.

Table 10 showed that best nitrogen source for *M.luteus* was peptone, for *B.cereus*,NaCl, for *S.aureus* , NaCland for *N.flavescenes* it was Peptone.

Table 11showed that best pH for *M.luteus* was pH- 6, for *B.cereus*, pH-6 for *S.aureus* , pH- 7 and for *N.flavescenes* it was pH- 6..

Table 12 showed that best metal ion for *M.luteus* was Ca, for *B.cereus* , Zn, for *S.aureus* , Mg and for *N.flavescenes* it was Ca.

Discussion

Bioremediation is a process in which the environment can cleanup through microbes. There are so many types of microbes which used to eliminate contamination in seawater. The contamination can be in the form of oil or some chemicals. This type of contamination may lead to adverse effect on the organisms, animals as well as plants mainly found in sea water.

The present study is carried out by isolation, production and characterization of oil degrading microbes from oil contaminated soil and also expression oil degrading genes from oil degrading microbes to non-oil degrading microbes. The various types of soil sample were

collected from different regions of Unnao and Lucknow. The soil was contaminated with oil because of this reason there are chances to get oil degrading microbes.

Total 15 cultures were isolated through serial dilution method out of which 4 cultures were characterized through Bergey's manual. These cultures were tested for oil degradation by test tube method in small scale and flask method in large scale method. Four types of oil were used: petrol, diesel, kerosene and vegetable oil. The best results were obtained for all these cultures against petrol. The identified cultures were: *M.luteus, S.aureus, B.cereus, N.flavescens.*

Further plasmid isolation was done by alkaline denaturation method and transformation process was completed by heat shock treatment. The host cells used as *E.coli* which was not oil degrading microbes and after transformation compared with *E.coli*(transformed) the oil degrading capacity was developed which shows positive results and also optimization was done to enhance the capacity of oil degrading microbes. The optimization parameters include carbon source (glucose, dextrose, maltose, sucrose, beef extract), nitrogen source (urea, peptone, KNO_3, NH_4Cl), metal ion (Zn, Pb, Ca, Mg) and pH (5,6,7,8).

The best conditions for cultures like suitable carbon source, nitrogen source, metal ion and pH was provided in the production media to get best results. For *S.aureus*suitable carbon source is lactose, nitrogen source is urea, metal ion is Zn and pH is 7.For *B.cereus*suitable carbon source is sucrose, nitrogen source is yeast, metal ion is Pb and pH is 6. For *M.luteus*suitable carbon source is maltose, nitrogen source is peptone, metal ion is Mg and pH is 6. For*N.flavescens*suitable carbon source is sucrose, nitrogen source is peptone, metal ion is Ca and pH is 7.

Conclusions and future perspectives

At the end of the experiment it was concluded that out of 15 cultures 4 cultures were identified. The culture A1 was identified as *Micrococcus luteus,* B2 as *Bacillus cereus,* B3 as *Staphylococcus aureus*and S1 as *Neisseria flavescens.* These cultures were subjected to check activity test for production of biosurfactant and finally were successful in degradation of oil.

Further the biosurfactant producing genes were expressed in non biosurfactant producing microbe and the cultures were later optimized to different parameters for better results. Thus, it was concluded that *E.coli* which was non biosurfactant producing microbe was transformed in away to develop oil degrading capability. Among the characterized cultures *Micrococcus luteus* was found to be the most active microbe in production of biosurfactant.

Application of biosurfactant and biosurfactant-producing bacteria in environmental technologies (bioremediation and phytoremediation) has been studied. Both organic and inorganic contaminants can be removed through different processes (physico-chemical and biological) in which bio surfactants are involved. Due to their biodegradability and low toxicity, they are very promising for use in environmental biotechnologies. The commercial success of bio surfactants is still limited by their high production cost. Optimized growth conditions using cheap renewable substrates (agro-industrial wastes) and novel, efficient methods for isolation and purification of bio surfactants could make their production more economically feasible. Another important aspect regarding biological remediation technologies is the use of biosurfactant in the process on a large scale. To felicitate this process, new techniques should be developed such as foams or micro-foams (colloidal gas aphrons-CGA) in conjunction with biosurfactants.

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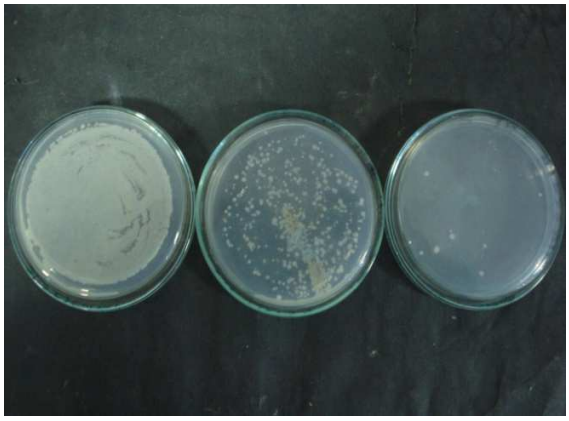


Fig1: Bacterial colonies in a mixed culture.

Fig 2: shows subculturing of culture B3, B2, A1 and S1.

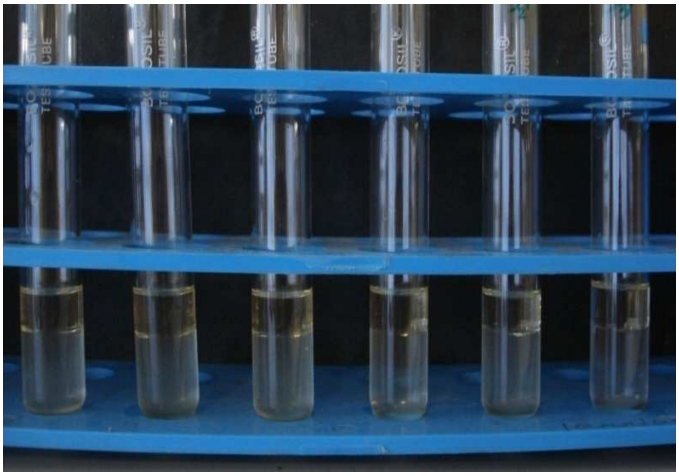


Fig 3: shows test tube method for activity test of sample with various substrates.

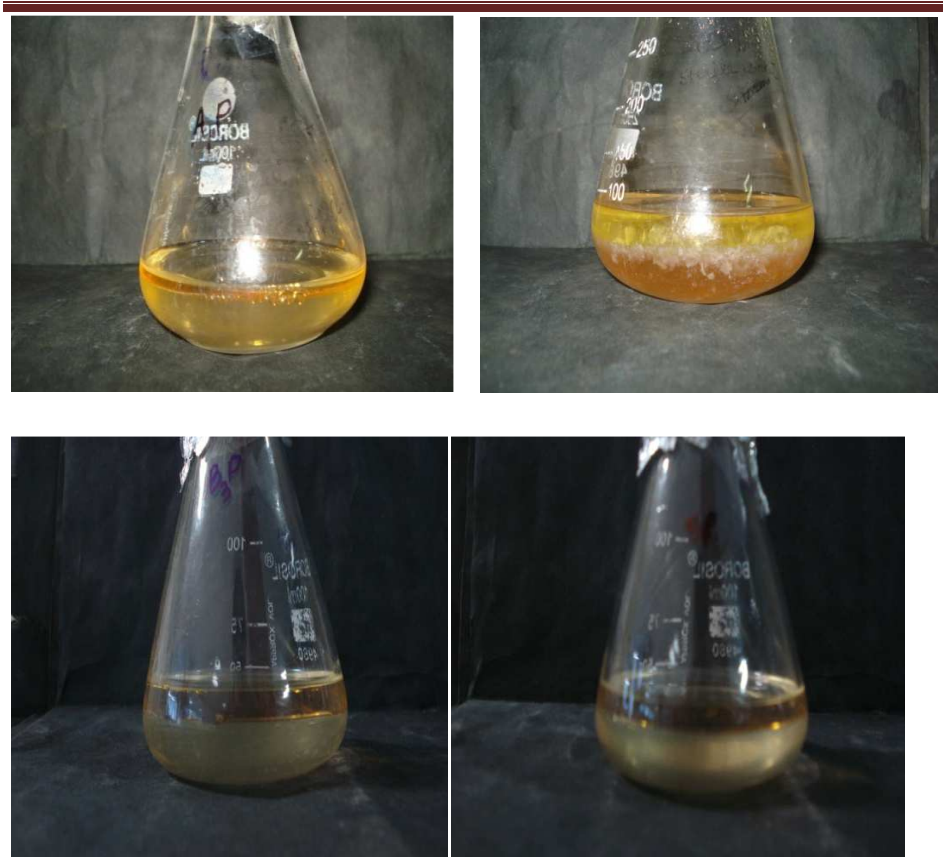


Fig 4: shows flask method for cultures A1, B2, B3 and S1 using petrol as substrate.

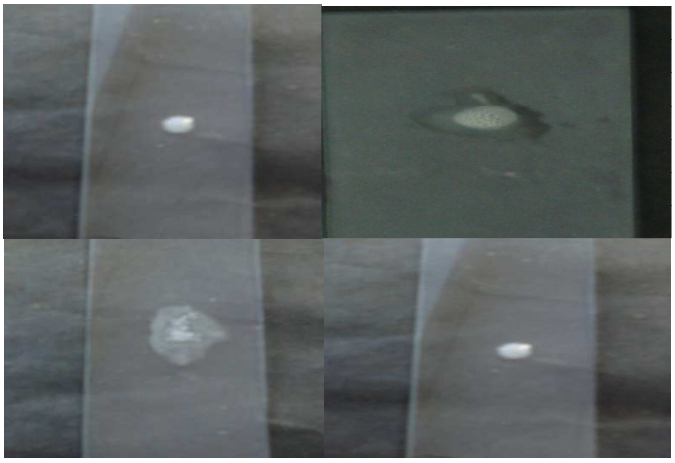


Fig 5: shows catalase test for culture A1, B2, B3 and S1.

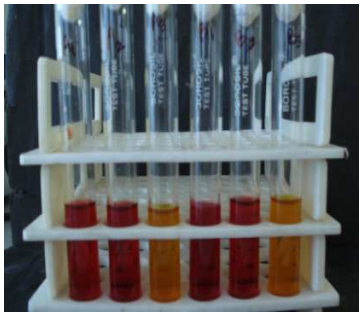


Fig 6: shows carbohydrate test for the isolated cultures

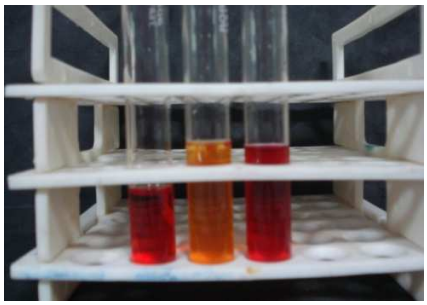


Fig 7: shows mannitol test for the isolated cultures.

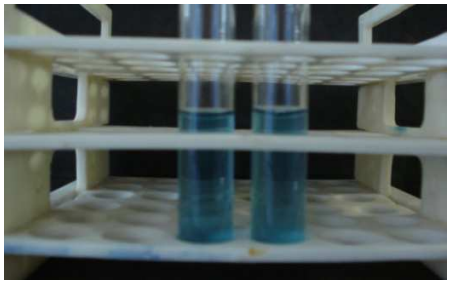


Fig 8: shows glucose test for the isolated cultures.

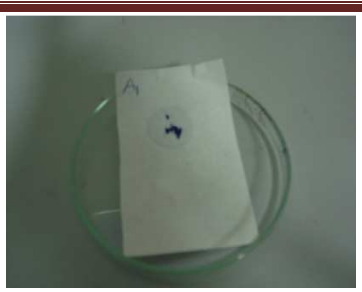


Fig 9: shows confirmation of *M.luteus* in A1 culture.

Fig 10: shows confirmation of *B.cereus* in culture B2.

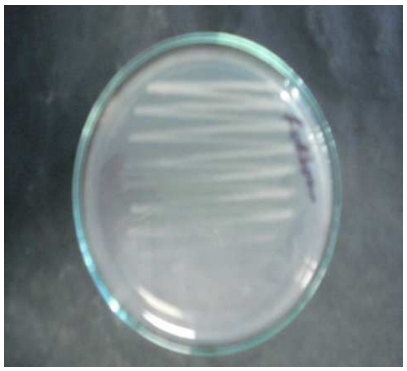


Fig 11: shows confirmation for *S.aureus* in culture B3.

Fig 12: shows confirmation for *S.aureus* in culture B3.

Fig 13: shows confirmation for *N.flavescens* in culture S1.

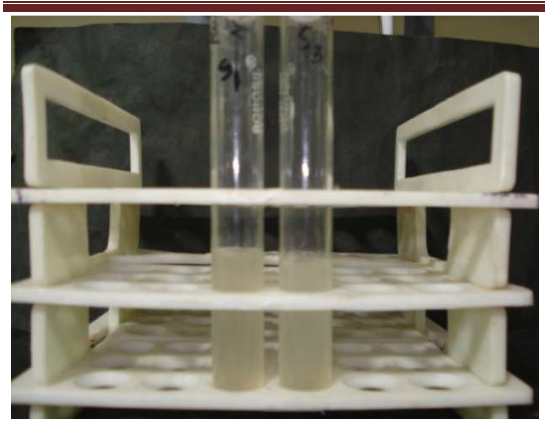


Fig 14: shows confirmation for *N.flavescens* in culture S1.

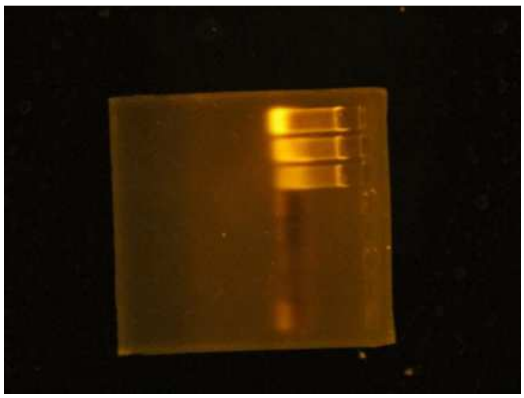
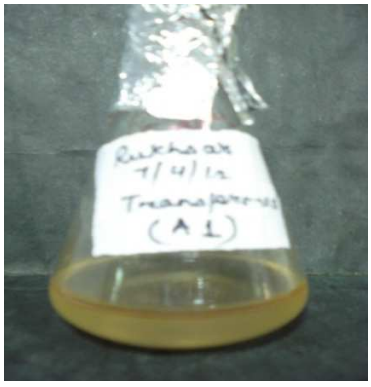


Fig 15: shows plasmid isolation for culture A1, B2,B3 and S1.



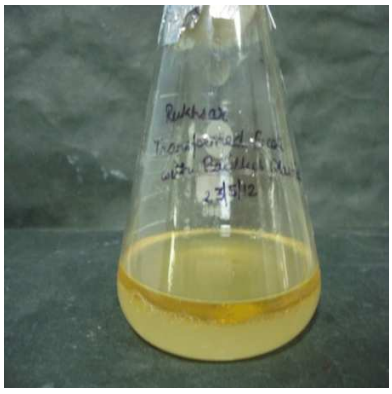


Fig 16: shows oil degradation in *E.coli* with plasmid of *M.luteus* and *B.cereus* after transformation.

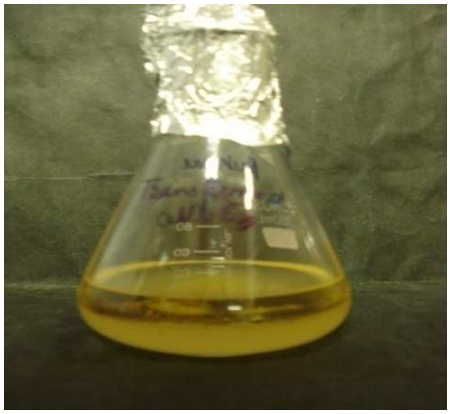
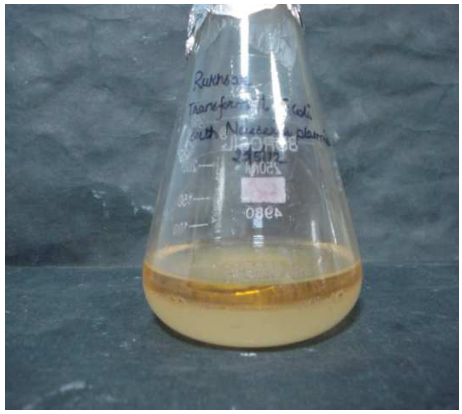



Fig 17: shows oil degradation in *E.coli* with plasmid of *S.aureus* and *N.flavescens* after transformation.

Table 2: illustrates decrease in level of substrate layer.

Sample	Blank (cm)	Diesel (cm)	Petrol (cm)	Kerosine (cm)	V.Oil (cm)
R1	1.0	1.0	0.4	1.0	0.9
R2	1.0	1.0	0.4	1.0	0.9
R3	1.0	1.0	0.4	1.0	0.8
S1	1.0	1.0	0.3	1.0	1.0
S2	1.0	1.0	0.3	1.0	1.0
S3	1.0	1.0	0.4	1.0	1.0
A1	1.0	1.0	0.2	1.0	0.9
A2	1.0	1.0	0.4	1.0	1.0
A3	1.0	1.0	0.4	1.0	0.9
B1	1.0	1.0	0.3	1.0	0.9
B2	1.0	1.0	0.3	1.0	1.0
B3	1.0	1.0	0.3	1.0	1.0
C1	1.0	1.0	0.3	1.0	0.6
C2	1.0	1.0	0.4	1.0	0.8
C3	1.0	1.0	0.4	1.0	1.0

Table 3: illustrates growth kinetics for the isolated cultures.

No. of Days	Blank	A1	B2	B3	S1
1	0.00	0.35	0.73	0.49	0.61
2	0.00	0.50	0.56	0.56	0.78
3	0.00	0.49	0.79	0.79	0.62

Table showed the stationary phases were obtained for all the cultures were 3 days.

Table 4: illustrates decrease in layer of petrol.

Sample	Blank (cm)	Petrol (cm)
A1	0.00	0.2
B2	0.00	0.4
B3	0.00	0.4
S1	0.00	0.5

Table 5: colony morphology of cultures A1, B2, B3 and S1.

Characteristics	A1	B2	B3	S1
Shape	Regular	Regular	Regular	Regular
Colour	Off-white	White	Golden brown	White
Texture	Smooth	Smooth	Smooth	Smooth
Margine	Lobate	Entire	Entire	Entire
Opacity	Translucent	Opaque	Opaque	Opaque
Elevation	Elevated	Elevated	Elevated	Elevated

Table 6: illustrates biochemical analysis of isolated cultures.

Biochemical test	A1	B2	B3	S1
Gram staining	+ve (cocci)	+ve (rods)	+ve (cocci)	-ve (cocci)
Catalase test	+ve	+ve	+ve	+ve
Endospore test	-ve	+ve	-ve	-
Carbohydrate test	-ve	+ve	-	-
Mannitol test	-	+ve	+ve	-
Glucose test	+ve	-	-ve	-ve

Table 7: illustrates the decrease in layer of petrol.

Blank (cm)	*E.coli* transformed with *M.luteus* plasmid (cm)	*E.coli* transformed with *B.cereus* plasmid (cm)	*E.coli* transformed with *S.aureus* plasmid (cm)	*E.coli* transformed with *N.flavescens* plasmid (cm)
0.00	0.2	0.5	0.5	0.6

Table 8: illustrates O.D of isolated cultures in production media.

No. of days	*M.luteus*	*B.cereus*	*S.aureus*	*N.flavescens*
1	0.48	0.03	0.17	0.61
2	0.62	0.08	0.26	0.75
3	0.77	0.08	0.50	0.78
4	0.69	0.01	0.66	0.62

Table 9:

Carbon sources	*M.luteus*	*B.cereus*	*S.aureus*	*N.flavescens*
Maltose	0.72	0.14	0.25	0.27
Glucose	0.61	0.21	0.28	0.18
Dextrose	0.60	0.08	0.29	0.33
Lactose	0.66	0.13	0.30	0.18

Table 10:

Nitrogen Sources	*M.luteus*	*B.cereus*	*S.aureus*	*N.flavescens*
Peptone	0.80	0.26	0.30	0.42
KNO_3	0.70	0.32	0.31	0.02
NaCl	0.68	0.35	0.38	0.27
$(NH_4)_2.SO_4$	0.68	0.08	0.37	0.35

Table 11:

pH	*M.luteus*	*B.cereus*	*S.aureus*	*N.flavescens*
pH 5	0.62	0.23	0.00	0.01
pH 6	0.74	0.28	0.00	0.18
pH 7	0.68	0.18	0.05	0.24
pH 8	0.65	0.19	0.00	0.02

Table 12:

Metal ions	*M.luteus*	*B.cereus*	*S.aureus*	*N.flavescens*
$CaCl_2$	0.26	0.06	0.00	0.39
$MgSO_4$	0.03	0.00	0.19	0.35
$Pb(NO_3)_2$	0.68	0.08	0.00	0.20
$ZnSO_4$	0.00	0.11	0.01	0.17

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