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Research Article

Analysis of the Behavior of oil degrading Bacteria Enriched Bioremediation of spilled oil

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ABSTRACT

Petroleum hydrocarbon contamination in soil is the major problems resulting from the activities related to petroleum industry, automobile garage/shops, and accidental release in soil. The present thesis work is carried out by isolation, identification, characterization & optimization of spilled oil degrading bacteria from spilled oil contaminated soil sites. Two oil degrading bacteria were isolated from different places of Lucknow, U.P.India. In this work the two oil degrading bacteria i.e. *Bacillus cereus* and *Streptococcus faecalis* were isolated, identified and Characterize by colony morphology,staining and biochemical characteristics based on Bergey's Manual .The used Mobil oil &2T oil is used to check oil degradation activity by the method of the Shake Erlenmeyer Flask Method, Agar well diffusion method & Spread Plate method. The Biosurfactant activity was measured till 7 days and further optimization was carried out for best carbon source, nitrogen source,pH, temperature and again Checked biosurfactant activities. Starch and Sucrose is the best Carbon sources in *Bacillus cereus* and *Streptococcus faecalis* respectively. Urea and Peptone is the best Nitrogen sources for *Bacillus cereus* and *Streptococcus faecalis* respectively. pH 7 is the best for both *Bacillus cereus* and *Streptococcus faecalis* and optimum temperature 37^0C was best for both cultures.

Key wards- Bio surfactant, Petroleum hydrocarbon, Oil degradation, Biosurfactant, optimization.

1. INTRODUCTION:-

Soil contamination due to spill oil is one of major environmental problem as they damage the soil groundwater, water ecosystems. Oil spill can be from petroleum industries, used engine oil from different mechanical shops, from different petrol pump which leads to adversely damage to the soil and ground water due to seepage into ground water. Accumulation of hydrocarbon into the soil is also due to leakage, accidental oil spillsbecause of exploration, production, refining, transport and storage of petroleum and petroleum products [1].

Spill in the environment originate a long-lasting damage to human health, aquatic ecosystems, soil and natural resources. Spills on land are easily absorbed into the food-chain through soil through plant roots [2]. Poly aromatic hydrocarbons (PAHs) are the product of incomplete combustion and it enters the body and accumulate fatty tissues such as liver [3]. Oil spill are causing a major problem to soil that reduce fertility and ground water and marine environment [4]. Bioremediation is becoming an increasingly popular technology for remediating contaminated sites. Bioremediation approach is currently applied to contain contaminants in soil, groundwater, surface water and sediments including air [5]. Microorganisms decompose most organic compounds into carbon dioxide, water and mineral matter , such as sulphate, nitrate and other inorganic compounds. The present study is carried out by isolation, identification, Screeing characterization and optimization of oil degrading microbes and also their growth kinetics study[6][7].

2. MATERIALS & METHODS:

Sterilization of glassware and media: glassware is sterilized in hot air oven at160^0C for 50-60 minutes. And all the media are firstly prepared in Erlenmayer flask and then kept in autoclave for sterilization at 15 psi pressure, 121^0C temperature for 15-20 minutes.

Collection of Soil samples: Soil samples were collected from three different oil contaminated soil sites. Sample from roadways bus workshop gomtinagar lucknow (S1), Samples from Anup auto service center gomtinagar Lucknow (S2),Samples from Vibhutikhand petrol pump gomtinagar lucknow (S3). Soil were collectected arbitrarily 5-10 cm

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underneath the surface using spatualla and were packed in sterized polybags and transferred to the laboratory (MRD LifeSciences, Gnomtinagar Lucknow) and stored at 4^0C unill the end of the work.

Microbial Isolation from soil samples: The samples S1, S2 and S3 were isolated for bacteriological examination by serial dilution method followed by agar well diffusion method and sub culturing. After incubation period of petriplates, nine different bacterial culture were selected for screening of biosurfactant production [8].

Screening of oil degradation activity: All the nine isolates were subjected to screening of oil degradation property where the oil degradation potential was decided based on agar well diffusion method, shake flask method and spread plate culture technique.

Agar well diffusion Method: Prepared sterilized Nutrient Agar plates and Spread 100 µl of oil (mobile oil and 2T Oil) and then prepared well into the NA Plates and load 50 µl of bacterial culture in well and then incubate at 37 ^{0}C for overnight and observe result, if culture showing growth in the presence of oil it means culture have property to degrade oil.

Shake Flask Method: Prepared Nutrient Broth and added half quantity of oil (mobile oil and 2T Oil) and autoclave it. After that cooled at room temperature and then inoculate bacterial culture and incubated at 37 ^{0}C for 7 to 10 days. Day by day measurement was done and growth was also checked by using colorimeter by taking OD at 620 nm.

Spread plate method: Prepare sterilized nutrient agar plates and spread 100 µl of oil and then spread 50 µl bacterial culture over it and incubate at 37 ^{0}C for overnight and observe result, if culture showing growth in the presence of oil it means culture have property to degrade oil.

Characterization of bacterial Isolates: Out of nine bacterial isolates two isolates were identified based on culture morphology, gram's staining and then biochemical test according to the Bergey's manual [9][10][11].

Growth Kinetics study of isolated strains: Growth kinetics study was applied to determine the time period at which cultures showed optimum activity. Prepared Nutrient broth in 250 ml Erlenmeyer flask and autoclave it, after that cooled at room temperature and inoculate isolated bacterial culture in medium. And then incubated at 37 ^{0}C for 7 to 10 days. Day by day measurement was done and growth was also checked by using colorimeter by taking OD at 620 nm. Growth study of strains was done at 4 different pH (5, 7, 9 and 11) and 5 different temperature (12^0C, 25^0C, 35^0C,37^0C and 50^0C).

Optimization: Optimization technique used to check growth activity of isolated microbes in different carbon source (sucrose, maltose, mannitol, glucose, starch, beef extract), nitrogen source (urea, peptone, ammonium chloride, and CH_3COONa), 4 different pH (5, 7, 9 and 11) and 5 different temperature (12^0C, 25^0C, 35^0C, 37^0C and 50^0C). Optimization is the term to provide suitable condition for the growth of isolated culture.

Optimization was done by Shake flask method. In this method best carbon, Nitrogen source, optimum pH and optimum temperature was used and culture was inoculated with oil and kept at 37 ^{0}C for 7 to 10 days and further result were observed[12][13].

3. RESULTS:

Serial Dilution Method:- The serial dilution technique were used to get pure and reduced number of bacterial colonies and mixed culture were was obtained and the purified by quadrant streaking method.

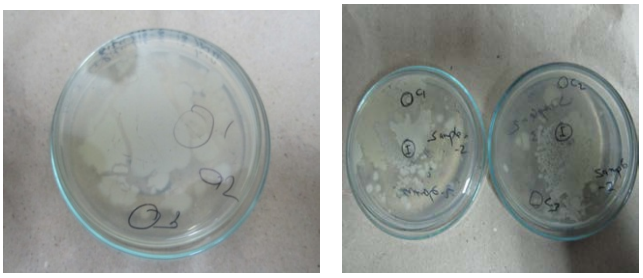


Fig 1: Mixed Culture in spreading

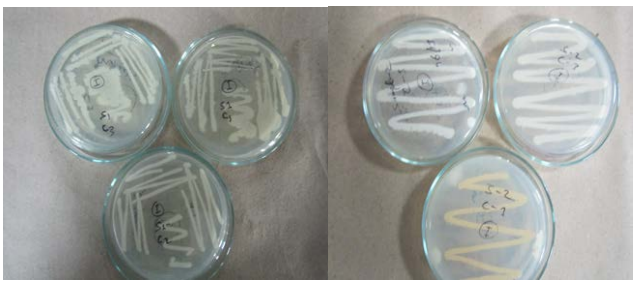


Fig 2: Subculturing by quadrant striking method

Screening of isolated colonies: All the isolated strains/ colonies (S1-C1,C2 & C3; S2-C1,C2 & C3 and S3-C1,C2,& C3) were selected for the conformation of having oil degradation capability in bacteria in which three strains/ colonies showed good result and these three (S1-C1,S2-C3 &S3- C1) were taken for the further study.

Agar well diffusion method:

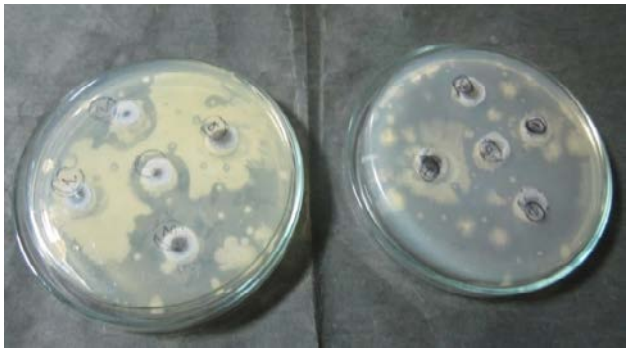


Fig 3: degradation of oil in agar well diffusion method.

Shake Erlenmeyer Flask Method:-

Oil degradation (mobil oil for S1-C1 and S3-C2).

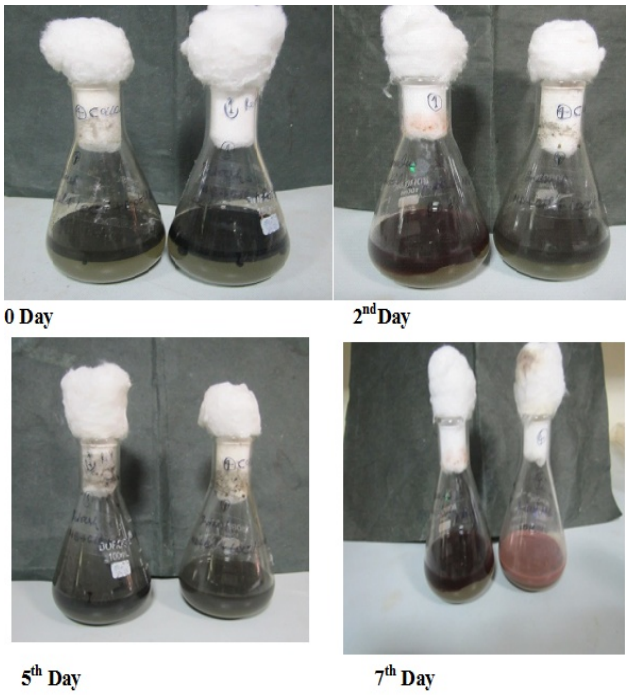


Fig 5: oil degradion in shake flask method.

Oil degradation(2T-oil for S1-C1 and S3-C2):

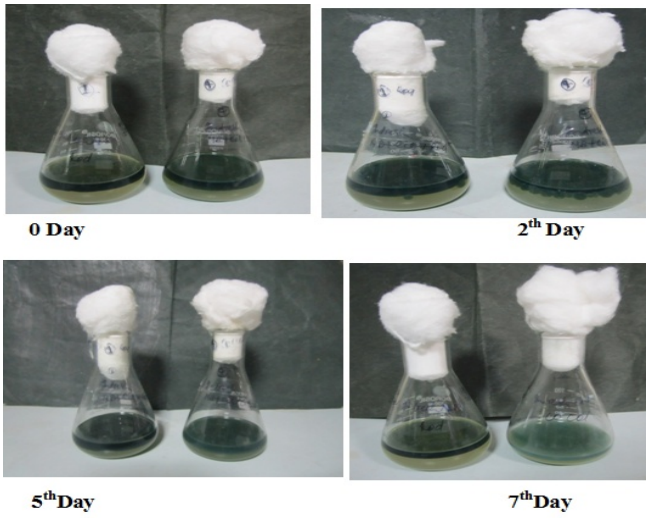


Fig 5: oil degradation in shake flask method.

Colony morphology study:

Characteristics	S1-C1	S3-C2
Size (cm)	0.2	0.4
Shape	Irregular	Regular
Color	White	Off white
Texture	Smooth	Smooth
Margin	Lobate	Entire
Opacity	Transparent	Opaque
Elevation	Flat	Elevated

Result of Gram staining of bacterial culture:-

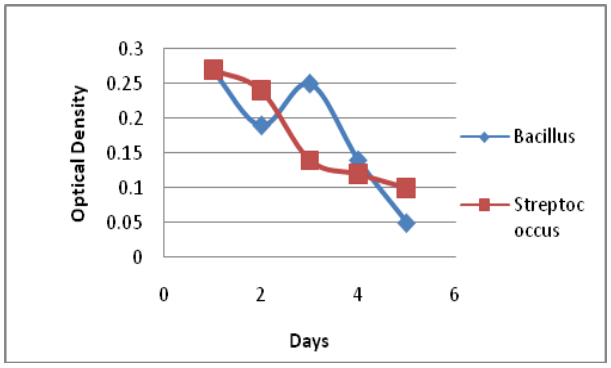
Culuture	Bacteria
S1-C1	Gram positive Cocci
S3-C2	Gram positive rod

Biochemical test for different culture:

Biochemical Test	*Bacillus cereus*	*S.faecalis*
Endospore Test	Positive	-
Catalase Test	Positive	Positive
Glucose Test	Positive	Positive
Mannitol Test	Negative	-
Growth in 6.5% Nacl	-	Positive

Day by day OD to check mobil oil degradation activity:

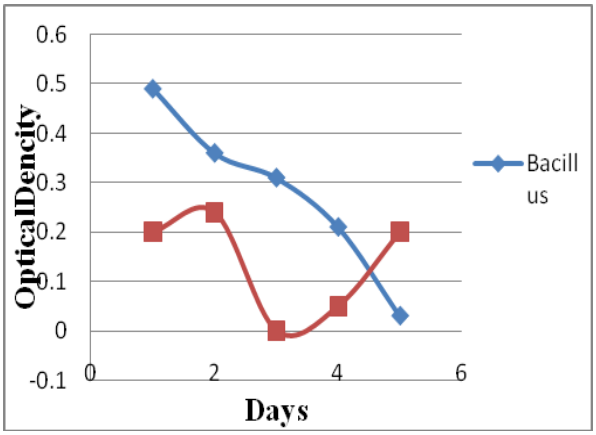
OD(Mobil oil)		
Days	***Bacillus***	***Streptococcus***
1st	0.27	0.27
2nd	0.25	0.24
3rd	0.19	0.14
4th	0.14	0.12
5th	0.08	0.10
6th	0.05	0.06
7th	0.03	0.02



Graph 1: Graph between days and OD (mobil oil degradation).

Day to day OD to check 2T-oil degradation activity:

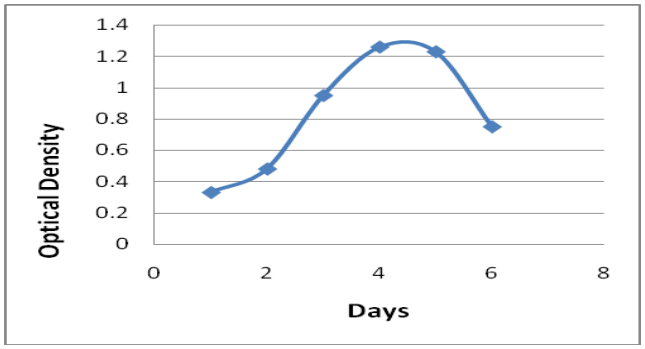
OD(2T-oil)		
Days	***Bacillus***	***Streptococcus***
1st	0.49	0.20
2nd	0.36	0.24
3rd	0.31	0.18
4th	0.21	0.16
5th	0.11	0.08
6th	0.06	0.05
7th	0.03	0.03



Graph 2: Graph between days and OD (2T-oil degradation).

Growth kinetic of *B. cereus*:

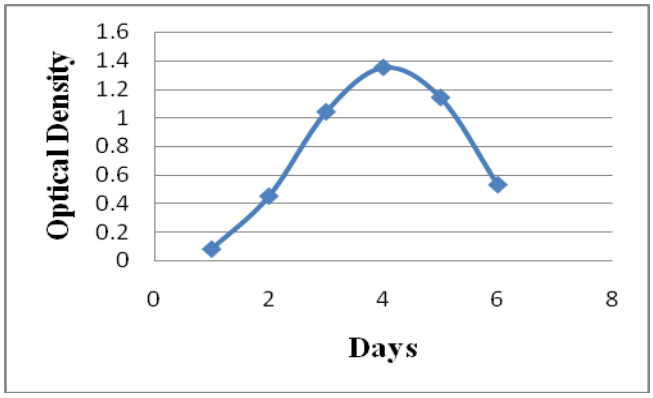
Days	**Optical Density(OD) 620nm**	**Phases**
1	0.33	Lag phase
2	0.48	Log phase
3	0.95	Log phase
4	1.26	Stationary phase
5	1.23	Stationary phase
6	0.75	Decline phase



Graph 3: Graph between days and OD (Growth of *B. cereus*).

Growth of *S. faecalis* OD:

Days	**Optical Density(OD) 620nm**	**Phases**
1	0.08	Lag phase
2	0.45	Log phase
3	1.04	Log phase
4	1.35	Stationary phase
5	1.14	Stationary phase
6	0.53	Decline phase

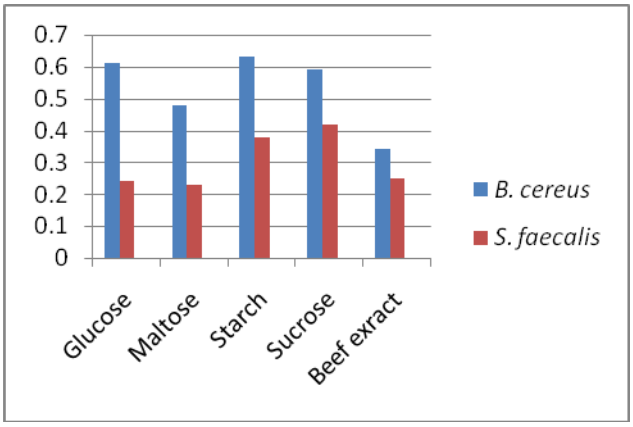


Graph5: standard Graph between days and OD (Growth of *S. faecalis*).

Optimization (To check growth Activity):

OD for different carbon sources.

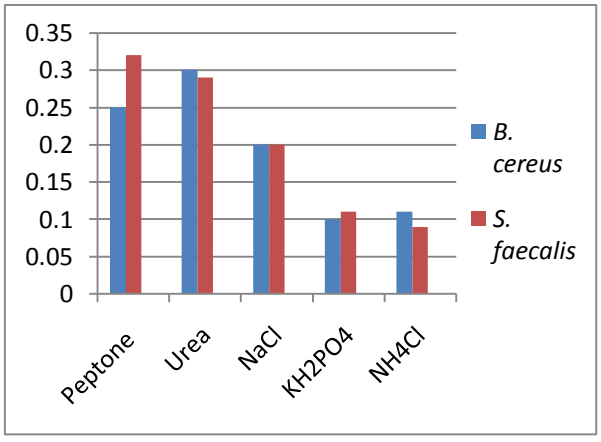
Sources	***B. cereus***	***S. faecalis***
Glucose	0.61	0.24
Maltose	0.48	0.23
Starch	**0.63**	0.38
Sucrose	0.59	**0.42**
Beef exract	0.34	0.25



Graph 6: It shows best carbon source starch for B. cereus and sucrose for *S. faecalis*.

OD for different Nitrogen sources.

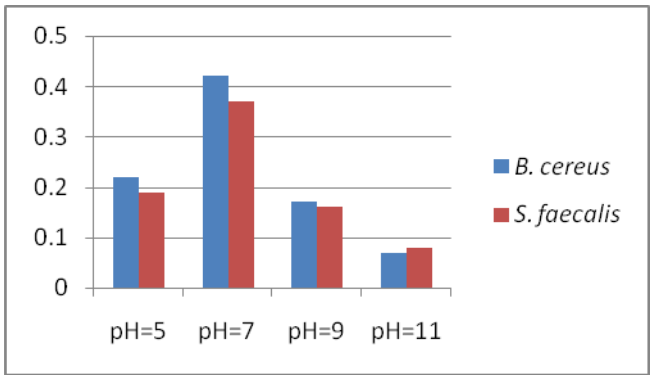
Sources	***B. cereus***	***S. faecalis***
Peptone	0.25	**0.32**
Urea	**0.30**	0.29
NaCl	0.20	0.20
KH_2PO_4	0.10	0.11
NH_4Cl	0.11	0.09



Graph6: It shows best nitrogen sources urea for B. cereus and peptone for *S. faecalis*.

OD for different p^H.

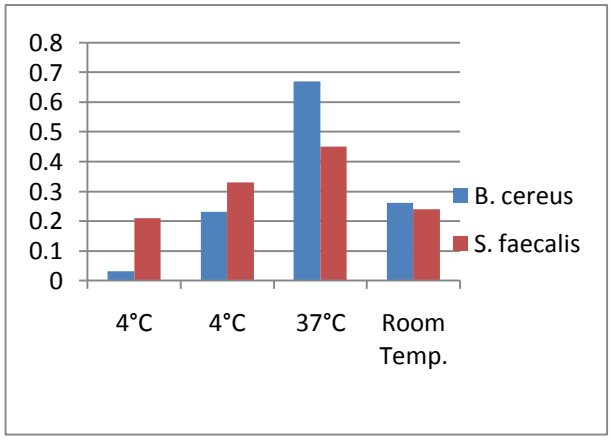
pH	B. cereus	S. faecalis
5	0.22	0.19
7	0.42	0.37
9	0.17	0.16
11	0.07	0.08



Graph 7: It shows best pH -7 for *B. cereus* and *S. faecalis*.

Optical Density for different temperature.

Temperature	*Bacillus cereus*	Streptococcus faecalis
12°C	0.03	0.21
25°C	0.23	0.33
37°C	0.67	0.45
50°C	0.26	0.24



Graph 8: optimum temperature 37°C for *B. cereus* and *S. faecalis*.

Cultures	Carbon source	Nitrogen source	pH	Tempe Rapture
Bacillus cereus	Starch	Urea	7	37°C
Streptococcus faecalis	Sucrose	Peptone	7	37°C

Mobil oil degradation activity at optimized condition:

OD(Mobil oil)		
Days	*Bacillus cereus*	*Streptococcus faecalis*
1st	0.29	0.33
2nd	0.23	0.27
3rd	0.28	0.17
4th	0.17	0.14
5th	0.11	0.13

2T-oil degradation activity at optimized condition:

OD(2T-oil)		
Days	*Bacillus*	*Streptococcus*
1st	0.53	0.23
2nd	0.39	0.26
3rd	0.34	0.03
4th	0.24	0.08
5th	0.05	0.24

4. DISCUSSION:

Bioremediation is a process of clean up the environment with the help of microbes. The thesis work is carried out by isolation, Identification , production and characterization of Bio surfactant producing microbes, the samples were collected from spilled oil contaminated soil sites from Lucknow region . Out of three soil samples total 9 bacterial culture were isolated and only 2 culture were identified (*Bacillus cereus*, *Streptococcus faecalis*) and characterized through Beregey's manual having the oil degradation capabilities. The Agar well diffusion method, spread plate method and shake Erlenmeyer Flask methods were used to check the degradation of layer. In the Agar well diffusion method, bacterial culture grows by using oil as a carbon source. In shake Erlenmeyer Flask methods, as the culture grows in the medium day by day the length of oil was decreased and completely degraded till 7th days and the degradation of oil was also confirmed by spectrophotometer by taking OD at 620nm.The value of optical density is high at first day and then decreased continuously till 7th days which indicate the transmittance of uv light is decreasing because as the bacterial growth occurs in the medium, most of the light is scattered by the cells no longer reaches the photoelectrical cell so that electrical signal (absorbance) decreased. In Spread plate method, culture growth observed in the presence of oil that means bacteria having property to degrade oil. The growth kinetics study showed Log phase of cultures. The oil was used as Mobil oil and 2T oil. The complete oil degradation obtained after 7 days for all samples. In case of growth kinetics the transmittance of light is considered which showed all the phase like lag phase, log phase, stationary phase and the decline phase.

Further production of bacterial culture through optimize media .Optimization was done for best carbon, nitrogen, pH, and temperature and the best result were obtained for carbon source in *Bacillus cereus* Starch and *Streptococcus faecalis* Sucrose was a best sources, for Nitrogen sources in *Bacillus cereus* Urea and *Streptococcus faecalis* Peptone was a best sources, for optimum temperature 37^0C was best for both culture and for optimum pH 7 was best for both culture.

5. CONCLUSION & FUTURE PROSPECTS:

The present study concluded that bioremediation is the best Ecofriendly method to clean up the environment using bacteria. The bio surfactant particles which reduced the surface tension and also the oil degradation is possible through this method .The culture were isolated from oil contaminated in soil region of Lucknow and their activity was checked by shake Erlenmeyer Flask & OD method. Further they were characterized through Bergay's manual. The optimization was done to get suitable Carbon, Nitrogen, Temperature and pH.

The future prospect may involved to identify the compound which are responsible for biosurfactant production and also to convert non biosurfactant producing microbe in to biosurfactant producing microbe by using transformation and other Gentic engineering methods.

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