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A study on Hydrocarbon Degradation by Biosurfactant Producing *Bacillus cereus* in Oil Contaminated Soil Samples

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ABSTRACT- Microorganisms are the important factors in the degradation of the toxic substances in our environment. Petrol and diesel oil is one of the complex mixtures which cannot be easily degraded. The *Bacillus cereus* was involved in the degradation of oil during, which the complex toxic substances were detoxified by the production of biosurfactants. In our study we have identified that the biosurfactant producing *B. cereus* have a high potential for hydrocarbon degradation. *B. cereus* was isolated from hydrocarbon contaminated soil and identified based on morphology and biochemical test according to the Bergey's manual of systematic bacteriology. The maximum hydrocarbon degrading biosurfactant producing *Bacillus cereus* was obtained by qualitative and quantitative methods. In optimization studies, the best results observed for *Bacillus cereus* were, Olive oil as the suitable carbon source, Sodium nitrate as the best Nitrogen source and Optimum pH is 7 and Optimum temperature is 37°C. The ability of these isolates to degrade hydrocarbons and survive in the oil contaminated soil is attributed to the development of resistance by mutation on the plasmid. It is also clearly evident that the specific gene was responsible for the production of biosurfactant and the degradation process. According to the results from the present study the *B. cereus* has high potential for hydrocarbon degradation and can be used especially for Microbial Enhanced Oil Recovery and bioremediation of hydrocarbons in near future.

Key-Words: *Bacillus cereus*, Biosurfactant, Hydrocarbon, Biodegradation, Plasmid DNA

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INTRODUCTION

India is suffering with ever increasing consequential environmental threats. Rapid industrialization can only move hand in hand with the efficient and optimum feasibility of transport, which results in increased use of automobiles [1]. Diesel oil contains 2,000 to 4,000 hydrocarbons, a complex mixture of linear, branched and cyclic alkanes and aromatic compounds obtained from the middle distillate fraction during petroleum separation [2]. The availability of nutrients, especially nitrogen and phosphorus significantly control microbial activities [3], and these nutrients are necessary to enhance the biodegradation of oil pollutants [4].

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Fuel and lubricating oil spills have become a major environmental hazard to date. The contamination of the environment with petroleum hydrocarbons provides serious problems for many countries [5].

Biodegradation is a biologically catalyzed reduction process of complex chemicals. This process is being performed by a variety of bacteria, fungi and yeast, transforms potentially toxic compounds into non-toxic compounds to obtain energy and nutrients [6]. These microorganisms are directly involved in biogeochemical cycling of many carbon sources, including petroleum hydrocarbons [7].

Biosurfactant are extracellular or membrane associated, heterogeneous group of low molecular weight surface active compounds produce by different microorganism such as bacteria, fungi and yeast. On the basis of chemical composition they are categorized into glycolipids, lipopeptides, phospholipids, neutral peptides, fatty acids [8,9]. They are mainly composed of hydrophilic and hydrophobic moiety. The Hydrophilic moiety consists of acids, peptides, mono, di, or polysaccharides while the hydrophobic moiety consists of saturated or unsaturated fatty acids. They have great potential to reduce surface and

interfacial tension of liquids, solid and gases, and enhance their solubilisation in liquid solution. They have shown good stability at extreme temperature, pH and salt concentration. Because of their unique characteristic and better performance than synthetic surfactants, they have gained attention and importance in various fields such as enhanced oil recovery, environmental bioremediation, food processing and pharmaceuticals [10]. In addition to lubrication, fixing dyes, making emulsions, stabilizing dispersions, preventing foaming, the main application is in the enhancement of oil recovery techniques, especially the trapped residual oil [11]. The oil bioremediation is due to their biodegradability and low critical micelle concentration (CMC) [12-13]. Among them, lipopeptide biosurfactants produced by *Bacillus species* are capable of generating the low interfacial tension between the hydrocarbon and aqueous phases required to mobilize the entrapped oil [14].

One of the most important bacteria producing biosurfactant is *Bacillus* sp, which is mainly used for biotechnological applications [15]. *Bacillus licheniformis* produce a variety of products such as extracellular enzymes, biosurfactants, biopesticides and are ecofriendly. They are used for enhanced oil recovery and inhibition of sulfate reducing bacteria. The biosurfactants produced from *Bacillus* sp. possess the property of functionality under extreme conditions of pH, temperature, salinity and in addition are from renewable resources and are ecofriendly [16-18]. Since the lipopeptide biosurfactants produced by *Bacillus* spp. have high ability to reduce the surface tension, they find wide use in the oil industry and bioremediation [19]. The aim of our research was to investigate the biosurfactant producing ability of *B. cereus* for hydrocarbon degradation in oil contaminated soil samples in and around the Erode district, Tamil Nadu, India.

MATERIALS AND METHODS

Soil samples

In this study totally twenty soil samples were collected to isolate the hydrocarbon degrading bacteria *Bacillus cereus* from oil contaminated soil. The soil samples were extending from the ground surface to a depth of 10-20 cm from petroleum contaminated areas near petrol station refining areas in Erode. Samples were then transported to laboratory under sterile conditions. The research work was carried out during the period of January 2014 to May 2014 in department of microbiology lab in Vivekanandha College of Arts and Sciences for Women (Autonomous), Tamilnadu (India).

Isolation of bacteria

One gram of soil sample was suspended in 10ml of normal saline to make tenfold serial dilution of the sample for the isolation of bacteria. The dilution factor is expressed in colony forming unit per ml (CFU/ml) [20]. The suspension was heated at 70°C for 15 minutes to reduce any vegetative bacterial cells and facilitate the isolation of *Bacillus* sp. Serial dilutions were prepared and plated on Nutrient agar medium supplied with 80 µg/ml of cycloheximide and incubated at 30°C for 2–3 days for the growth of the spore forming bacterial colonies. The bacterial colonies were purified and sub-cultured on the same medium for further identification and biosurfactant production.

Identification of bacteria

The preliminary identification of the isolates was done by the colony morphology, grams staining, motility and spore staining. The biochemical reactions include oxidase test, catalase test, gelatin liquefaction, starch hydrolysis, lipid hydrolysis, sugar fermentation tests, IMViC test, H2S and Nitrate reduction test. The identification of the *Bacillus cereus* isolates was based on Bergy's Manual of Determinative Bacteriology [21].

Cultivation media for biosurfactant production

Mineral Salt Broth (MSB, pH 7.2) composed of (g/l) KH_2PO_4 0.5g, K_2HPO_4 1g, $NaNO_3$ 4g, $MgSO_4.7H_2O$ 0.5g, KCl 0.1g, $FeSO_4.7H_2O$ 0.01g, Yeast extract 0.01g, Carbon source (2%), Crude oil, Trace element 0.05 ml, 2ml contained (g/l) $NaMo_4$ 0.1g, $MnCl_2$ 1.75g, $ZnSO_4.7H_2O$ 3.1g, $CuSO_4.5H_2O$ 2g, H_3BO_3 1.5g for biosurfactant production [22-24].

Screening of biosurfactant producing *Bacillus cereus*

The bacterial strains maintained on nutrient agar at 4°C was inoculated in 50 ml of seed medium (Nutrient broth) in a 100 ml Erlenmeyer flask and incubated in a rotary shaker at 120 rpm at 30°C. After growth for 18 hrs, standard inoculum 5% (v/v) was used in the fermentation medium which is equivalent to $1x10^8$ CFU/ml. For crude oil biodegradation mineral salt broth supplied with 2% crude oil was used as the sole carbon and energy source. The media inoculated with 5% (v/v) inoculum of the isolate was incubated at 30°C on a rotary shaker at 120 rpm for 7 days [25]. The culture was centrifuged (Eppendorf cooling centrifuge) at 6000 rpm for 15 minutes at 4°C and supernatant was collected for extracellular biosurfactant assay by different methods.

Biosurfactant assay

Drop collapse method

The slightly modified drop-collapse test was performed [26]. In this method, the supernatant from each bacterial isolate was placed onto a glass slide. Then, petrol and diesel was added onto the surface of the supernatant. If the drop of oil on the supernatant became flat in 1 min after adding the oil, the result was taken to be positive. If the drop remained beaded, the result was scored as negative indicating the lack of biosurfactant production.

Chemotaxis

Chemotaxis was tested with drop assay [27]. For this assay, 40 ml of cells were harvested in the logarithmic phase of

growth and resuspended in 12 ml of chemotaxis buffer (100 mM Potassium phosphate [pH 7.0], 20 mM EDTA). A small amount of a test attractant was added to the center of a petri dish containing 10 ml of the chemotaxis buffer. A positive chemotactic response of cells was indicated by the formation of a ring of turbidity near the center of the petri dish.

Culture media for biodegradation

Culture media for biodegradation is Mineral Salt Medium (MSM, pH 7.2) composed of (g/l) $(NH_4)2SO_4$ 2 g, KH_2PO_4 2.4 g, K_2HPO_4 4.8 g, $MgCl_2$ 0.08 g, (NH4)$Mo7O_2$, $4H_2O$ 0.01 g, $CaCl_2.2H_2O$ 0.03 g, Citric acid 0.4 g, Carbon source (2%) crude oil in one liter, Trace element solution 2ml contained (g/l) $FeSO_4.7H_2O$ 1 g, $NaMO_4$ 2 g, $MnCl_2$ 1 g, $ZnSO_4.7H_2O$ 0.03 g, $CuSO_4.5H_2O$ 0.25 g, $CoCl_2.6H_2O$ 0.25 g.

Detection of hydrocarbon degradation by turbidity method

In the present study, turbidometry is used to determine the growth of *B. cereus* by utilizing the hydrocarbons (2% petrol and diesel) given as sole carbon sources in MSM broth. This shows whether the bacterium possess the degrading ability of hydrocarbons like petrol and diesel. The degrading activity of each isolate was obtained by using Mineral Salt Broth (MSB) in which 2% of each hydrocarbon (petrol and diesel) was added and incubated based on the optimization parameters for 4 days. The growth of the bacterium was measured by taking the O.D readings at 595nm from 0 hrs to 4 days at regular intervals of 1 day against mineral salt medium as blank.

Optimization of physical parameters

The production of biosurfactant by the organism determines the hydrocarbon degrading capacity of the organism. Hence optimal conditions for the production of the biosurfactant were determined at different Inoculum size, pH, Temperature, Carbon source and Nitrogen source.

Inoculum size

For determination of optimum inoculum size (250 µl, 500 µl, 750 µl, 1000 µl) the bacteria were grown in MSM at optimized pH and incubated at 37˚C for 4 days.

pH

For determination of optimal pH, the standardized inoculum was inoculated in MSM at different pH (5.0, 6.0, 7.0, 8.0, 9.0), then incubated at 37˚C for 4 days.

Temperature

After optimal pH had been determined, the bacteria were grown in MSM at optimized pH and incubated at different temperature (32˚C, 37˚C, 42˚C) for 4 days.

Carbon sources

Bacterial inoculum was inoculated in MSM at optimized pH, to which was added different carbon sources comprising of olive oil, palm oil, almond oil at 2% (v/v), and then incubated at the predetermined optimized temperature (32°C) for 4 days. The carbon source that induced the highest biosurfactant production demonstrated by showing the lowest surface tension was subsequently chosen. Different concentrations of different carbon source starting from 0.7, 1, 2 ml were taken up for the study.

Nitrogen sources

To determine the best nitrogen source for optimized production of biosurfactant, the total amount of nitrogen in MSM, which contained NaNo 3 0.4 g/100 ml was replaced with the same amount of total nitrogen. Standardized bacterial inoculum was inoculated in MSM at optimized pH with different nitrogen sources, namely Ammonium chloride, Sodium nitrate, Potassium nitrate and incubated at the predetermined optimized temperature (32°C) for 4 days. The nitrogen source that induced the highest level of biosurfactant production as demonstrated by the lowest surface tension activity was further chosen. The different concentration ranging from 0.7, 1, 2 g were chosen for this study.

Isolation of plasmid DNA by alkaline denaturation method

5 ml LB medium containing proper antibiotics were inoculated with a single bacterial colony. The tube was incubated at 37 °C overnight with vigorous shaking at 360 rpm. Pellet bacteria from the culture at 10,000x g for 5 minutes at room temperature. Discard the supernatant**.** Re-suspend the bacterial pellet in a total of 1 ml ice-cold solution I (50 mM). Pipette up and down or vortex as necessary to fully re-suspend the bacteria. Add 2 ml of 0.2 N NaOH/ 1.0% SDS to the suspension. Mix thoroughly by repeated gentle inversion. Do not vortex. Add 1.5 ml ice-cold Solution III to the lysate. Mix thoroughly by repeated gentle inversion. Do not vortex. Centrifuge at 15,500x g for 30 minutes at 4°C. Recover the resulting supernatant. Add 2.5 ml isopropanol to precipitate the plasmid DNA. Mix thoroughly by repeated gentle inversion. Do not vortex. Centrifuge at 15,500x g for 30 minutes at 4°C for the removal of supernatant. The pellet is plasmid DNA. Rinse the pellet in ice-cold 70% Ethanol and air-dry for about 10 minutes to allow the Ethanol to evaporate. Add double distilled H_2O or TE buffer to dissolve the pellet. After addition of 2 µl RNase A (10 mg/ml), the mixture was incubated for 20 minutes at room temperature to remove RNA.

Alkaline Solution I (Lysis buffer I): 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0. Store at 0°C.10ml 500 mM Glucose was added with 2 ml 500 mM EDTA pH 8.0. Then it will be added with 2.5ml 1M Tris pH 8.0 and 85.5 ml H2O. The prepared solution was autoclave and store at

4°C for further use.

Alkaline Solution II (Lysis buffer II): Freshly prepared 0.2 N NaOH, 1% SDS. Store at room temperature (RT). Isopropanol: Stored at -20°C.

Alkaline Solution III (Lysis buffer III): 3M KOAc, pH 6.0. This solution was prepared with 60 ml 5M Potassium acetate (49.07 g Potassium acetate in 100 ml H_2O) was added with 11.5 ml glacial acetate and 5 ml H_2O.

Agarose Gel Electrophoresis

Electrophoresis is a process of separation of charged particles based on charge/mass ratio, carried out in the presence of an electric field. Agarose is a linear polysaccharide which acts as solidifying agent. 1% (w/v) agarose gel was made by adding 1 gm of agarose to 100 ml of 1x TBE buffer solubilized by heating at boiling temperature, then the agarose was left to cool at 55°C before pouring in a tray to solidify. A comb was placed near one edge of gel, and gel was left to harden. 1x TBE was poured into gel tank and the gel tray was placed horizontally in electrophoresis tank, 3 µl of loading buffer was mixed with 10 µl DNA sample, and then samples were added carefully to individual wells. Power was turned on at 45 Volts for 15 minutes and 85 Volts for 4-5 hours to run DNA. Agarose gel was stained with ethidium bromide by immersing them in distilled water containing the dye of final concentration of 0.5 µg/ml for 30 to 45 minutes. DNA bands were visualized by U.V illumination at 366 nm wavelength on U.V transilluminator.

RESULTS AND DISCUSSION

The present study was carried out to determine the biosurfactant producing *B. cereus* for hydrocarbon degradation in oil contaminated soil samples. In this study, twenty soil samples were collected from hydrocarbon contaminated sites, correct engineering work, Ramesh engineering work at Petrol bunk in Tindal around Erode district. These areas were selected as there were more chances to get oil degrading microbes.

Isolation and identification of *B. cereus*

The *B. cereus* generally produced large colonies with a dull or frost-glass surface and undulate margin on Nutrient agar. In preliminary test, the isolates were identified to be gram positive, spore forming rods, catalase and oxidase positive. Further species level confirmation was done using biochemical test. Totally four isolates were identified as *B. cereus* and results were documented (Fig. 1 & 2).

Fig. 1: *Bacillus cereus* on Nutrient agar

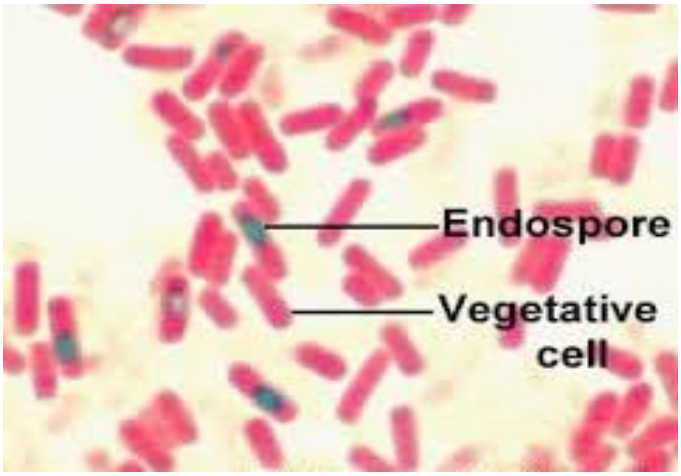


Fig. 2: Endospore staining of *Bacillus cereus*

Biosurfactant production

Biosurfactants are produced by many bacterial strains that showed a strong ability to grow on hydrocarbon as sole carbon source and degrade or transform these compounds [28]. Many microorganisms produce biosurfactants. Bacteria such as *Bacillus* sp. were shown high biosurfactant productivity [29]. In this study a biosurfactant producing bacteria, *B. cereus* was obtained from hydrocarbon contaminated soil. The results showed that this strain could utilize 80% of crude oil in MSM during 7 days as the carbon source. The survival of microorganisms in petroleum hydrocarbon medium after their inoculation is an important factor in the biodegradation of hydrocarbons in liquid medium [30].

Drop collapse method

The result of drop collapse test performed with petrol and diesel oil in glass slide showed positive reaction (++) between drop of surfactant producing culture and oil surface causing flattening of the oil surface (Fig. 3). Full spreading was observed due to the reduction in surface tension between the liquid drop and the hydrophobic

surface whereas with non biosurfactant producing culture the drops remained stable [16].

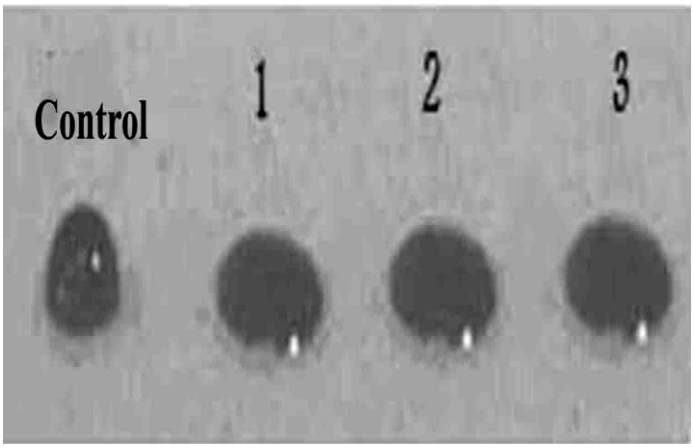


Fig. 3: Drop collapse method- Control, Collapsed droplets 1, 2, 3 -Positive

Chemotaxis

In this study all the isolates show positive chemotaxis to petroleum oil. Recent results with the biodegradation of hydrocarbon have shown the potential of chemotaxis to enhance biodegradation in laboratory-scale microorganisms. Indeed chemotaxis have already been studied in bacteria able to degrade a wide variety of organic pollutants such as naphthalene, BTEX (Benzene, Toluene, Ethylbenzene, and Xylene), and pesticides. The similar result was showed toluene-degrading bacteria are chemotactic towards the environmental pollutants, benzene, toluene, and trichloroethylene [31]. In addition, the chemotaxis of *Ralstonia* to herbicide [32]. Bacterial chemotaxis towards environmental pollutants have important role in bioremediation (Fig. 4).

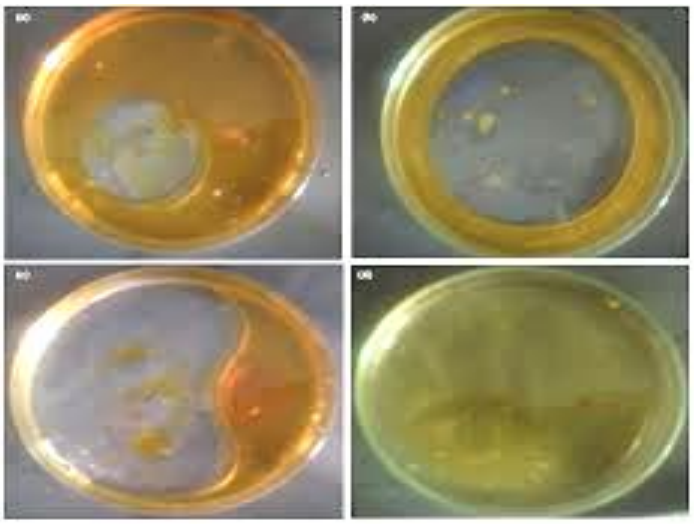


Fig. 4: Chemotaxis activity of *Bacillus cereus* towards oil droplets

Degradation of hydrocarbon

The optical density (OD) reading of biodegrading activity of each isolate on hydrocarbon (Petrol and diesel) based on the turbidity of MSM broth at regular intervals of 4 days gives the degradative activity on hydrocarbons by bacteria. The result demonstrated that *B. cereus* have the greatest ability to degrade petrol and diesel. Our results showed that all the *B. cereus* isolates maximally utilized the hydrocarbon substrates (Petrol and diesel) when supplied as the sole source of carbon and energy although, the level of utilization differs from one to another (due to differences in their growth) and from one hydrocarbon substrate to the other, due to the obvious differences in their molecular sizes. The degrading capabilities on different hydrocarbons revealed that the *B. cereus* isolated from the soil were able to degrade hydrocarbons. The cells were able to multiply within the days of study, indicating that they were able to degrade and utilize the hydrocarbons for their growth and development; hence the concomitant increase in the concentration of the broth (turbidity) was observed. It was also noticed that as the incubation hours increased there was gradual increase in the concentration of the broth, and degradation of hydrocarbons. Bioremediation is proposed as an alternative to various physicochemical treatments at many hydrocarbon contaminated sites [33]. Extensive hydrocarbon exploration activities often result in the pollution of the environment, which could lead to disastrous consequences for the biotic and abiotic components of the ecosystems if not restored. Remediation of hydrocarbon contaminated system could be achieved by either physicochemical or biological methods [34]. Surfactants and biosurfactants can increase the pseudo solubility of petroleum components in water. Moreover, biosurfactants can be as effective as synthetic chemical surfactants due to their high specificity and their biodegradability.

Optimization Parameters

Inoculum size

The results showed that, the utilization of hydrocarbon gradually increased with the growth of *B. cereus* in different volume of inoculum. It was determined by the optical density value of *B. cereus* culture. Totally four different inoculum sizes such as 250 µl, 500 µl, 750 µl and 1000 µl were analyzed in different incubation time intervals such as 24 hrs, 48 hrs, 72 hrs and 96 hrs and the O.D. values were noted. The 750 µl of inoculam size favored the growth of *B. cereus* and the gradual increase of *B. cereus* cells to degrade the oil in medium at different time intervals. The O.D. values of 0.048, 0.090, 0.112 and 0.148 were also recorded (Table 1). It was also noticed that as the incubation time was extended, bacterial concentration increased. Low inoculum size required longer time for cells to multiply and produce the desired effect [35]. A small amount of inoculum can lead to insufficient number of microbial cells and a reduced amount of the secreted enzymes, while a much higher inoculum could lead to or cause a lack of oxygen and depletion of nutrients in the culture media [36].

Table 1: Degradation of Hydrocarbon using various inoculum size of *B. cereus*

S. No	Inoculum size (µl)	OD Reading (nm)/ Incubation (Hrs)			
		24	48	72	96
1.	250	0.028	0.012	0.108	0.098
2.	500	0.064	0.005	0.077	0.097
3.	750	0.048	0.090	0.112	0.148
4.	1000	0.069	0.019	0.098	0.143

Optimum pH

Degradation of Hydrocarbon at different pH by *B. cereus* showed the production of biosurfactant at a wide range of pH from 5.0 to 9.0 in different incubation time intervals such as 24 hrs, 48 hrs, 72 hrs and 96 hrs as shown in Fig. 5. The pH 7.0 was selected as the best pH for biosurfactant production because it produced the highest surface tension reduction. It was also observed that as the incubation time was extended the biodegradation was at the maximum. At an acidic pH (5.0) and extreme alkaline pH (9.0), these isolates produced lower levels of biosurfactant. The pH range of the experimental samples (6 to 7) observed in this study is within the favourable range for biodegradation of oil in polluted soil. Similar observations have been documented [37-38].

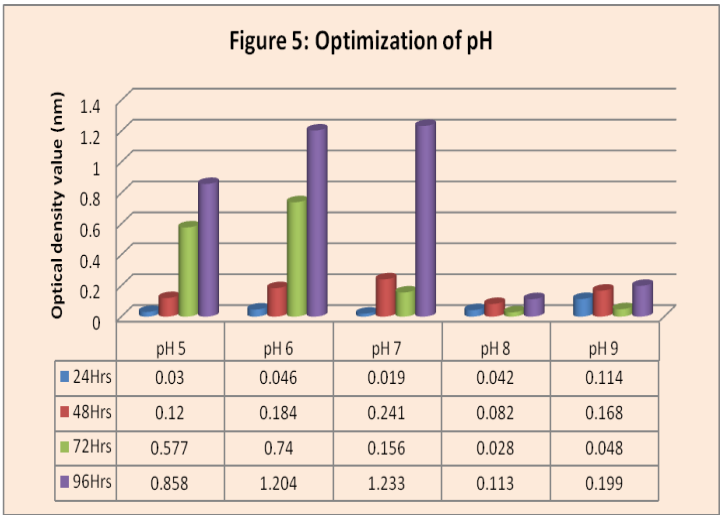


Fig. 5: Optimization of pH by *Bacillus cereus*

Temperature

Degradation of hydrocarbon by *B. cereus* at different temperatures at 32°C, 37°C and 42°C at various time intervals such as 24 hrs, 48 hrs, 72 hrs and 96 hrs at pH 7.0 was studied. *B. cereus* grown in MSM broth at pH 7.0 produced maximum biosurfactant when incubated at temperature 37°C, which was significantly different from cultures grown at 32°C and 42°C. At 37°C it produced the highest biosurfactant production of 1.233 O.D. representing a reduction in surface tension. When the incubation temperature increased to 42°C, bacterial growth and biosurfactant production were significantly decreased, indicating that the biosurfactant produced by *B. cereus* was temperature dependent (Fig. 6).

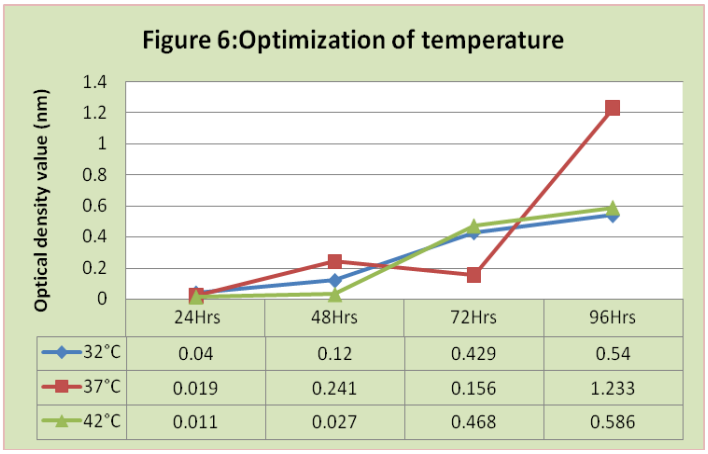
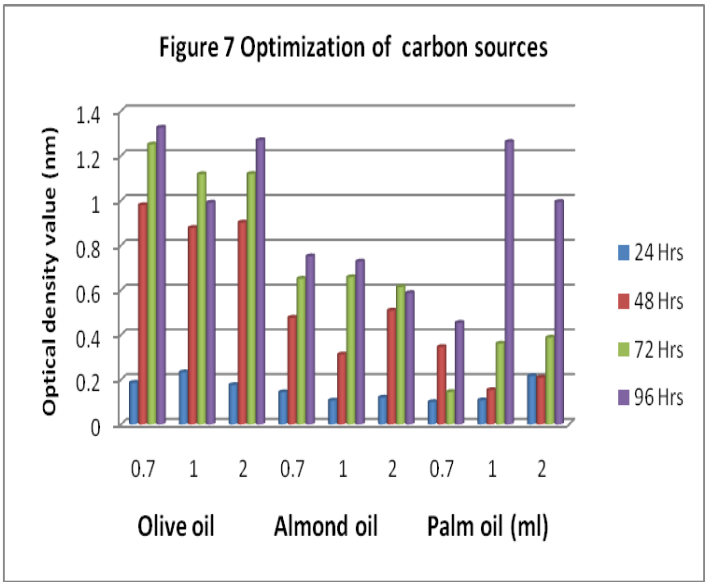


Fig. 6: Optimization of Temperature by *B. cereus*

With the increase in enzymatic activity of mesophilic and thermophilic microorganisms associated with increasing temperatures, it is expected that biodegradation rates can be enhanced to a certain extent, typically in the range of 30°C to 40°C. Above 40°C, the membrane toxicity of hydrocarbons is increased, thus hindering biodegradation [39].

Carbon source

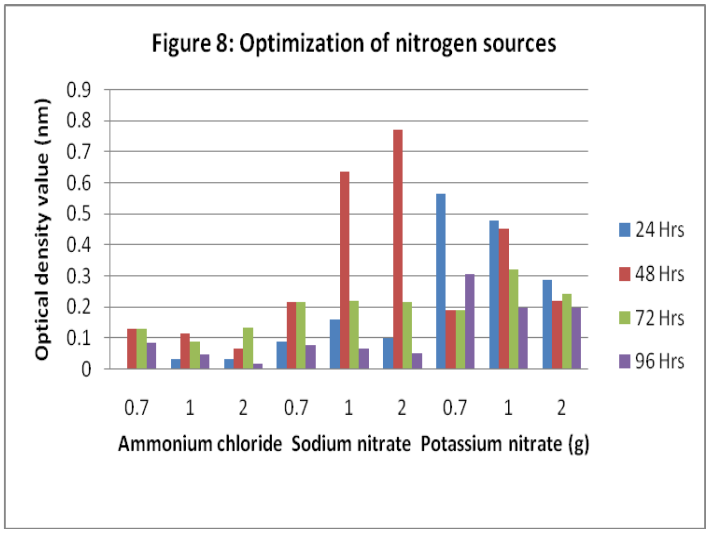
The ability of *B. cereus* to utilize various types of carbon sources for biosurfactant production was tested with Olive oil, Almond oil and Palm oil at 0.7, 1.0, 2.0 ml. All oils were gradually degraded showing increased O.D values at different incubation hours (24 hrs, 48 hrs, 72 hrs and 96 hrs) at optimized temperature 37°C and pH 7. Among these carbon sources tested, Olive oil has highly favoured the *B. cereus* growth in concentration 0.7 ml with the O.D. value of 1.325 in 96hrs was shown in (Fig. 7). Increase in the concentration of olive oil has not shown maximum growth. The appropriate reason for this needs further investigation. The next carbon source which supported maximum growth was Palm oil at 1 ml concentration on 96 hrs incubation. From the results it is evident that almond oil supported least growth.



Biosurfactants are secondary metabolites that are produced by microorganisms as they grow on suitable carbon sources [40]. Owing to this, it was important to select a carbon source that could instigate a high growth density leading to an increase in biomass and a subsequent increase in the concentration of biosurfactants produced.

Nitrogen sources

The ability of *B. cereus* to utilize various types of Nitrogen sources for biosurfactant production was tested with Ammonium chloride, Sodium nitrate, Potassium nitrate at 0.7, 1.0, 2.0 g concentration at different incubation hours (24 hrs, 48 hrs, 72 hrs and 96 hrs) at optimized temperature 37°C and pH 7. Among these, *B. cereus* was able to utilize Sodium nitrate at the maximum when given at 2 mg concentration and showed OD reading of 0.768 at 48 hours. The next nitrogen source which was utilized to the maximum was Potassium nitrate at 0.7 g concentration and showed an OD value of 0.565 at 24 hrs. Ammonium chloride was least utilized by *B. cereus* for growth as a nitrogen source. While there was significant difference in bacterial concentration and hydrocarbon degradation between ammonium chloride, sodium nitrate and potassium nitrate as nitrogen sources, sodium nitrate was selected as the optimal nitrogen source since it produced the highest surface tension (Fig. 8). From the results it is evident that the degradation is dependent on concentration of the given nitrogen source and the time of incubation.



The optimization parameters included different concentration of carbon sources (olive oil, almond oil, palm oil), different concentrations of nitrogen sources (ammonium chloride, sodium nitrate, potassium nitrate), different pH 5, 6, 7, 8, 9 and different temperature 32°C, 37°C and 42°C. The best conditions for culture like suitable carbon source, nitrogen source, pH was provided in the production media to get best results. For *B. cereus* suitable carbon source is Olive oil, nitrogen source is sodium nitrate, optimum temperature is 37°C and optimum pH is 7.

Plasmid DNA isolation

All the isolates were screened for the presence of hydrocarbon degrading ability on mineral salt medium with 2% of the hydrocarbons as the sole carbon sources (petrol and diesel). Hydrocarbons are needed as a carbon source but it can be toxic to microorganisms due to the solvent effects of petrol and diesel that could destroy bacterial cell membrane but only resistant bacteria can survive in that region due to mutation in the plasmid. The presence of plasmid DNA was investigated in the isolates of *B. cereus*. The extracted plasmid DNA was resolved in 0.7% agarose gel using TBE buffer. Plasmid DNA profile indicated that the four isolates harboured the plasmid DNA, which was confirmed by agarose gel electrophoresis. The sizes of the extracted plasmid from *Bacillus cereus* were found to be between 2.322 kb to 23.130kb. This is similar to that 47 of the *B. cereus*, isolated from animals in Lagos harbored detectable plasmids which ranged in sizes from 0.564 kb to >23kb [41] and also reported plasmids of sizes between 3.9 kb and 50 kb in *B. cereus* strains isolated from traveler diarrhea [42]. 92% of *B. cereus* serotype 0164 strain possessed two small plasmids of molecular size 9.06kb and 7.046 kb. From the above findings, it is suggested that the presence of plasmid in all *B. cereus* could be responsible for the degradation of total petroleum hydrocarbon.

CONCLUSIONS

From this study, we have concluded that the biosurfactant producing *B. cereus* has the ability to degrade the hydrocarbon at optimized parameters in laboratory condition. In our study, we have to clear that the *B. cereus* utilized the hydrocarbon as sole carbon source for their growth and multiplication in the soil by degrading the hydrocarbon due to the production of biosurfactant, and also it helped to maintain the soil fertility by the removal of the hydrocarbon pollutant. Moreover, it is also evident that the plasmids are responsible for hydrocarbon degradation. It is believed that these plasmids may confer the resistance to the isolates to survive in the hydrocarbon rich environment. The biosurfactants produced by them are nontoxic, biodegradable, do not cause any harm to the environment and can be produced by utilizing cheaper substrate. These unique characteristics of biosurfactant produced by *B. cereus* make them better candidate than chemical and physical methods for the removal of hydrocarbons, and have gained more importance for industrial and environmental application. So, the problem of environmental pollution due to hydrocarbon can be addressed with such biosurfactant producing strains of *B. cereus*.

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