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RESEARCH ARTICLE

SCREENING AND EXTRACTION OF BIOSURFACTANT PRODUCING BACTERIA FROM OIL CONTAMINATED SOILS.

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Abstract

Biosurfactants produced by bacteria are surface active compounds involved in the degradation of hydrocarbons. They are heterogeneous group of surface active molecules produced by microorganisms, which adhere to the cell surface or are excreted extra cellularly in the growth medium. The biosurfactants producing microbes are helpful in bioremediation of heavy metals, pesticides and hydrocarbon contaminated sites. They are also used as bio control agent to protect plant against various diseases, resulting in higher crop yields. The present study was aimed to isolate potential biosurfactants producing bacteria from five different oil contaminated sites and tested for biosurfactant production using different techniques includes oil spreading test, emulsification test, haemolysis test and drop collapse assay.

Introduction:-

Now a day's pollution is considered as one of the major problems of the world which could be either organic or Inorganic in nature. The most common are petroleum derivatives which includes alkanes and other aliphatic and aromatic compounds and other minor constituents (Chikere,et al.,2011; Atlas ,et al., 2005). The impact of environmental pollution to man, animals and plants cannot be over emphasized. The release of contaminants into the environment such as petroleum derived products, is one of the main cause of global contamination (Rahman, et al., 2003).

Such pollution has led to decrease in agricultural produce, loss of aquatic lives, increase in the incidents of disease and loss of aesthetic value of the natural environment. It's also a risk for human and animal health since many of these contaminants have demonstrated to be toxic and carcinogenic (Chayabutra et al., 2001). Most environmental pollution are known to be persistent for quite a long period of time. Hydrocarbon molecules that are released into the environment are difficult to remove, since they absorb to surfaces and are trapped by capillarity in a water immiscible phase. Petroleum derived hydrocarbons are among the most persistent soil contaminants and some hydrocarbon degrading microorganisms can produce biosurfactants to increase bio availability and degradation. Bioremediation has proven to be an alternative to reduce the effects caused by hydrocarbon contamination of soil and water, using the metabolic capacities of microorganism that can use hydrocarbon as source of carbon and energy. The efficiency of removal is directly related to the compounds chemical structure, to its bio availability and to physiochemical condition present in the environment (Christofi,et al., 2002).

Biosurfactant are surface active compounds of a heterogenous group of surface active molecules produced by microorganism, which either adhere to cell surface or are extracted extracellularly in the growth medium

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(Fietcher,1992; Cameotra, et al., 1998 ; Zajic, et al., 1994). These biosurfactants molecules reduce surface tension. Several types of biosurfactants have been isolated and characterized, including glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids and lipopolysaccharides. Chemically synthesized surfactants have been used in the oil industry to aid clean-up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment. Due to the amphiphilic structure of biosurfactants they increase the surface area of hydrophobic water insoluble substances and change the properties of the bacterial cell surface. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agent (Desai, et al., 1997).

In comparison to their chemically synthesized equivalents, they have many advantages: -They are environmentally friendly, biodegradable, less toxic and non-hazardous. They have better foaming properties and higher selectivity. They are active at extreme temperature, p^H and salinity as well, and can be produced from industrial wastes and form by-products. This last features make cheap production of biosurfactants possible and allows utilizing waste substrate and reducing their polluting effect at the same time (Kosaric, 2001). The biosurfactants 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 (Soberon-Charaz ,et al 2011).

Biosurfactants activities depends on the concentration of the surface-active compounds until the critical micelle concentration (CMC) is obtained (Whang,et al ., 2008). Very often the growth of microorganism on hydrocarbon is accompanied by the emulsification of the hydrocarbon in the medium and in most cases this has been attributed to the production of surface active compounds. Biosurfactants producing microorganism are naturally present not only in hydrocarbon pollutes soils but are also present in pristine environment rich in organic matter suitable for the growth of diverse organism. Most known biosurfactants are synthesized by microorganisms grown on water immiscible hydrocarbon, but some have been produced on such water-soluble substrates as glucose, glycerol and ethanol (Abu-Ruwaida,et al., 1991).

The microorganism that produce biosurfactant are abound in nature, they inhabit both water (fresh water, ground water and sea) and land (soil, sediment and sludge). In addition, they can be found in extreme environment (oil reservoirs) and thrive at wide range of temperature, p^H values and salinity (Chirwa,2015). Furthermore, they can be isolated from undisturbed environment where they have physiological roles, not involving the solubilisation of hydrophobic pollutants, such as antimicrobial activity, biofilm formation or process of motility and colonization of surfaces (Van Hamme, et al., 2006). However, hydrocarbon degrading microbial communities remain the most implicated environment on white spread capability for biosurfactant production. Hence, the study evaluated the biosurfactant production capacity of bacteria from hydrocarbon polluted and pristine environment.
Materials and methods:

Sterilization:-
Media and Glassware were sterilized in autoclave at 121°C with 15 lbs pressure for 20 mins.

Soil sample collection:-
Surface soil samples were collected from various oil contaminated sources including Central railway station, Egmore railway station, Perembur logo, Avadi yard, Moolakadai bus depot using sterile spatula at a tillage depth of 1-2 cm , randomly from different points. The soil samples were collected into sterilized glass bottles, properly sealed labelled and wrapped with foil to prevent any further light reaction. All collected samples were stored in ice box at 40C and then transferred to the laboratory for further analysis. Temperature of collected soil ranged from 35-360C.

Media:-
Bushnell hass medium, nutrient broth, nutrient agar medium, Bushnell hass broth, Tryptone broth, MRVP broth, Carbohydrate broth, Simmon citrate agar.

Isolation, screening and identification of biosurfactant producing bacteria:-
Biosurfactant producing bacteria were isolated by successive enrichment culture technique from the petroleum contaminated soil using minimal salt medium containing diesel oil (2%) as a sole carbon source. The minimal salt medium used consist of (g/l): MgSO4:0.2, CaCl2: 0.02, KH2PO4:1, K2HPO4:1, NH4NO3:1, FeCl3.6, H2O:0.05,

Ph: 7. The isolation was done on solidified minimal salt medium where diesel oil is introduced as sole carbon source and incubation was carried out at room temperature for 5 days.

The isolated colonies were identified by following microbiological and biochemical tests: indole, MRVP, citrate TSI, H2S production, oxidase, catalase, urease, starch hydrolysis, gelatine hydrolysis, spore staining, motility test and gram staining.

Extraction of biosurfactant:-
For studying the biosurfactant activity, the selected isolates were inoculated into nutrient broth containing mixture of oils (petrol+kerosene+diesel) in 1:1:1 ratio and incubated overnight for 10 days at 300C. All the bacterial cells were removed by centrifugation at 12000 rpm for 30 mins. Cultural supernatant was acidified with 6N HCL to obtain the ph of 2. The extraction was performed twice with an equal volume of ethyl acetate. White precipitate formed culture was used for further experiments.
Screening of biosurfactants producing organisms:

Haemolysis test:-
Screening of biosurfactant producing bacteria was done by using haemolysis test (Carillo et al.,1996). Pure culture of bacterial isolates was streaked on the freshly prepared blood agar and incubated at 370C for 48-72 hrs. Results were recorded based on the type of clear zone observed.

Drop collapsing test:-
Screening of biosurfactant production was performed using the qualitative drop collapse test described by Bodour and Maier 1998. Crude oil was used in this test. Two microliters of oil were applied to the well region delimited on the covers of 96 well microtiter plates and these were left to equilibrate for 24 hrs. 5 micro litres of the 48 hrs culture, before and after centrifugation at 12000 rpm for 5 mins to remove cells, was transferred to the oil coated well regions and drop size was observed after I min with the aid of a magnifying glass. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production (Youssef et al., 2004).

Oil spreading assay:-
Oil spreading experiment was performed using the method described by Morikawa et al. 20 ml of distilled water was added to a plastic petri plates followed by addition of 20 microliters of crude oil to the surface water. 10 microliters of the cell free culture broth were then added to the oil surfaces. If the biosurfactant is present in the cell free culture broth, the oil will be displaced with an oil free clearing zone and diameter of this clearing zone indicates the surface activity, also called oil displacement activity. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed and triton X-100 was used as positive control.

Emulsification Index measurement:-
Emulsification index activity was measured according to the method of Cooper and Goldenberg (1987) with the slight modification. 1ml of cell free culture broth was added to the 5ml of 50millimolar tris buffer (p^H 8.0) in a 30ml screw capped test tubes. 5ml of hydrocarbon was added to the above solution and vortex shaken for 1min and the emulsion mixture was allowed to remain upright for 20min. The absorbance of the aqueous phase was measured by spectrophotometer, at the wavelength of 400nm. The emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed as percentage. The percentage of E24 indexes calculated by the following formulae

E24 = Height of the emulsified layer(cm) / total height of the column(cm) * 100

Results & discussion:-

This study was designed to isolate biosurfactant producing bacteria from oil contaminated soil and extract the surfactant produced. Five different soil samples were collected from different places like Central railway station, Egmore railway station, Perembur logo, Avadi yard, Moolakadai bus depot and were taken to microbiology lab for processing.

Standard microbiological and biochemical test were used to isolate, characterize and identify the bacterial isolates. From the five samples analysed, total of 10 isolates were obtained. On characterization, 3 of the isolates produced

biosurfactants after screening. Of the 3 organism isolated, 1 is gram negative rod and were identified as Pseudomonas sps, one was gram positive rod and identified as Bacillus and other was gram positive cocci identified as staphylococcus. These organisms demonstrated biosurfactant production to an extent.

Although gram negative rod-shaped bacteria are more frequently reported to produce biosurfactants. It can efficiently degrade the diesel engine oil faster than that of the other organisms. As incubation period increases the rate of degradation of diesel engine oil also increases. But it was seen that till 15 th day, the rate of degradation was much faster. This was probably due to the exponential phase of the cell growth. Pseudomonas will be effective in degrading the fuel when compared to other bacterial isolates in this study.

Most researchers have used maximum 2 to 3 screening methods before selecting biosurfactant producers. It is suggested that a single method is not suitable to identify all types of biosurfactants (Yousef et al., 2004). Therefore, combination of various methods is required for effective screening. Occurrence of biosurfactant producing bacteria in hydrocarbon polluted environments was reported by many researchers (Yateem et al.,2002., Bodour et al., 2003., das & Mukherjee., 2005) . Kiran et al., 2010 suggested that the single screening method is unsuitable for identifying all types of biosurfactants and recommended that more than one screening method should be included during primary screening to identify potential biosurfactant.

Blood haemolysis:-

Blood haemolysis is also usefull in testing for the presence of biosurfactantsas it has been previously reported that biosurfactants ca cause the lysis of erythrocytes (Mulligan, et al., 1984). From our study we discovered that all the isolates were haemolytic. Jain et al., reported the methods have some limitations because it is not specific as lytic enzymes can also lead to cleaning zone. Schultz et al., showed that some biosurfactants do not show haemolytic activity at all. Youssef et al., and plaza et al ., also reported the poor specificity of this methods. Mullaigain et al., recommended the blood agar method as a preliminary screening method which should be supported by other techniques based on the surface activity measurement.

Oil spreading method:-

Oil spreading method were conducted for the primary screening of biosurfactant production. The oil displacement method is the indirect measurement of surface activity of a surfactant sample tested against the oil, a larger diameter represents the higher activity of the testing solution (Rodrigues et al., 2006). The presence of biosurfactants results in displacement of oil and clearing zone formation. The diameter of clearing zone on the oil surface correlated to surface activity. Surfactant has a linear correlation between quality of surfactant and clearing zone diameter. among these five soil samples, Pseudomonas isolated from Egmore railway station and Perembur logo showed strong positive results. (table 1)

Table 1

s.no	Soil samples	Organisms isolated	Oil spreading results
1	Central railway station	*Bacillus*	Neg
2	Egmore railway station	*Pseudomonas*	Positive
3	Perembur logo	*Pseudomonas*	Positive
4	Avadi yard	*Bacillus*	Negative
5	Moolakadai bus depot	*Streptococcus*	Negative

Drop collapse test:-

Drop collapse test were conducted for the primary screening of biosurfactant production. These qualitative tests are indicative of surface and wetting activities (Youssef, et al.,2004). Among these soil samples collected bacillus isolated from CRS & AY showed positive results and streptococcus isolated from MBS showed positive and pseudomonas isolated from ERS &PL showed strong positive result.

A positive drop collapse test showed a preliminary identification of the biosurfactant activity of the bacterial cells that clearly indicated the production of biosurfactants by the bacterial cell. The positive drop collapse assay also revealed about the extracellular production of the biosurfactant and its surface-active nature. The study conducted by (Das and Chandran 2010, is in accordance with the present investigation. (table 2)

Table 2

s.no	Soil samples	ORGANISMS	Drop collapse results
1	Central Railway Station	*Bacillus*	+
2	Egmore Railway Station	*Pseudomonas*	++
3	Perambur Logo	*Pseudomonas*	++
4	Avadi Yard	*Bacillus*	+
5	Moolakadai Bus Depot	*Streptococcus*	+

Emulsification index activity:-

Emulsification activity gave indication on the presence of biosurfactant. Higher emulsification index indicated a higher emulsification activity of the tested biosurfactant . the finding of the present study revealed about the surface-active nature of pseudomonas strains screened to show emulsification activity as a property of biosurfactant produced by them. Formation of emulsion usually results from the dispersion of liquid phase (Desai and Bennet., 1997). similar study was conducted by (Aparan et al., 2011) (29) reported maximum emulsification activity of pseudomonas at 72 hrs (80%)

Among these isolates, pseudomonas isolated from Prembur logo showed a very good emulsification activity of 75%). (table 3)

Table 3

s.no	Soil samples	ORGANISMS	Height of emulsion layer	Height of the liquid column	Activity
1	CRS	*Bacillus*	-	-	-
2	ERS	*Pseudomonas*	0.6	1.0	60%
3	PL	*Pseudomonas*	0.9	1.2	75%
4	AY	*Bacillus*	0.3	1.2	25%
5	MBD	*streptococcus*	0.5	1	50%

Conclusion:-

In this era of green technology biosurfactant have led considerable interest for present and future application. Application of biosurfactant and biosurfactant producing bacteria in environmental cleaning is a potential area of more research as revealed from the present study. Both organic and inorganic contaminants can be removed through different process in which biosurfactants are involved. Thus, pseudomonas is considered to be the best biosurfactant for degrading the oil contaminated soil. These microbes are very promising for use in environmental biotechnologies.

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