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BIOREMEDIATION OF CRUDE OIL AND STUDY OF THE HYDROCARBON DEGRADING ISOLATES, THEIR BIOSURFACTANT ACTIVITY AGAINST HUMAN PATHOGENS

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ABSTRACT
Oil spill is a major threat to marine environment in recent years. Bioremediation of crude oil is of significant importance as chemical dispersants are highly toxic. Crude oil is mainly composed of hydrocarbons, which cause environmental pollution and health hazards to all living beings. Hydrocarbon-degrading microorganisms were isolated from sea water sample and percentage of degradability was estimated by gravimetric analysis. The gravimetric analysis study revealed that 53.4% and 51.9% of crude oil added to the medium were degraded by *Bacillus* and *Pseudomonas aeruginosa* respectively. Production of biosurfactant by isolated hydrocarbon degrading microorganisms were analysed by primary, secondary and quantitative test such as blood haemolysis test, oil displacement test, drop collapse test and emulsification test. Biosurfactants were extracted from all four bacterial isolates *Bacillus, Pseudomonas aeruginosa, Micrococcus luteus,* and *Serratia marcescens* by acid precipitation method. The crude biosurfactant was tested for its antimicrobial activity against human pathogens such as *Bacillus sp., Pseudomonas sp., Staphylococcus aureus, E. coli* and *Shigella spp.,* by agar well diffusion method. Maximum antimicrobial activity was reported by Biosurfactant extracted from *Bacillus* and *Serratia marcescens* against all tested pathogens. Isolated hydrocarbon degrading microorganisms were identified by 16s rRNA sequencing and standard biochemical testing. The isolates C1, C2, C3 and C4 were identified to be *Bacillus, Pseudomonas aeruginosa, Micrococcus luteus* and *Serratia marcescens* respectively.

KEYWORDS: Bioremediation, Hydrocarbons, Gravimetric analysis, Biosurfactants, Antimicrobial agent, laundry detergent.

INTRODUCTION

Crude oil is a naturally occurring unrefined petroleum product composed of hydrocarbon deposits and other organic compounds as defined by James G. Speight, 1999. It is also known as unprocessed oil or petroleum. Crude oil can be refined to produce usable products such as gasoline, diesel and various forms of petrochemicals. Crude oil is extracted from earth which is formed naturally from the fossil of animal and plants. Hydrocarbons are organic molecules that contain hydrogen and carbon comprising of various length and structures forming straight chains to branching chains to rings. It is the major constituent of crude oil. Though there are different types of hydrocarbons alkanes are the most common ones present in crude oil. Oil spill is generally the release of a liquid petroleum hydrocarbon into the environment especially in marine ecosystem during shipment of crude oil across countries. This is a major cause of pollution to marine environment. Though spills can occur in land, marine oil spills are has adverse effects on the environment. It is considered to be a global environmental problem worldwide.

The wide spread pollution caused to the marine environment is due to petroleum products discharge or oil spillage which is hazardous to the surroundings as well as live forms. Petroleum enters into the marine environment either through disposal, accidental spillage, leakage tankers and losses during transport or storage. Crude oil possess toxic effects on plants, animals, humans, and marine environment. Also dissemination of oils are comparatively very faster in marine spillage when compared to land resulting in serious effects on marine ecosystems. Toxicity of crude oil to the environment is mainly due to the presence of soluble fraction which consists of low boiling point aromatic hydrocarbons including benzene, toluene, xylene, naphthalene, pentrathene etc. (Hassein *et al*., 2016).

Bioremediation refers to removal, degradation or transforming of contaminants into harmless or less harmful substances with the help of microorganisms. The process relies on the enzymatic activity that determines the efficiency of microorganism to degrade the hydrocarbon contents in crude oil. Biosurfactant are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly and contain hydrophilic and hydrophobic moieties that reduces surface tension and interfacial tension between individual molecules at the surface and interface respectively. Application of bio-surfactants in medicine (Mukherjee *et al*., 2006) elucidated on the wide range of applications of bio-surfactants in medicine they include:

Antimicrobial activity: The diverse structures of bio-surfactants confer them the ability to display versatile performance. By its structure, bio-surfactants exerts its toxicity on the cell membrane permeability bearing the similitude of a detergent like effect. Nikhil Shah *et al.,*

2016 reported that several bio-surfactants have strong antibacterial, antifungal and antivirus activity; these surfactants play the role of anti-adhesive agents to pathogens making them useful for treating many diseases as well as its use as therapeutic and probiotic agent. A good example is the bio-surfactant produced by marine *B. circulans* that had a potent antimicrobial activity against Gram positive and Gram negative pathogens and Semi pathogenic microbial strains including MDR strain.

MATERIALS & METHODS

Isolation of hydrocarbon degrading microorganisms

Hydrocarbon degrading microorganisms were isolated from sea water sample using selective media called Bushnell Hass broth which is supplemented with 1% crude oil as sole carbon source. The isolated colonies were sub-cultured on to fresh nutrient media with 1% NaCl and stored at 4°C.

Bio-degradation assay

Biodegradation of crude oil is the process carried out to degrade crude oil added to the medium along with enriched culture. The amount of crude oil degraded is directly proportional to the degradation ability or efficacy of the microorganisms in degradation of hydrocarbons. Four different isolates were selected to test its degradation efficacy by estimation under gravimetric analysis.

The four culture isolates were enriched with Nutrient broth supplemented 1.5% $Nacl_2$ for 1hour.100ml of Bushnell Hass broth was prepared and sterilized at 121°C at 15lbs pressure for 15 minutes. The broth was cooled to 35°c and 1% of crude oil sample was added to it which acts as the sole carbon source.0.5ml of enriched culture isolate was added to the broth under aseptic condition and the flasks were kept in shaker incubator for 21 days at 37° °C and 120rpm. The procedure was repeated for all other four isolates and incubated. At the end of 21days the flasks were subjected to gravimetric analysis to estimate the degradation efficacy of isolated hydrocarbon degraders.

Extraction of residual crude oil

After degradation assay the residual oil present in the medium is extracted by solvent extraction procedure using separating funnel. The solvent used in this procedure is chloroform in ratio of 3:1 (Chloroform: Sample) the amount of residual oil is calculated by gravimetric analysis and percentage of degradation is estimated.

Gravimetric analysis

Gravimetric analysis is the easier and convenient method for estimating the amount of degradation using mathematical formula. Gravimetric assay is carried out by the technique through which the amount of analyte is determined based on the mass. Amount of hydrocarbons present prior to degradation studies are compared to the contents after degradation and the efficiency of microorganism's ability is accessed using the formula

$$\text{Percentage of degradation} = \frac{\text{Amount of crude oil added to the medium}}{\text{Amount of residual crude oil}} \times 100$$

SCREENING OF BIO-SURFACTANT PRODUCING ISOLATES

Haemolytic assay (Anandaraj and Thivakaran, 2010)

Haemolytic assay is the primary screening of bio-surfactant production. This assay is to confirm that the organisms are capable of bio-surfactant production as they lyse blood added to the medium. Beta haemolysis lyses the erythrocytes present in the blood and show a clear zone around the colonies. Bio-surfactant producing microorganisms will show characteristic beta haemolysis.

Drop collapse test

Drop collapse test is based on the principle that biosurfactant produced by microorganisms has the ability to reduce the surface tension between the hydrophilic and hydrophobic molecules. 24 hours enriched culture isolates were prepared and centrifuged at 10000 rpm for 5 minutes at 4°c and supernatant are collected. Single drop of crude oil was placed on the glass slide.20µl of cell free supernatant was added and the results were noted within 2 minutes for collapsing of oil drop.

Oil displacement test

Oil displacement test is the screening procedure for confirming bio-surfactant producing microorganism. This test works on the principle that the bio-surfactant reduces interfacial tension between the hydrophobic and hydrophilic moieties. Oil displacement test is similar to drop collapse test which is carried out in sterile petriplates. 15 ml of distilled water was poured on to the petriplate. 500µl of crude oil was added in the centre of the plate and allowed to spread throughout the plate. 200µl of cell free supernatant was added above it and results were observed for oil displacement within 2 minutes.

Emulsification assay

Quantitative tests are performed to quantify the rate of bio-surfactant produced by microorganisms by emulsification assay (E_{24})

$$E_{24} = \frac{\text{Height of emulsion layer}}{\text{Total height}} \times 100$$

A sterile test tube was taken and cell free supernatant was added to it. Equal volume of vegetable oil was added and the tube was vortexed vigorously at high speed for 2minutes.The tubes were allowed to stand for 24hours and the results were recorded on the next day.

Extraction of biosurfactant

100ml of Bushnell-Hass broth was prepared and sterilized by autoclaving at 121°c at 15lbs pressure for 15 minutes.1ml of vegetable oil was added aseptically to the broth which acts as carbon source 0.5ml of enriched culture was added to the broth and incubated at 37°C in

shaker water bath for 7days. After 7 days of incubation the culture was centrifuged at 10,000 rpm for 10minutes at 4℃ and cell free supernatant was collected. The supernatant was transferred to sterile test tube and pH was adjusted to 2 by addition of 1N HCl**.** To 1ml of supernatant 1ml of methanol and 2ml of chloroform was added and allowed to stand for few minutes. Intermediate layer between methanol and chloroform indicates presence of biosurfactant. The precipitant layer was aseptically transferred to sterile fresh tube and is allowed to evaporate overnight. The crude biosurfactant was collected and suspended in sterile distilled water and adjusted to pH 7.0**.** These procedures were repeated to collect enough amounts of biosurfactant.

Antimicrobial activity of biosurfactant

The antimicrobial activity of the extracted biosurfactant was tested against human pathogens by agar well diffusion method. The selected human pathogens were *E. coli, Pseudomonas, Bacillus, Salmonella, Shigella*

Identification and characterization of cultures

In biodegradation assay the microorganisms showing maximum degradation ability and those isolates that yielded maximum amount of biosurfactant was characterized by 16S rRNA sequencing. Other organisms were confirmed using Gram staining, motility and biochemicals.

RESULTS

Isolation of hydrocarbon degrading microorganisms

Sea water sample was serially diluted and hydrocarbon degrading micro-oganisms were isolated using Bushnell-Hass medium (selective medium) with 1% crude oil as sole carbon source. Four different isolates were selected for biodegradation assay.

FIGURE 1.a) *Bacillus spp.* (C3)

FIGURE 1.b) *Psuedomonas aeuroginosa* (C2)

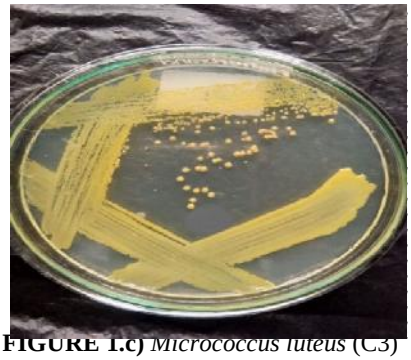

FIGURE 1.c) *Micrococcus luteus* (C3)

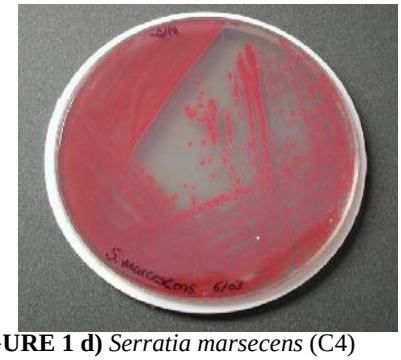

FIGURE 1 d) *Serratia marsecens* (C4)

FIGURE 1: Isolated microrganisms from sea water sample by serial dilution and spread plating on BHA

Biodegradation assay

Biodegradation of the crude oil was performed with the selected isolates (CI, C2, C3, C4) which were found to exhibit degradability. By the day 1 crude oil added to the medium was found to float on the surface of broth. After 21 days of degradation it was found to settle or well mixed with the broth indicating presence of degradation with respect to the culture isolate added to the medium.

Fig 2a) Biodegradation assay-Before incubation **Figure 2b)** After 21 days of incubation

Extraction of residual crude oil

After degradation study, residual crude oil present in the broth was extracted using solvent extraction procedure to estimate the amount of degradation. The hydrocarbon layer forms as last layer along with the solvent and the above layer is the broth. (Fig 5.3a) Hydrocarbon layer is decanted using separating funnel and allowed to evaporate to obtain the residual crude oil. The collected residual crude oil was subjected to gravimetric analysis.

Figure 3 a) Extraction of residual oil **Figure 3 b)**Extracted residual oil

Gravimetric analysis

Gravimetric analysis is the weighing method to calculate % of crude oil degradation by respective isolates. The percentage of degradation was estimated and tabulated.

Weight of empty container =1.20 g

Weight of 1ml of crude oil added to the medium = weight of crude oil with the container (-) weight of empty container =3.80-1.20 = 2.6 g

Weight of residual oil added to media= weight of container with residual oil (-) weight of empty container = 2.41-1.20 =1.21 g

Amount of oil degradation by culture 1= amount of oil added to the media (-) amount of residual oil =2.6-1.21

$$\% \text{ of degradation by culture 1} = \frac{\text{Amount of oil degraded}}{\text{Amount of crude oil added to the medium}} \times 100$$

$$= \frac{1.39 \times 100}{2.6} = 53.4\%$$

Thus % of degradation by culture 1 is estimated to be 53.4%. Similarly degradation of other three culture isolates were calculated by the above formula and tabulated.

Culture isolates	Percentage of degradation
Bacillus spp.,	53.4%
Pseudomonas aueroginosa	51.9%
Micrococcus luteus	47.6%
Serratia marcesens	39.6%

Screening of biosurfactant

Primary screening

Blood haemolysis was performed for primary screening to detect biosurfactant producing microorganisms. All the four isolates were found to produce clearance when grown on 10% blood agar plates. These isolates produced haemolysis as biosurfactants produced by these microorganisms produced amphiphilic molecules which has the ability to lyse blood

Secondary screening

Secondary screening is the confirmatory procedure to screen for biosurfactant producing microorganism. Oil displacement test and drop collapse test were the tests performed under secondary screening procedure.

- Oil displacement test

Culture isolate	Extent of displacement
C1	+++
C2	+++
C3	++
C4	+

+++(bigger displacement) ++(medium displacement) +(smaller displacement)

Figure 4 a) oil displacement by Bacillus spp.,(C1)

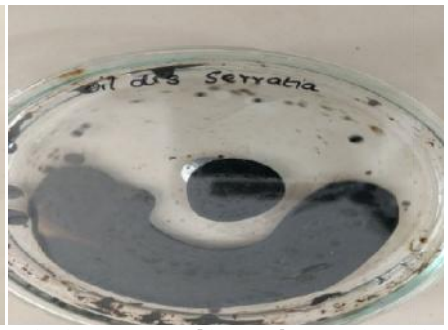

Figure 4 b) Pseudomonas spp.,(C2)

Figure 4.c) *Micrococcus luteus*

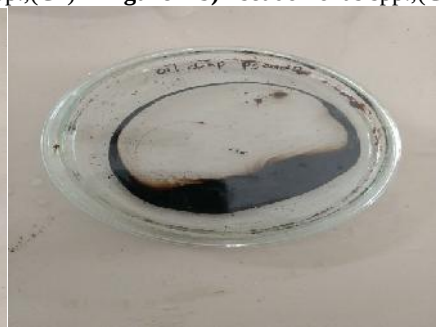

Figure 4.d)*Serratia marsecens*

C1	55.8
C2	52.0
C3	49.7
C4	41.3

Emulsification index: Emulsification index was tested to check the emulsification activity of isolated microorganisms. Higher emulsification index indicates the ability of microorganism in emulsification of hydrocarbons and other contaminants in higher rate.

Culture isolates	Result
C1	Positive
C2	Positive
C3	Negative
C4	Positive

All the four isolates were found to be positive for emulsification index. It was calculated with the formula,

$$E_{24} = \frac{\text{Height of emulsion layer}}{\text{Total height}} \times 100$$

Emulsification index was calculated using the above formula and the results were tabulated

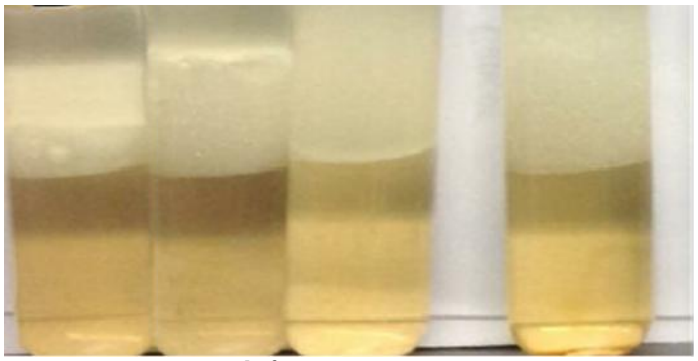

FIGURE 5. -Emulsification -C1, C2, C3, C4

Drop Collapse Test

Extraction of biosurfactant

From the above results microorganisms that produce efficient biosurfactant was screened. By solvent extraction Procedure biosurfactant was extracted and its antimicrobial activity was tested against human pathogens. The intermediate layer between the solvent indicates the presence of biosurfactant and it is precipitated and allowed to evaporated and crude biosurfactant was collected.

Antibacterial assay

Highest antibacterial activity was observed by biosurfactant extracted from *Bacillus spp.*,(C1) and *Serratia marsecens* with increased zone of inhibition.

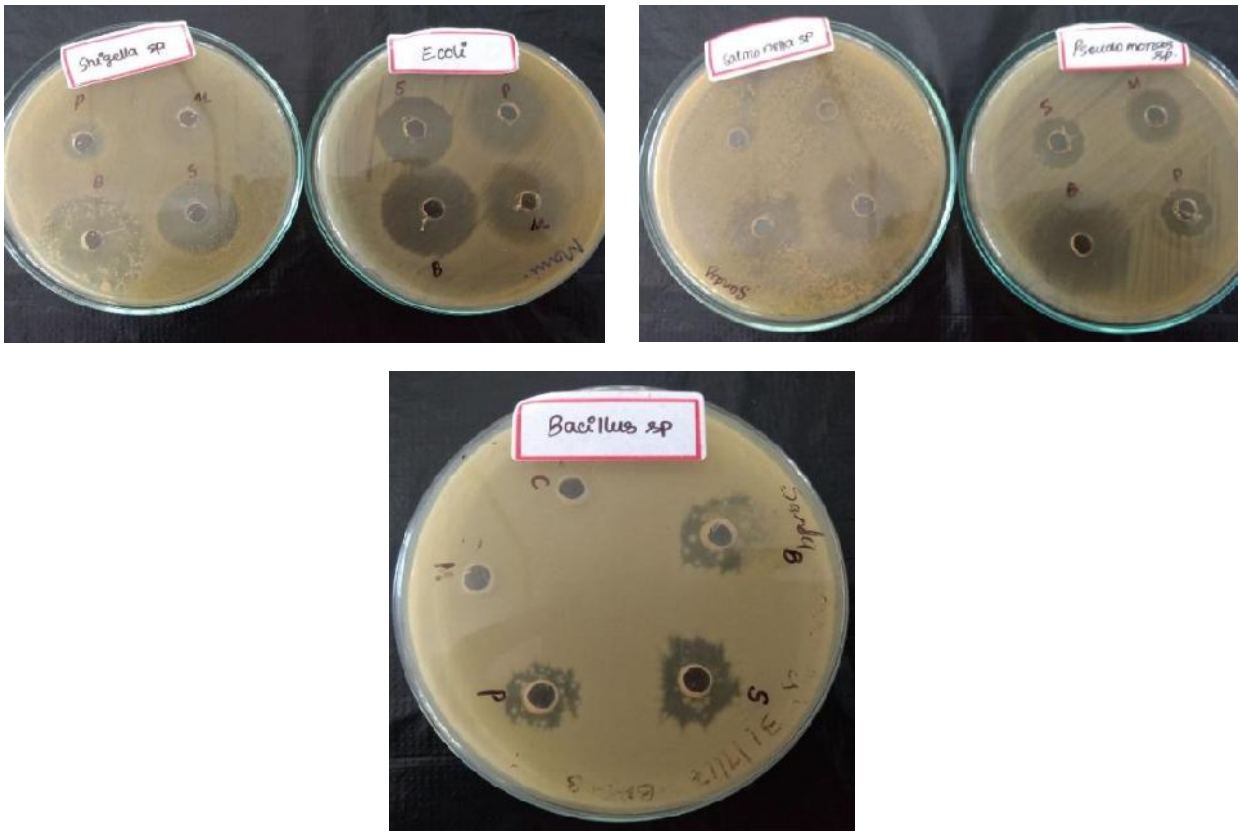

FIGURE 6. Antimicrobial activity of extracted biosurfactant against pathogenic isolates

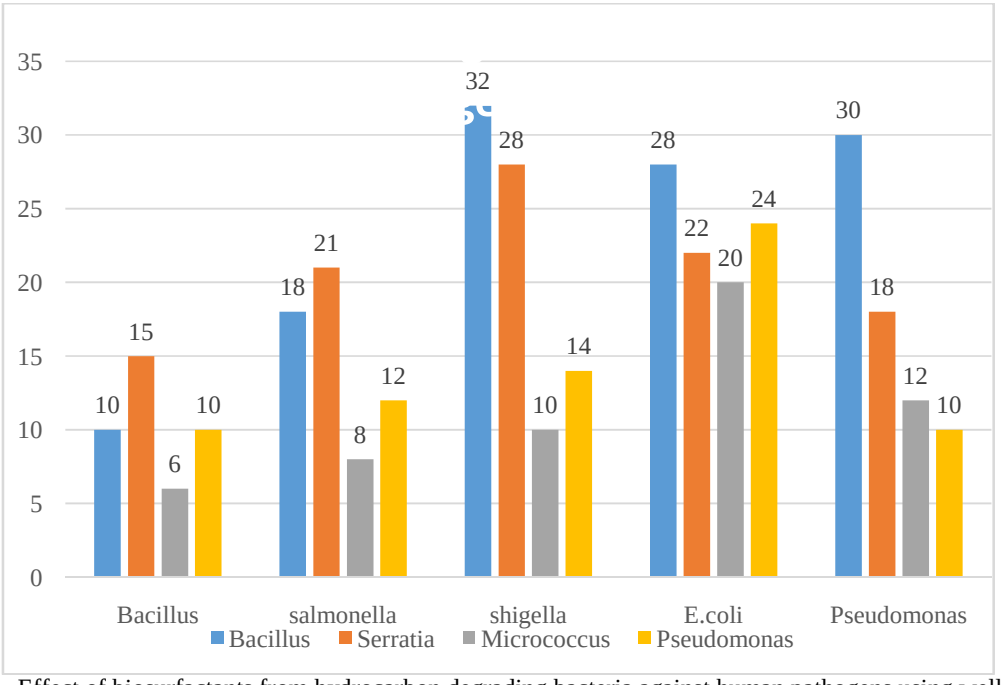

CHART 1 -Effect of biosurfactants from hydrocarbon degrading bacteria against human pathogens using well diffusion method for antibacterial activity (in mm)

Identification of isolates and characterization of isolates by 16S rDNA sequencing

Isolates microorganisms were identified by colony identification and 16S rDNA sequencing. Maximum degradability was reported in culture C1 and C2. Thus both the isolates were confirmed by 16S rDNA sequencing and rest of the two isolates was identified by Gram's staining, motility and biochemical testing. C3 and C4 were identified and reported as *Micrococcus luteus* and *Serratia marcesens* respectively.

DISCUSSION

Hydrocarbons are considered to be major environment pollutant and toxic to marine and terrestrial environment. Hydrocarbons accidently enter into marine ecosystem by oil spillage during shipment and transportation. Marine environment is rich in diverse group of microorganisms which may exhibit the property of biodegradation. Chemical surfactants are chemically active molecules is considered to be toxic to marine ecosystem and live forms. Thus biosurfactant produced by these hydrocarbon degrading microorganisms are considered to effective and alternative solution in oil spill clearance and bioremediation of hydrocarbons (Roger Marchant Ibrahim M. Banat 2012). In this study hydrocarbon degrading microorganisms were isolated from sea water sample using selective medium called Bushnell-Hass agar. Biodegradation ability of the isolated cultures was estimated by gravimetric analysis (Chitra S *et al.,* 2014) Highest degradation was reported in *bacillus* and *pseudomonas spp*.

Gravimetric analysis is the method to estimate the amount of crude oil degradation. By the end of incubation period the residual crude oil is extracted using solvent. The extracted crude oil is weighed to estimate the % of degradation. The amount of degradation was studied and maximum degradation was reported by *Bacillus spp.*, and *Micrococcus spp.*,

Biosurfactants are amphiphilic compounds produced by microorganisms which serves as an alternate source for oil spill clearance. Biosurfactant producing microoganisms were screened using haemolytic assay which is the primary screening procedure (Tabatabaee *et al*., 2005). This test was done to primarily screen for biosurfactant producing microorganisms. All the four tested isolates exhibited haemolytic activity indication production of biosurfactant.

Secondary screening procedure was performed to confirm the ability of the microorganism to produce biosurfactant. The confirmatory procedures include drop collapsing test and oil displacement test. Drop collapse test was performed to test the ability of biosurfactant produced by microorganisms to reduce surface tension and oil displacement test was performed to test the extent of interfacial tension (Nikhil Shah *et al.,* 2016).

Emulsification index is the quantitative test performed to test the extent of emulsification by microorganism. This is the procedure to quantify the amount of biosurfactant produced highest emulsification index was reported by *bacillus* and *micrococcus spp.*,

Biosurfactant was extracted using solvent extraction. The culture free supernatant was acidified to pH 2 and extracted using methanol chloroform mixture. The resulted biosurfactant was allowed to evaporated and stored under -20℃. Maximum yield of biosurfactant was reported in *Bacillus spp.,* 1.5 grams of biosurfactant was extracted from every 100ml of culture free supernatant.

Biosurfactants are studied for their antimicrobial activity against human pathogens by agar well diffusion method. Maximum activity was reported by biosurfactant extracted from *bacillus* and *serratia spp.,* maximum antimicrobial activity of biosurfactant extracted from bacillus spp., might be due to the presence of Rhamnolipids . Rahman et al., Some earlier workers have reported that the biosurfactant production by P. aeruginosa DS10-129 and P. aeruginosa LBM10 have

Remediation of petroleum contaminated sites pose serious threat to the environment being highly toxic. The remediation of contaminated sites can be achieved by physicochemical or biological methods. Crude oil cannot be completely cleaned up with physicochemical methods. Thus, more attention is given to biological alternatives. Biosurfactants play an important role in remediation agents as well as their environmentally friendly characteristics, such as low toxicity and high biodegradability. (Silva E.J. *et al*., 2014) Biosurfactants have applications in different industrial processes as well as novel uses and expected to become known as multifunctional materials of 21st century.

Currently the major market for biosurfactant in the petroleum industry, in which these compounds can be used in the clean-up of oil spills, removal of oil residue from storage tanks, microbial enhanced oil recovery, and the remediation of soil and water (Sobriho H.B. *et al.,* 2015). The economics of biosurfactant production merit particular attention. The total production of surfactants in 2012 was ~ 12 million tons, of which 3.5 million tons were biosurfactants. Industries are currently seeking to replace some or all chemical surfactants with sustainable biosurfactants, but high production cost is major drawback.

Antimicrobial activity of biosurfactant was reported in several studies. In the present study antibacterial activity of biosurfactant against disease causing human pathogen was tested and highest antibacterial activity was reported by *Bacillus* and *Serratia spp.,* with increased zone of inhibition in agar well diffusion method. This antimicrobial activity of biosurfactant is reported as they possess anti-adhesive property to pathogens, making them useful in treating many diseases and serving as therapeutic agents (Eman Zakaria 2013). The lipopeptide surfactants produced by bacillus genus present a great potential for biotechnological and biopharmaceutical applications due to their biological properties.

Sheppard *et al.* (1991) showed that various interesting biological properties of lipopeptide biosurfactants were presumed to be the result of interactions with the membranes of target cells. One explanation of the antimicrobial activity of biosurfactant is the adhering property of the biosurfactants to the cell surfaces caused deterioration in the integrity of the cell membrane and breakdown in the nutrition cycle.

Another explanation is the amphiphilic structures of biosurfactants, insertion of fatty acid components of

biosurfactants into the cell membrane caused an increase in the size of membrane and significant ultra structural changes in the cells such as ability of the cell to interiorize plasma membrane. Alternatively it is possible that the insertion of the shorter acyl tails into the cell membrane causes a disruption between the cytoskeletal elements and the plasma membrane (Desai and Banat 1997).

CONCLUSION

Bioremediation is an eco-friendly process on converting toxic compounds into toxic products by clearing pollutants. This process is of significant importance in treating oil spill clearance which is a major threat to marine and terrestrial ecosystem. Biodegradation ability of microorganisms is closely related to the biosurfactant produced by their cell walls. These biosurfactants play an important role in oil spill clearance. There are numerous chemical surfactants which are harmful for the environment when it is used for bioremediation process.

In the present study highest degradation of crude oil was reported by *Bacillus subtilis* and *Pseudomonas aeruginosa* as 53.4 and 51.9%. Bio-surfactant was extracted from the cell free supernatant and it was analysed through series of test to estimate its efficiency. Antimicrobial activity of bio-surfactant wastested against different human pathogens by agar well diffusion method. Highest antimicrobial activity was reported in *Bacillus subtilis* and *Serratia marcesens.* Thus importance of biosurfactants produced by microorganisms and their role in bioremediation of oil spill clearance and antimicrobial property was analysed by the present study. More biosurfactants can be produced by optimising the production medium and selecting active strains to increase the yield. These biosurfactant also plays an important role in application as laundry detergent and in removal of heavy metal pollution.

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