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RESEARCH ARTICLE

Bioemulsification of Crude Oil from Contaminated Soil using Fungal Biosurfactant Produced by *Aspergillus fumigatus* (PN1) and its Impact on Plant Growth

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ABSTRACT

Oil industries, refineries and automobiles are unavoidably generates large quantities of oil and viscous residue of sludge, which are formed during various production, transportation and refining. It is adversely affecting human health and posing several environmental problems. The hydrocarbon degrading microorganisms occur in most environments, where hydrocarbons may serve as organic carbon sources. The present study aimed to elaborate a new strategy of soil column process by the application of biosurfactant and synthetic surfactant in soil decontamination. Biosurfactant and chemical surfactants removed crude oil content about 53 and 78% respectively. Emulsified water severally influenced the plant germination and chromosomal aberrations of allum cepa roots.

Key words: *Crude oil, Bioremediation, Aspergillus fumigatus, Biosurfactant, Synthetic surfactant*

HIGHLIGHTS

- Potential biosurfactant producing fungal strain was obtained and used
- The biosurfactant has been used as an emulsifying agent to remove the crude oil in contaminated soil.
- A comparative approach was carried out with chemical surfactant for their efficiency
- Impact of treated and untreated soil was tested for the growth of green gram

INTRODUCTION

Environmental contamination by petroleum derivatives has been a subject of study over the past 4 decades. Petroleum hydrocarbons especially crude oil and its bi-products are serious environmental pollutants because of their persistence and high toxicity to all biological systems. India is both major energy producer and consumer. Currently India ranks as the world's eleventh greatest energy producer, accounting for about 2.4% of the world's total annual energy production, and as the world's sixth greatest energy consumer, accounting for about 3.3% of the world's total annual energy consumption (Kandasamy 2002). Oil is both the principle source of energy for man and an important environmental pollutant (Ferrari, *et al.* 1996; Vasudevan and Rajaram 2001). Crude oil is a complex mixture of many petroleum hydrocarbons like alkanes, aromatics, resins and asphaltenes associated with other organic compounds containing sulfur, nitrogen and oxygen. Among the petroleum hydrocarbons, aromatic compounds constitute a major fraction containing 30 polyaromatic hydrocarbons (PAHs) (Kumar, *et al.* 2011).

As petroleum exploration and commercialization continues to increase, routine and accidental spills are causing greater damage to the environment. The persistence of these contaminants can cause irreversible damage to the soil, air, rivers, oceans and groundwater (Vieira, *et al.* 2009). Leakage and accidental spills occur regularly during the exploration, production, refining, transport and storage of petroleum and its related products. Hydrocarbons are important soil pollutants because of the high toxicity of the polycyclic aromatic hydrocarbon (PAH). According to the environmental protection agency (EPA), 16 PAHs have been reported as carcinogenic and mutagenic compounds (Verdin, *et al.* 2004). In recent years, researchers are

working hard to find an effective and efficient way to remove the oil contaminants from the environment (Wei, *et al.* 2004).

Several authors have reported the physical and chemical treatment methods to handle crude oil contamination. Recovery of spilled crude oil by physical and chemical methods (booms, skimmers, adsorbents, chemical surfactants, oxidants and etc) over the marine water is possible only to 10-15% (Rahman, *et al.* 2002; Song, *et al.* 2013). The utility of physical and chemical processes has been limited due to their expensive operation and subsequent disposable problem of generated chemical sludge. Owing to these limitations in the management of crude oil contamination, the most versatile and widely used technology is biological process. Microorganisms play an important role in the petroleum contaminated sites. The hydrocarbon degrading microorganisms occur in most environments, where hydrocarbons may serve as organic carbon sources. Similar to microorganisms, application of biosurfactants to treat the crude oil contamination are attractive process since they are biodegradable and relatively non-toxic, making it an alternative compound to be released in bulk at a remediation site (Singh and Cameotra 2004; Rahman and Gakpe 2008). Biosurfactants act by emulsifying hydrocarbons, increasing the solubilization of crude oil and subsequent availability for microbial degradation (Menezes, *et al.* 2005; Zhang, *et al.* 2005).

In the present study an attempt was made to remove crude oil from contaminated soil using potential fungal strain and fungal biosurfactant. Chemical surfactants were applied comparing with biosurfactant to emulsify the crude oil from contaminated soil. Toxicity of the crude oil emulsified waste water was tested through the growth of onion plant and its chromosomal aberrations.

MATERIALS AND METHODS

Collection and Characterization of Crude Oil Degrading Fungi:

The fungal strain *Aspergillus* sp. (PN1) was obtained from the Bioremediation laboratory in the Department of Microbiology, Periyar University, Salem. The strain was identified by lactophenol cotton wet mount and maintained at 4^{o}C on Sabrouad Dextrose Agar ar slants in each month to maintain the viability of the strain.

For primary characterization about 100 ml of Bushnell-Hass (BH) medium containing (g/l) 0.2g of $MgSO_4$, 0.02g of $CaCl_2$, 1g of KH_2PO_4, 1g of K_2HPO_4, 1g of NH_4NO_3, 0.5g of $FeCl_3$amended with 1% (v/v) crude oil was prepared. To it, about 20mg/ml of 2, 6-Dichlorophnol indo phenol was incorporated as an indicator and sterilized. The flasks containing medium was enriched with 1% spore (10^6) suspension of *Aspergillus* sp. (PN1)and kept under agitation (120 rpm) at 35.0 ± 0.5^{o}C. Every 12 hrs the broth was centrifuged at 5000 rpm. The spectrophotometric value of the supernatant was taken at 600 nm (Hanson *et al.* 1993). In case of secondary characterization, every 12 hrs the oil content was extracted by solvent extraction method by using separating funnel. The residual oil was measured by using pre weighed watch glass by gravimetric analysis.

Effect of Various Carbon Sources on Crude Oil Removal in Aqueous Medium:

There are six different carbon sources namely; dextrose, sucrose, cellulose, fructose, glucose and starch were used find out their efficiency on fungal growth and the rate of crude oil degradation. Mineral salts medium amended with 1% respective carbon sources and 1% crude oil was prepared and sterilized. Spore suspension of 7 days old culture (PN1) was inoculated in each flask and agitated at 120rpm. Every day the samples were withdrawn aseptically and crude oil content was determined by gravimetric analysis. In this study, the dextrose at 1% was selected as optimum based on the rate of crude oil degradation.

Production and Characterization of Biosurfactant:

The fungal strain (PN1) was inoculated in sterile mineral salts medium amended with 1% dextrose (presumptively selected) and 1% crude oil. The flasks were kept under agitation at 120 rpm for 96 hrs. After incubation the culture broth was centrifuged at 5000 rpm and the cell free supernatant was used to determine the surfactant production and characterization by oil spread assay drops collapse method and emulsification index (E_{24}).

Emulsification Index (E_{24}):
The emulsification index was determined using the method described by Cooper and Goldenberg (1987) whereby 3 ml of a hydrocarbon (petrol, diesel, kerosene, used and fresh engine oil) was added to 3 ml of the cell-free culture broth in a graduated screw-cap test tube and vortexed at high speed for 2 min. Emulsion stability was determined after 24 h. The emulsification index was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100.

Oil Spread Assay:
About 20 ml of distilled water was added to a petri dish with a diameter of 15 cm. Then, 20 l crude oil was dropped onto the surface of the water, followed by the addition of 10 l of cell free biosurfactant. After 30 sec, the visualized clear zone was observed under visible light (Rodrigues *et al.* 2006).

Foaming Index:
The foaming index was done by adding 5 ml of cell free supernatant in a graduated tube, vortexed at high speed for 1 min. Foam activity was detected based on the duration of foam stability and foam height in a graduated cylinder (Dehghan, *et al.* 2010).

Mass Production and Extraction of Biosurfactant:
The mass production of biosurfactant was carried using mineral salts medium enriched with 1% dextrose and 1% crude oil. After inoculation of fungal strain (PN1), the enrichment was continuously mixed using a magnetic stirrer and the setup was maintained for 120 hrs. On final day, the surfactant was extracted and purified by solvent extraction method. The cell-free culture broth was acidified with 6M HCl to pH 2.0 and precipitated with two volumes of methanol. After 24 h at 4°C, the sample was centrifuged at 5000 rpm for 30 min, washed twice with cold methanol and dried at 37°C for 24-48 hrs. Biosurfactant yield was expressed as g/l. Known amount of crude precipitate was re-suspended in distilled water and used for further studies.

Soil Column Approach on the Removal of Crude Oil from Contaminated Soil:
The removal of crude oil from contaminated soil was carried out using a lab scale column packed with crude oil contaminated soil. This is a modified set up of biotransformation process reported by Ayyasamy and Lee (2009). Glass column's approximately 35cm in length and 4cm in diameter were used in this study and the experimental set up was made as shown in the (Fig. 1). There are eight emulsifiers were used to remove the crude oil from contaminated soil. The name and concentration of the emulsifiers are given in table 1. The emulsifiers from the reservoir were passed through soil column using a peristaltic pump at a flow rate of 20 ml per hour. The emulsified water was collected from the column outlet and used for analysis and pot culture experiment. The amount of crude oil content was estimated in each emulsified by toluene extraction method at 12 hrs time intervals. This study was carried out for the period of 120 hrs and on final day the soil samples were collected and checked the amount of crude oil content by spectrophotometric method.

Phyto and Genotoxicity Assays of Soil Column Extracts:
In phytotoxicity assay, the fertile soil was mixed properly with microbially remediated soil at 1:0, 1:1, 1:2, 1:3 and 1:4 ratios and used for the plantation. The prepared soil was filled in separate cups and irrigated with tap water. Four seeds of green gram (*Phaseolus aureus*) were sown in each cups and the set up was kept under open space. All the pots were irrigated with tap water to maintain moisture content. The seeds were allowed to germinate and germination percentage was assessed on every day of the experiment. On 10th day of the experiment, the plants were uprooted and shoot and root length was measured.
The onion was grown using soil column extracts. The growth and chromosomal aberrations were checked by root tip assay. The onion was grown in a 15 ml-Falcon tubes, filled with various extracts collected from soil columns. The base of the onion was kept reaching the surface of aqueous medium. The tube-stand was covered with aluminium foil to keep the onion

roots in dark during growth. The set up was kept at 25°C in an ultivator with light cycle. Control was maintained with the set up was irrigated with tap water. After exposition the plant with the poorest root growth was excluded. Approximately 10mm size of the root tip was chopped in each experiment and placed them into 10ml glass tube with 2 ml acetic acid/ HCl solution. The root tip was heated for 5 minutes at 50ºC. Hereby, the root cells became fixated and macerated. Thereafter, the root tip was placed on a microscope slide on a black background and cut off the terminal tips (1-2 mm) for further preparation. The rest of root material and liquid were removed from the slide. Then 2 drops of orcein solution was added and mixed it with the roots properly by stirring and knocking with a stick of stainless steel (stirring spattle). A cover slip was placed on the root cells. After that the cells were squashed by placing to layers of filtrate paper on the cover glass and pressing slightly down with thumb. The cover slip was fixed to the slides by applying clear nail varnish on the margin. The microscopic analysis includes mitotic index, micronuclei presence in interphase cells and chromosomal aberrations in late anaphase and early telophase cells score.

Table 1: Types of emulsifier and their concentrations

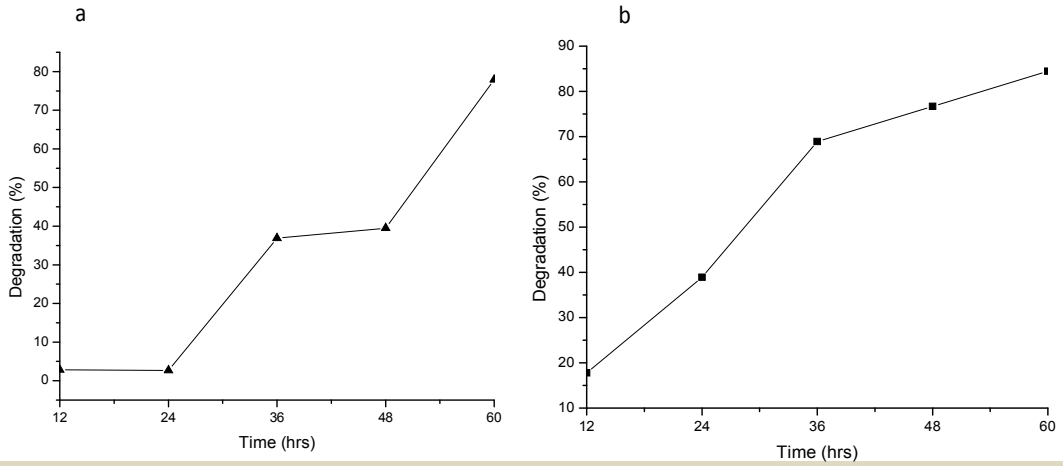
S. No	Name of the Emulsifiers	Concentrations (%)
1.	Fungal biosurfactant	0.5
2.	Fungal biosurfactant	1.0
3.	Fungal biosurfactant	1.5
4.	Sodium dodecyl sulphate	0.5
5.	Sodium dodecyl sulphate	1.0
6.	Sodium dodecyl sulphate	1.5
7.	Triton X 100	0.5
8.	Triton X 100	1.0
9.	Triton X 100	1.5
10.	Distilled water	Raw

Fig. 1: Bioremediation of crude oil contaminated soil by soil column process

STATISTICAL ANALYSIS

Statistical analyses were carried out using a statistical package (SPSS, Version 16.0) to find out relationship between the biosurfactant and synthetic surfactants. Tests of significance, was carried out at 95% level of confidence using the statistical package. P-Values were used to determine the significance levels between various treatments and data obtained during the experimental study.

Fig. 2: Screening of crude oil degradation **a.** DCPIP Study **b.** Gravitational Analysis



RESULTS AND DISCUSSION

Culture Collection and Confirmation:

The fungal strain *Aspergillus fumigatus* (PN1) was collected from bioremediation laboratory, Department of Microbiology, Periyar University, Salem. This strain was identified by morphology in fungal media plate and lacto phenol cotton blue staining method. This strain was produced colonies showing typical blue-green surface pigmentation with a suede-like surface consisting of a dense felt of conidiophores. The microscopic appearance shows uni seriate and columnar conidial heads with the phialides limited to the upper two thirds of the vesicle and curving to be roughly parallel to each other.

Toxic Resistant Study:

In the toxic resistant study, the result showed that the more or less similar colony forming units in control and test. The strain *A. fumigatus* (PN1) was confirmed that it has crude oil tolerant and capable to utilize the oil from the contaminant. In a previous evalution, Bartha and Atlas listed 14 genera of fungi isolated from an aquatic environment which had been demonstrated to contain members which utilize petroleum hydrocarbon. The evolution of the hydrocarbon mixture depends on the nature of the oil, microbial community and environmental factors which impact microbial activities.

Screening of Crude Oil Degrading Ability of *A. fumigates* (PN1):

The DCPIP study was evidence for taking out the effective bacterial strains from the isolates. Basically this study was owned by Hanson, *et al.* (1993) this technique had also been employed by several workers (Cormack and Fraile 1997; Mariano, *et al.* 2008). Similar method was used by Cormack, *et al.* (1997) to degrade n-hexadecane. They used n-hexadecane as carbon source and degradation capability of the strain was confirmed by the colour change of DCPIP from blue to colourless 12 h after inoculation. Balba, *et al.* (1998) reported those even native cultures (*S. hominis*and, and *K. palustris*) and the genera *Bacillus* and *Pseudomonas*, known to be responsible for oil degradation. The degradation was determined on the basis of colour change of the indicator. Crude oil removal by *A. fumigatus* (PN1) was investigated and attained 77.9% degradation (Fig. 2a). This results agreed with the results of Gesinde, *et al.* (2008) who indicated that *Aspergillus* sp. have very active degradation capabilities of four kinds of oil compounds, Durb oil, Escravos light, Arabian light and Bonny light. Yakubu, *et al.* (2009) studied crude oil degradation by bacterial species in soil which was in significant when compared to fungi. In this study, the degrading ability was confirmed by secondary screening of gravimetric analysis. Most of the recent researchers were used this method for quantitative analysis of crude oil degrading microbes in lab scale method. The strain *A. fumigatus* (PN1) removed the maximum level (84.4%) of crude oil in liquid medium within 60 hrs of incubation period (Fig. 2b).

Effect of Various Carbon Sources on Crude Oil Degradation in Aqueous Medium:
Out of 6 carbon sources used dextrose at 1% showed very good efficiency (97%) on the degradation of crude oil. There is no significant removal of crude oil was recorded using other carbon sources. Hence, dextrose was selected as potential carbon source for biosurfactant production.

Production and Characterization of Biosurfactant:
In this study the *A. fumigates* (PN1) showed as potential representative to produce very efficient biosurfactant based on the results of emulsification index, foaming index and oil spread assay. The emulsification index was done by comparing biosurfactant (cell free supernatant (CFS) with synthetic surfactants (SDS and Triton X 100) and water using different hydrophobic phases (Petrol, Diesel, Kerosene, Used engine oil and fresh engine oil). The CFS emulsifies 50% of petrol and fresh engine oil and 23% of kerosene and used engine oil, 16% in Diesel. The synthetic surfactants gave a very good result compared to the biosurfactant. The synthetic surfactant was toxic to natural ecosystem even though it shows significant efficiency.

Effect of Synthetic and Biosurfactant on Oil Emulsification in Column Study:
Remediation of crude oil contaminated soil was done by soil column process. In this study, the initial concentration of crude oil was noted about 7000 ppm which was checked by toluene extraction method (Fig. 4). Emulsification was noted in each treatment process. Biosurfactant showed less emulsification when compared to synthetic chemical surfactants. At the end of the study, oil content in each sample and the soil was checked. Here, the biosurfactant produced by *A. fumigates* (PN1) and two chemical surfactants (SDS and Triton X100), significantly showed as very good emulsifying action. From the starting the oil content was emulsified and comes out. Biosurfactant slowed very good emulsification activity. In control (distilled water), there was no oil content run out from the column. Based on this process 53% of crude oil content was removed from contaminated soil. The chemical surfactants removed the oil content up to 78% within 120 hrs (Fig. 5). Poindexter and Lindemuth (2008) reported that chemical substances normally emulsified crude oil at maximum level. They confirmed that the concentration of surfactants influencing the remediation process. When the concentration increases (0.5-1%) the oil removal was slightly increased, but the same time the 1 to 1.5%, the remediation process slowed and the oil content of the soil also insignificantly emulsified. In the synthetic surfactants, Triton X 100 was showed higher emulsifying activity when comparedto the SDS. Correspondingly both chemical surfactants showed higher emulsifying activity than the biosurfactant. Significance between various concentrations biosurfactant and synthetic surfactants was analyzed using SPSS package Version 16.0. In this study, the significance between biosurfactant and synthetic surfactant found to be very less. However, SDS and Triton X 100 emulsified crude oil at 5% significant level (Table 2 and 3).

Fig. 3: Optimization of carbon source for crude oil removal in aqueous medium

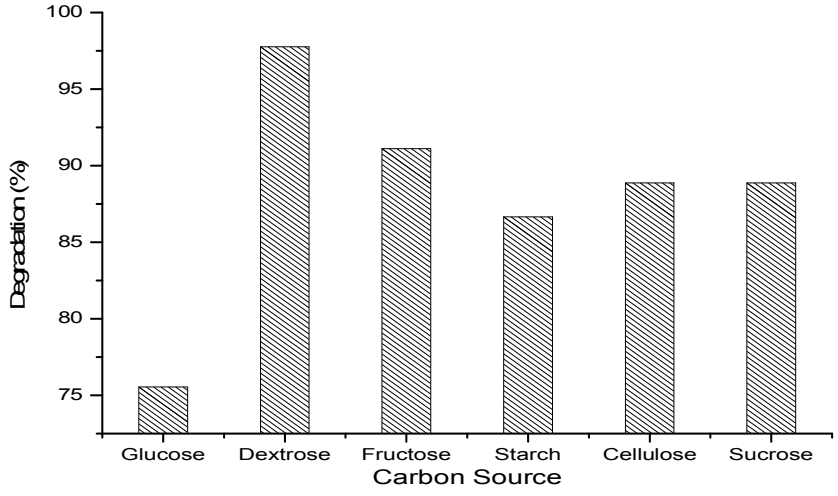


Fig. 4: Emulsification of crude oil from soil column using (a) various concentration of biosurfactant, (b) SDS and (c) Triton X100

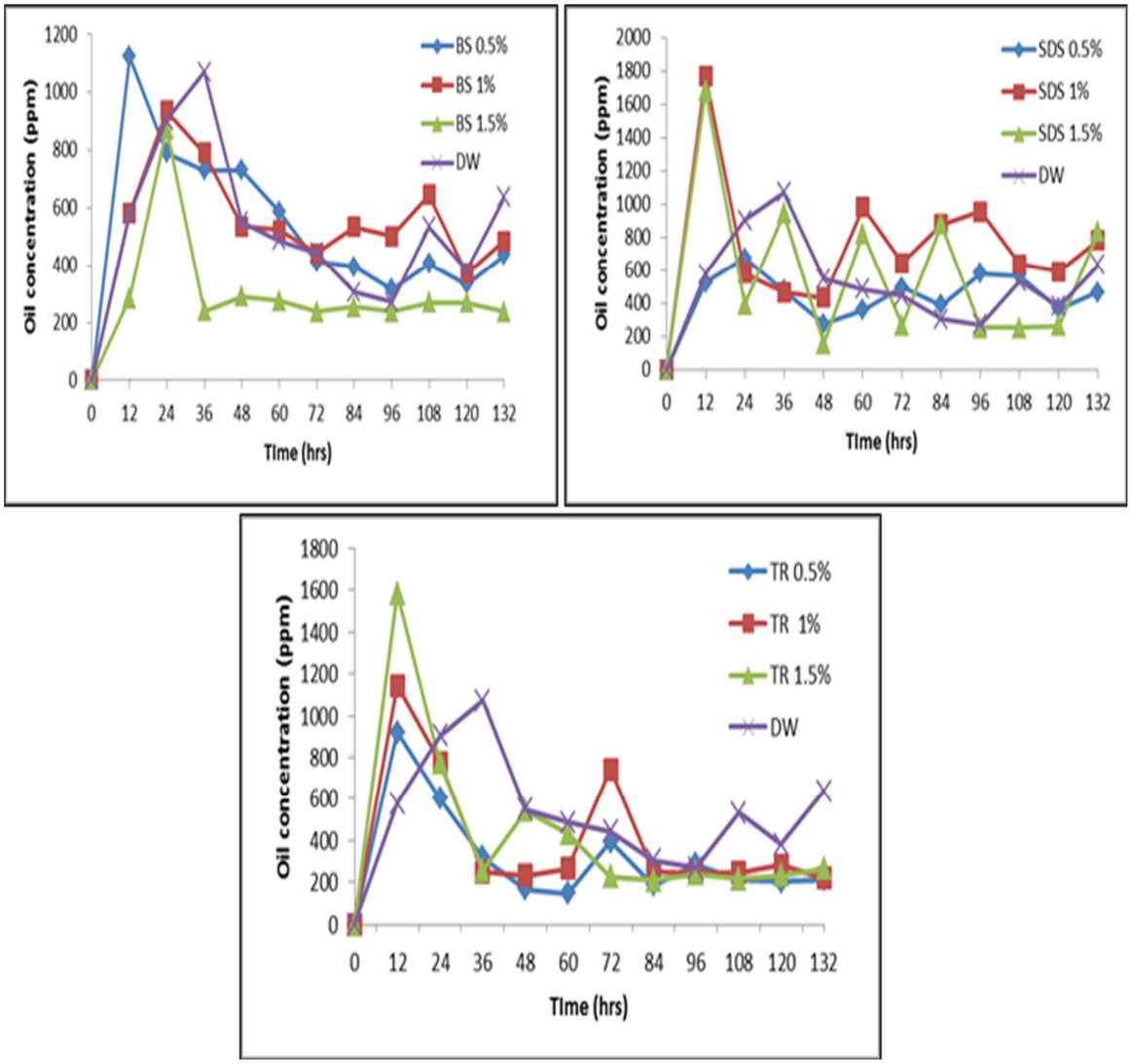


Fig. 5: Bioremediation of crude oil contaminated soil by soil column process

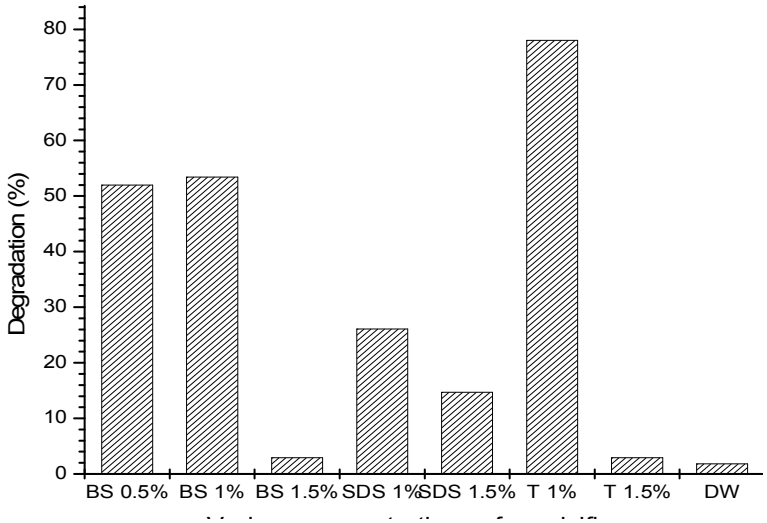


Fig. 6: Phytotoxicity assay by the irrigation of soil column extract in 12-120 hrs, a. BS 0.5%, b. BS 1%, c. BS 1.5%, d. Tr 1%, e. Tr 1.5%, f. DW

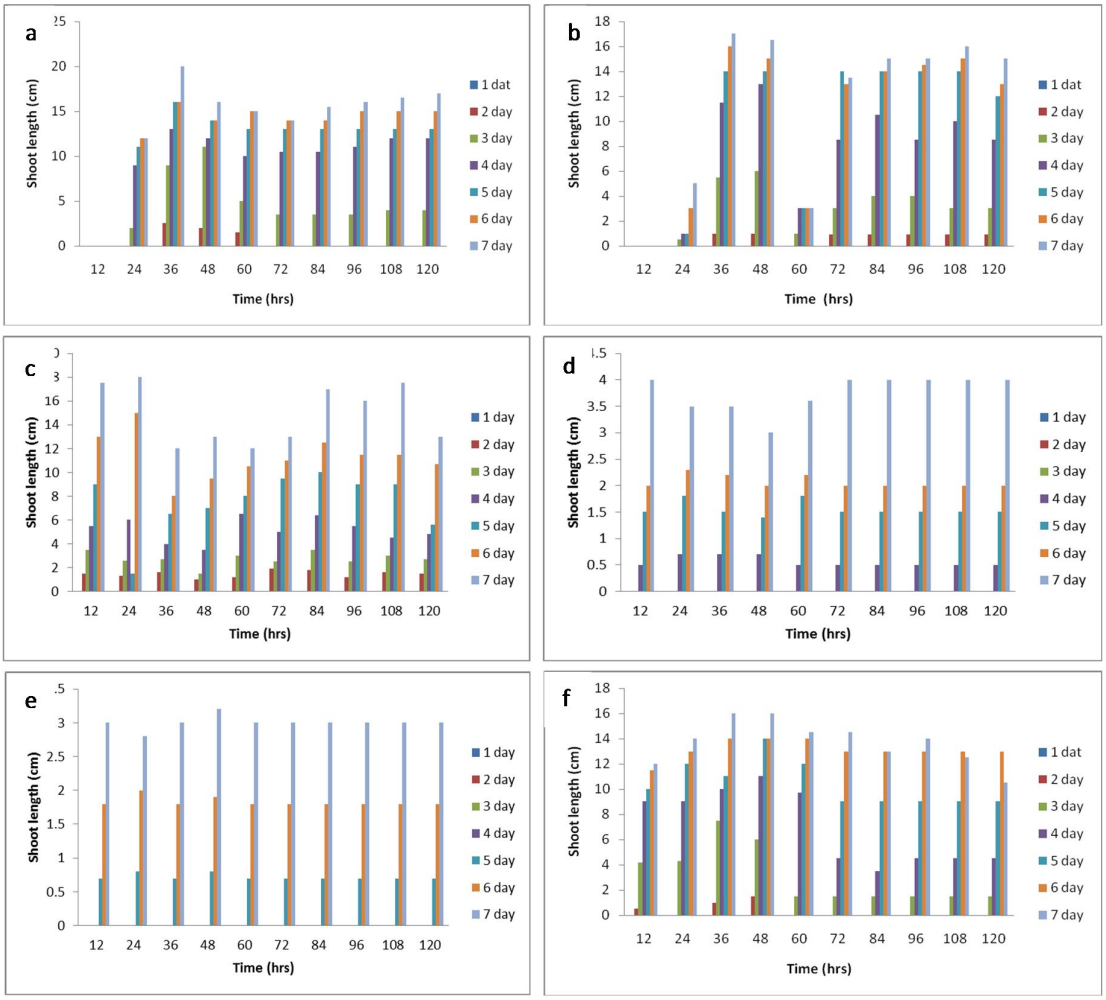


Table 2: Significance between various concentrations biosurfactant and synthetic surfactants

Emulsifiers	Control (0%)	BS 0.5%	BS 1.0%	BS 1.5%
Control Vs. various concentration of BS				
Control (0%)	1.000	0.702[b]	0.868[a]	0.596[b]
BS 0.5%	0.702[b]	1.000	0.703[b]	0.511
BS 1.0%	0.868[a]	0.703[b]	1.000	0.777[a]
BS 1.5%	0.596[b]	0.511	0.777[a]	1.000
SDS Vs. BS				
Control (0%)	1.000	0.702[b]	0.868[a]	0.596[b]
SDS 0.5%	0.605[b]	0.500	0.819[a]	0.819[a]
SDS 1.0%	0.128	0.626[b]	0.305	0.128
SDS 1.5%	0.394	0.693[b]	0.351	0.050
TR Vs. BS				
Control (0%)	1.000	0.702[b]	0.868[a]	0.596[b]
TR 0.5%	0.455	0.797[a]	0.527	0.500
TR 1.0%	0.349	0.727[a]	0.404	0.492
TR 1.5%	0.345	0.875[a]	0.430	0.405

[a]. Correlation is significant at the 1% level (2-tailed).
[b]. Correlation is significant at the 5% level (2-tailed)
Where, BS: Biosurfactant, SDS: Sodium dodecyl sulphate, TR: Triton X 100

Fig.7: Phytotoxicity assay by irrigation of soil column extracts

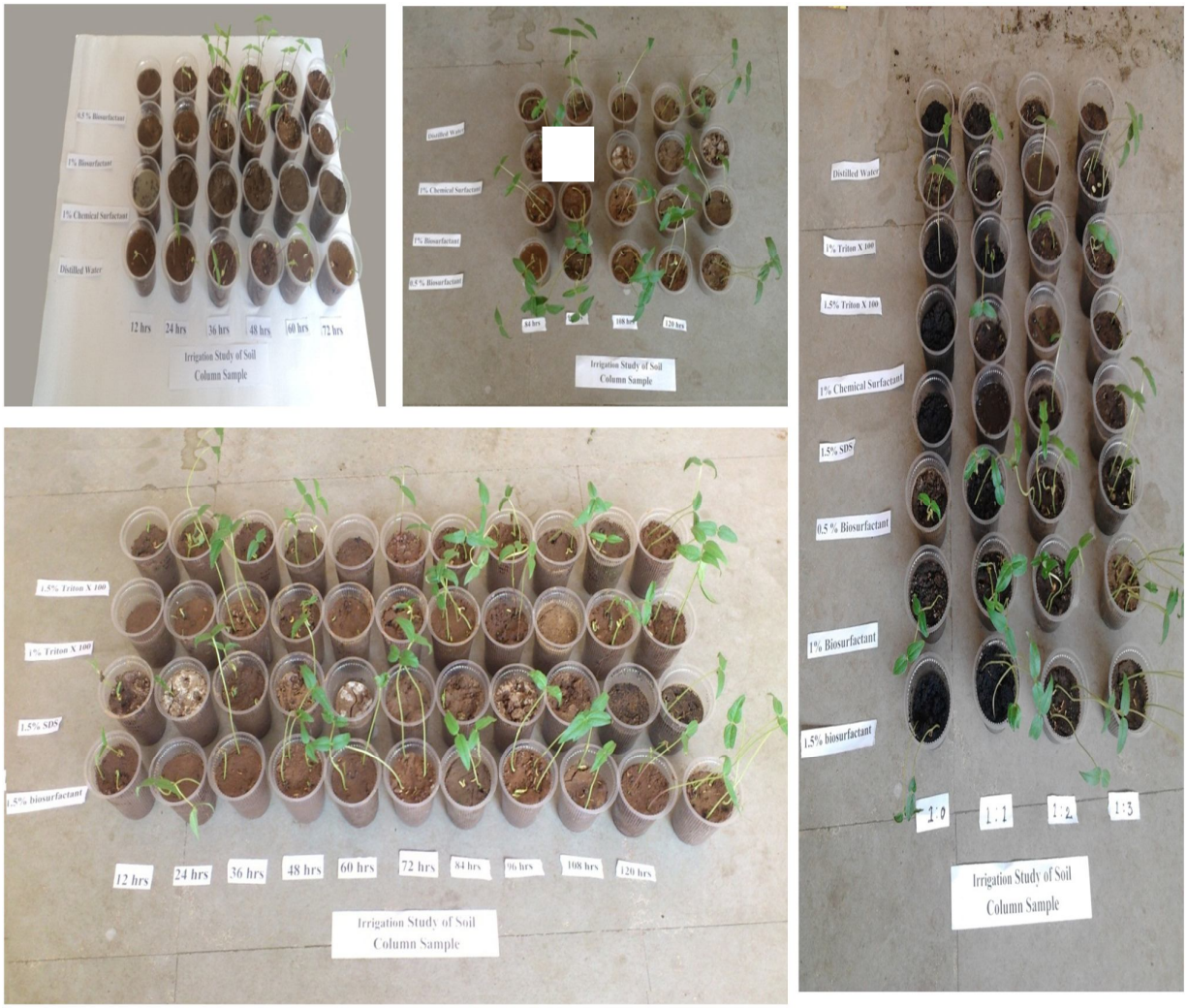


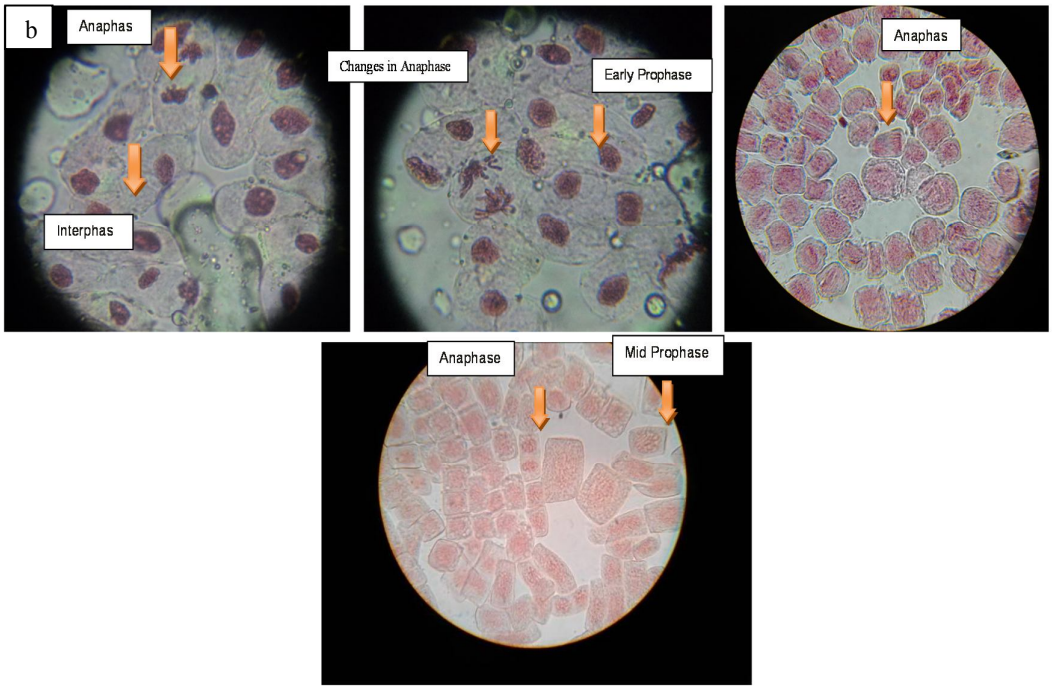
Table 3: Significance between synthetic surfactants [SDS and Triton X100(TR)]

Emulsifiers	Control (0%)	SDS 0.5%	SDS 1.0%	SDS 1.5%
Control Vs. various concentration of SDS				
Control (0%)	1.000	0.605[b]	0.128	0.394
SDS 0.5%	0.605[b]	1.000	0.506	0.299
SDS 1.0%	0.128	0.506	1.000	0.796[a]
SDS 1.5%	0.394	0.299	0.796[a]	1.000
Control Vs. various concentration of TR				
Control (0%)	1.000	0.455	0.349	0.345
TR 0.5%	0.455	1.000	0.952[a]	0.885[a]
TR 1.0%	0.349	0.952[a]	1.000	0.836[a]
TR 1.5%	0.345	0.885[a]	0.836[a]	1.000
SDS Vs. TR				
Control (0%)	1.000	0.455	0.455	0.345
SDS 0.5%	0.605[b]	0.624[b]	0.554	0.367
SDS 1.0%	0.128	0.712[a]	0.665[b]	0.752[a]
SDS 1.5%	0.394	0.630[b]	0.681[b]	0.531

[a]. Correlation is significant at the 1% level (2-tailed).
[b]. Correlation is significant at the 5% level (2-tailed).
Where, BS: Biosurfactant, SDS: Sodium dodecyl sulphate, TR: Triton X

Fig.8: Effect of soil column extracts on the growth and genetic characteristics of onion, **a.** Growth of onion (*Allium cepa)*

b. Microscopic observation of various phases of *Allium cepa* chromosomes



Toxicity Study:

The germination index, of relative seed germination and the onion root tip assay was used to evaluate the toxicity of the soil column processed sample to green gram. In the study of germination of green gram, the germination index was noted about 80% which has been used as an indicator of the absence of phytotoxicity in biosufactant mediated sample and also indicate that the biosurfactant solutions tested did not have an inhibitory effect on seed germination or root elongation. Moreover, leaf growth and the elongation of secondary roots occurred under all conditions tested. In this study clearly proved the chemical surfactants was very toxic to the plants. The 90% of the plant growth was inhibited by synthetic surfactants (Fig. 6 and 7).

In this study, the potential cytotoxic and genotoxic effects of soil column extractson *Allium cepa* were evaluated. There was a linear relationship between macroscopic and microscopic

parameters for all the extracts. There was concentration-dependent decrease in root growth and the order of induction of root growth inhibition was indicates the samples were toxic; Our results showed among other aberrations, induction of sticky chromosomes, bridges and disturbance of spindle fibers at different stages of mitotic division in the onion root cells. Based on the previous reports related to this assay, the extracts from chemical surfactants were inhibiting the root growth compare to the control and extracts from biosurfactant. Based on the microscopic assessment, the root tips from extracts of chemical surfactants were had a changes in morphological aberrations (Fig. 8). In *A. cepa*, whenever chromosome aberrations occurred, there were almost always certain growth restrictions (Fiskesjo, 1997). Most of these aberrations are lethal which can cause genetic effects, either somatic or inherited (Swierenga, *et al.* 1991).

CONCLUSION

Based on the observation, it could be concluded that the biosufactant produced by *Aspergillus fumigatus* (PN1) is a potential biological resource. It can be used for remediation of soil contaminated with crude oil pollution. The toxicity of the soil column extracts from bio and synthetic surfactants was carried out. There is no germination recorded in the extract of Sodium dodecyl sulphate. Triton X 100 extracts produce poor shoots but the soil column extracts by fungal biosurfactant shows the germination of green gram plants in more or less equal to the control plants using distilled water. The onion root tip assay also proved the genotoxicity of the extracts. The biosurfactants shows normal chromosome aberrations; however, the chemical treated extract shows some deviations in anaphase of the onion root chromosomes.

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