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Production of a non-cytotoxic bioflocculant by a bacterium utilizing petroleum hydrocarbon source and its application in heavy metal removal

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A bacterium isolated from the activated sludge of an oil refinery of Assam, India retained efficient bioflocculating activity through production of bioflocculant when it was grown on crude oil amended media avoided of any other carbon source. The bioflocculating activity gained from the optimized media broth was 86.2%, which could be enhanced up to 89.1% with the purified bioflocculant. In course of bioflocculant production, the bacterium utilized about 77% of the petroleum hydrocarbons after incubation for 168 h when the activity was found to be the highest. The bioflocculant was efficient in flocculating Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+}. The bioflocculant was characterized as a glycoprotein complex by biochemical tests, FT-IR, SEM-EDX and LC/MS analyses. The bioflocculant showed negligible cytotoxicity on testing with L292 cell line indicating tremendous possibility of its use in bioremediation.

Keywords: Bioflocculant, Crude oil, Heavy metals, Negligible cytotoxicity

1 Introduction

In the field of bioremediation and bioprocesses, microbial involvements are receiving wide attention and the search for robust microorganisms with desirable results has been actively pursued. Microorganisms can produce surfactants, emulsifiers, enzymes, proteins and exopolysaccharides, which participate in the bioremediation processes through both physical and biochemical mechanisms. Microbial flocculants are considered as a key asset in remediating water contaminated with inorganic and organic loads through the process of "Bioflocculation" considered as more efficient than the common chemical flocculation process [1]. Several bacterial species like *Alcaligenes latus*, *Paenibacillus* sp., *Bacillus* sp., *Pseudomonas aeruginosa*, *Rhodococcus* sp., *Acinetobacter* sp. etc., are known for their capacity to generate bioflocculants. One notable advantage in the use of a bioflocculant is its innocuous nature to the ecosystem [2]. The bioflocculants consist of microbial metabolic products that may include glycoprotein, polyoses, proteins, etc.[3]. In obtaining effective bioflocculant, different media

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including brewery wastewater, soybean juice, fishmeal wastewater, etc., are in use [4].

Bioremediation of petroleum and petroleum based pollutants has been a prime concern during the last few decades and many pure and mixed bacterial consortia have undergone scientific scrutiny and evaluation for practical and systematic application [5]. Often, oil-degrading microorganisms produce extracellular surface-active products to enhance the utilization of oily substrates through increased oil solubility or dispersion. *Pseudomonas aeruginosa* has been a prime candidate in this regard and has been widely tested for in situ or ex situ degradation of petroleum hydrocarbons leaving behind beneficial or harmless metabolites[6]. Many microorganisms cannot produce biosurfactants but are still able to degrade oil substrates effectively via formation of extracellular or cell membrane-bound bio-emulsifiers such as exopolysaccharides (EPS). The EPS-producing bacteria can utilize petroleum hydrocarbons as the sole carbon source for cell growth [7]. On the other hand, microorganisms such as *Rhodococcus* are known to utilize n-hexadecane more efficiently at low temperatures through biosurfactant or bioflocculant production and in the process, can degrade linear and branched alkanes[8]. Microbial cells may produce such extracellular substances in the form of capsules or mucoid secretions that may interact with hydrophobic substrates, such as hydrocarbons. *Achromobacter* sp. has also been reported recently as a potential source for bioflocculant production [9].

The following work was designed to evaluate the appropriate and optimum conditions for production of an efficient bioflocculant by a potent bacterial strain isolated from activated sludge of the effluent treatment system in petroleum refineries thriving on petroleum crude oil and this is the first report of such investigation. Further, the efficiency of such bioflocculant was determined *in-vitro* for possible use in removing heavy metals from water which was also approved to be a non-cytotoxic biopolymer.

2 Experimental

2.1. Collection of samples and chemicals

Activated sludge samples were collected from effluent treatment plants of the oil refineries at Guwahati, Bongaigaon and Numaligarh, Assam, India. Petroleum crude oil was collected from Numaligarh Refinery Limited, Golaghat district, Assam, India. All t chemicals were purchased from HiMedia chemicals, Sigma-Aldric., SRL India and MERCK, India.

2.2 Isolation and screening of bioflocculant producing bacteria

Bioflocculant producing bacteria were isolated from the activated sludge samples following the methodology reported earlier through enrichment culture technique and screened by spread plate technique with selective media agar plates [10]. The isolates were further grown in production media and their bioflocculati activities were determined by flocculating kaolin suspension with the supernatants of the production media broth as reported earlier by Kurane et al [11]. 1.0 litre of the production medium contained 5.0 g yeast extract, 5.0 g peptone, 2.0 g K_2HPO_4, 5.0 g KH_2PO_4, 1.0 g NH_4Cl, 1 % glycerol, 0.5 g $MgSO_4$, 2.5 g NaCl, 0.2 % $CaCO_3$. The initial pH of the medium was maintained to 7.2 and incubated for 72 h in an orbital shaker at 37± 2°C with a rotation at 150 x *g*. 0.04 g of kaolin clay was suspended in 9.45 ml distilled water and 0.5 ml of 1% $CaCl_2$ solution was mixed thoroughly with the kaolin suspensio 0.05 ml of the culture supernatant (taken as the raw bioflocculant) was added to the mixture and the pH of the mixture was adjuste to 7.0. The mixture was vortexed in a test tube for 1 min and th n

was kept at room temperature for 5 min. The flocculating activity was calculated using the formula,

Bioflocculating activity = $((A_s - A_{550}) / A_s) \times 100$ %

where, A_{550} and A_s are the absorbances of the supernatant and the blank without any treatment respectively at 550 nm (UV-VIS spectrophotometer, Shimadzu 1601, Japan). The most prominent isolates that exhibited maximum bioflocculating activity were allowed to grow in the production medium (mentioned above) amended with crude oil (1 %) in order to screen their ability to produce bioflocculant utilizing the petroleum hydrocarbons. Incubation conditions were maintained similar to those in the aforementioned experiment. The bacterial isolate *Pseudomonas aeruginosa* IASST201 identified by 16s r RNA sequencing, exhibited the highest bioflocculating activity and was therefore selected for further experimentation. Studies on growth pattern along with flocculating activity assays were performed up to 240 h after inoculation. The pattern of flocculation of kaolin clay was determined with the advancement of time after treating with bioflocculant.

2.3 Production and purification of the bacterial bioflocculant

Bioflocculant produced by the selected bacteria, *Pseudomonas aeruginosa* IASST201, was extracted from the bacteria-grown production media broth (crude oil as carbon source) when the bioflocculating activity was found highest, by pouring onto two volumes of ice-cold ethanol at 4 °C and kept for 12 h to separate the bioflocculant content. The resulting precipitate was collected by centrifugation at 6000 x *g* for 30 min. The bioflocculant sample was then lyophilized and solublized with deionized water (at concentration, 10 mg ml^{-1}) and purified with a column packed with DEAE-cellulose-52 (26 mm × 150 mm). The purified bioflocculant was eluted with deionized water and a grade of (0.1-1 M) NaCl in phosphate buffer (pH 7) with a flow rate of 0.5 ml min^{-1} [12]. The optimum dose of purified bioflocculant to obtain highest flocculating activity was determined as described earlier by taking a content of 10-150 µg. To retain the exopolysaccharide (EPS) content of the bioflocculant for LC/MS analysis, protein content was separated by chloroform: butyl alcohol (5:1) extraction mixture. The material was then dialyzed against de-ionized water overnight and vacuum-dried to obtain the purified EPS of bioflocculant [13].

2.4 Degradation of crude oil hydrocarbons

In order to determine the utilization of petroleum hydrocarbons in production of the bioflocculant by the bacteria, the residue of the optimized production media broth was extracted (V/V) with dichloromethane (DCM) when the bioflocculating activity was found highest [14]. The treated portion along with an untreated control was subjected for FT-IR analysis (NICOLET 6700 FTIR-Spectrophotometer, USA). The DCM extracted proportion of crude oil (control) and degraded oil (test) were analyzed though a triple quadruple Gas Chromatograph-Mass Spectrometer (GC/MS TQ8030, Shimadzu, Japan) with an autoinjector (AOC 20I, GC-2010, E). The GC program was optimized for detection of petroleu hydrocarbons and all analyses were carried out with the split ratio of 20:1. Helium was used as the carrier gas with a flow rate of 1.0 ml min^{-1}. Injection temperature was set at 300°C. The column ov temperature was set at 60^0C with a hold time of 5 min and was subsequently increased to 300^0C with a ramp of 10^0C min^{-1}. The

final hold was for 44 min. The ion-source temperature was set at 230^{0}C for MS using an interface temperature of 310^{0}C. Mass range (*m/z*) was selected from 45-800 for the entire analysis. The chromatograms were analyzed with GC-MS solution software (version 4). For compound identification, NIST 11 library database was used. The percentage degradation of the hydrocarbons was calculated by the formula, [(mHc – mH)/mHc] x 100, where mHc and mH were the sum of the total areas of peaks for the control sample and the test sample respectively.

2.5 SEM and EDX study of the bioflocculant

The purified bioflocculant from the DEAE column was powdered with a lyophilizer (Thermo modulo D, USA) and was subjected to Field Emission Scanning Electron Microscopy (FE-SEM). Dry bioflocculant was mounted on a stub over adhesive tape and was coated with gold-palladium powder by Sputter Coater (SC-7625, EMITECH, India) before finally attaching the stub over the microscope support. Scanning electron microscopic images were taken at 5 kV in a FE-SEM (Zeiss, ∑-Sigma, Germany) Scanning Electron Microscope. The energy dispersive X- ray analysis (EDX) measurements were done with an X-ray Detector and were analyzed with INCA 4.15 EDS software (Oxford Instruments).

2.6 Characterization of the bioflocculant

Bioflocculant composition of the column purified sample was determined with respect to polysaccharide and protein contents. The protein concentration of the bioflocculant was measured by the Bradford method while the total carbohydrate content was determined by the Anthrone method [15,16]. The DNS reaction, carbazol–sulfuric acid and Elson-Morgan assays were used to determine the contents of reducing sugars, uronic acids and amino sugars respectively [17]. Purified bioflocculant was subjected to FT-IR in ATR mode within a range of 500 to 4000 cm^{-1}. For the characterization of the bioflocculant for its monosaccharide composition, 10 mg of the purified EPS sample was hydrolyzed at 100°C (6 h) with 2N trifluoroacetic acid (TFA) in a hydrolysis tube [18]. Excessive TFA was removed by vacuum evaporation. Denatured protein debris was precipitated by the addition of 5 ml of 80% (w/v) trichloroacetic acid, followed by incubation in ice for 30 min and further centrifugation (10000 x *g* for 30 min at 4^{0}C). EPS was precipitated from the supernatant by the addition of ice-cold ethanol and the pellets were collected through further centrifugation (10000 x *g* for 30 min at 4^{0}C). Determinative tests such as Benedict's reaction, Seliwanoff's test, Pyrocatechol reaction, Ketose test and Bial's test were performed for characterizing the monomers of the EPS. The hydrolysate was precipitated and reconstituted with 1 ml of Acetonitrile/water (80:20 v/v). The EPS fraction was separated and analyzed for different structural analogues by liquid chromatography-mass spectrometry (LC/MS, 1260 Infinity LC and 6410 Triple Quadrupole MS, Agilent Technologies, USA). A 2 µL sample aliquot was injected into a ZORBAX C18 column (2.1 × 50 mm^2) using a gradient of water + 0.1% formic acid (solvent A) and acetonitrile (solvent B) at 40°C at a flow rate of 0.2 ml min^{-1} with a linear increase from 10 to 90% solvent B addition within 25 min, a modification to the method by Francois et al.[19]. ESI-MS spectra was obtained in positive ion mode and was analyzed using Agilent ChemStation Software. Full scan data were obtained by scanning from *m/z* ratio of 50–950 with a fragmentor voltage calibrated at 135.0 V.

2.7 Study of heavy metal removal efficiency by bacterial bioflocculant

Heavy metal removal efficiency of bacterial bioflocculants was determined using heavy metal solutions without kaolin clay. For this purpose, aqueous solutions containing Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+} at concentrations of 10 mg L^{-1} each were prepared. Bioflocculation experiment was done with both raw bacterial culture supernatant and the purified bioflocculant as per the methodology mentioned in section 2.1. The samples were maintained at pH 7 and the final volume was made up to 10 ml. After the bioflocculant was added, the samples were vortexed and kept in rest for 5 min. The metal concentrations in the upper layer of the solution (3 ml) were measured with an Atomic Absorption Spectrometer (Shimadzu A7000, Japan) as well.

2.8 Cytotoxicity study of the bioflocculant

For determining toxicity level of the bioflocculant, 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) dye conversion assay was carried out on mouse L929 fibroblastic cell line (obtained from NCCS, Pune) [20]. L929 cells were cultured at a density of 1×10^4 cells per well in a 100 µL volume of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum in a 96-well cell culture plate. The MTT is a colorimetric cytotoxicity and proliferation detection assay, based on the metabolic activity of viable cells in reducing tetrazolium salts (MTT) [21] .After 24 h, cultured cells were treated with a series of different doses (10, 30, 50, 70, 90, 110, 130 and 150 µg) of column purified bioflocculant in 100 µL DMEM without serum per well plate and was incubated further for 24 h. This was followed by removal of the media and treatment with MTT dye at a final concentration of (0.5mg ml^{-1}) and further incubation for 4 h. Finally, 100 µL of dimethylsulfoxide (DMSO) was added to each well to dissolve blue formazan precipitate, and absorbance was measured at 570 nm using a microplate reader (Bio-Rad Model 680; Bio-Rad). The cell viability was expressed as a percentage of the control by the following equation,

$$\text{Viability}(\%) = Nt/Nc \times 100$$

where, Nt is the absorbance of the cells treated with bioflocculant and Nc is the absorbance of the untreated cells.

2.9 Statistical analysis

All the experiments were done in triplicates and error bars in figures represents standard deviations of the data. The software, Origin (version 8.5), was used for developing graphs supporting the experiments. Petroleum hydrocarbons, derivatives in crude oil and degraded compounds of crude oil found after utilization by the selected bacteria, were detected with GC/MS analysis and were depicted by a Venn diagram with the help of Vennture software (NIA Bioinformatics Software) [22].

3 Results and discussions

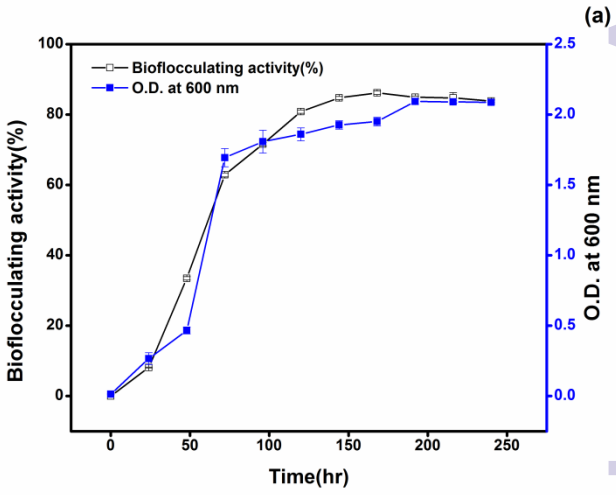
3.1 Isolation of the bioflocculant producing bacteria

Innumerable bioflocculant producing microorganisms have been isolated from oil contaminated soils and activated sludge with profound biological importance[23]. The present study was therefore an attempt to characterize bioflocculant producing bacteria from activated sludge deliberated in biological effluent treatment plants in oil refineries. The perspective behind was to generate

significant achievement that can be reverberated for large scale effluent treatment in oil refineries worldwide. The bacteria isolated from such sites usually have the potential for surviving in toxic and extreme environments. When the most prominent isolates out of the selective 37 bioflocculators were subjected to crude oil amended media without any other carbon source, a bacterium, IASST201, was found to exhibit the most efficient bioflocculating activity and was therefore chosen for further experiments. The molecular identity of the isolate was established as *Pseudomonas aeruginosa* strain IASST201 through 16s rRNA sequencing. The generated 16s rRNA gene sequences were submitted to genbank with an accession number of KF583972 [10].

3.2 Production of bioflocculant and the study flocculation pattern

As for routine analyses, the selected strain depicted 78.8% efficiency in flocculating kaolin suspension in the initial production medium amended with 1% crude oil as the sole carbon source. Crude oil concentration was found 2% as optimum to get enhanced bioflocculating activity of the selected isolate in the production media. Several workers have reported and studied the capacity of bioflocculant producing bacteria in utilizing uncommon sources as nutrient for better production of efficient bioflocculant. A bacterial species, *Rhodococcus erythropolis*, was targeted to utilize a cheap medium composed of sludge and live-stock wastewater for obtaining active bioflocculant with an yield of 1.6 g L^{-1} after 72 h with an activity of 83.6% with the raw supernatant [24]. In the present case, the bacterial growth curve obtained from the production medium indicated the emergence of stationary phase after 192 h of incubation (Fig.1a) and the yield of bioflocculant was found to be 1.48 g l^{-1} with an activity of 86.2 ± 0.51%. Study of the growth rate and the bioflocculating activity in the production medium showed a maximum activity after 168 h of inoculation with formation of clump or aggregating flocs of crude oil at around 36th h of incubation. The comparatively longer time required for showing maximum bioflocculating activity may be attributed due to the use of petroleum crude as the carbon source since *Pseudomonas* species shows maximum growth behaviour in presence of glucose as sole carbon source. Utilization of petroleum hydrocarbons by bacteria as carbon source for producing extracellular metabolites has not been reported much and the pattern of growth vs. the production has been found to vary in various cases. However, it has been known that certain species of bacteria produce exopolysaccharides during the hydrocarbon degradation process [25]. The flocculation of kaolin suspension varied from 20.0, 40.2, 58.2, 82.5 to 86.2 % after the flocculant was applied for a time period ranging from 1 to 5 min with an interval of 1 min respectively (Fig. 1b) .



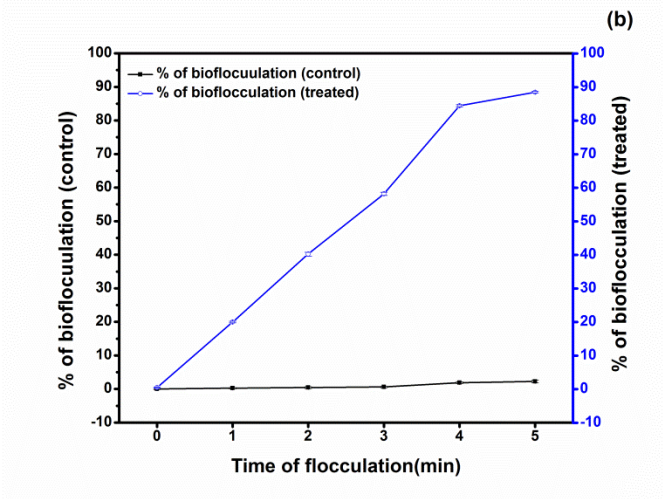


Fig. 1 (a) Bioflocculating activity obtained from optimized production in relation to growth pattern shows highest activity during 168th hr of incubation. **(b)** Figure showing course of flocculation of kaolin suspension after treatment with bioflocculant through the time of flocculation compared with a control kaolin suspension without any treatment.

3.3 Utilization of the petroleum hydrocarbons during bioflocculant production

Comparison of the FTIR spectra of the control (original crude oil sample) and the test sample (crude oil sample used as the carbon source for bioflocculant production by the bacteria) clearly indicated the persistence of degradation process. The control and the test sample showed the presence of aromatic C–H, substituted aromatic ring, aromatic C=C, C–C stretch, CH_3 and CH_2 (750 cm^{-1}, 812 cm^{-1}, 878 cm^{-1}, 1455 cm^{-1}, 1598 cm^{-1}, 2930-2850 cm^{-1} and 2960-2870 cm^{-1} respectively). However, two bands around 1602 and 1697 cm^{-1} indicating carbonyl C=O stretching in the control sample disappeared in the test sample, which, instead, had a strong absorption band at 1673 cm^{-1} for –C=C– stretching or >C=O group indicating possible presence of ketone or carboxylic acid formed as degradative intermediates. Interestingly, the triplet of 750, 812 and 878 cm^{-1} had lower intensity in the degraded sample. A small but expanded band around 3265 cm^{-1} in the test sample indicated –OH stretching bands, which might have resulted from alcohols and acids produced due to mineralization of aliphatic and aromatic components of crude oil during microbial action [26] (Fig. 2).

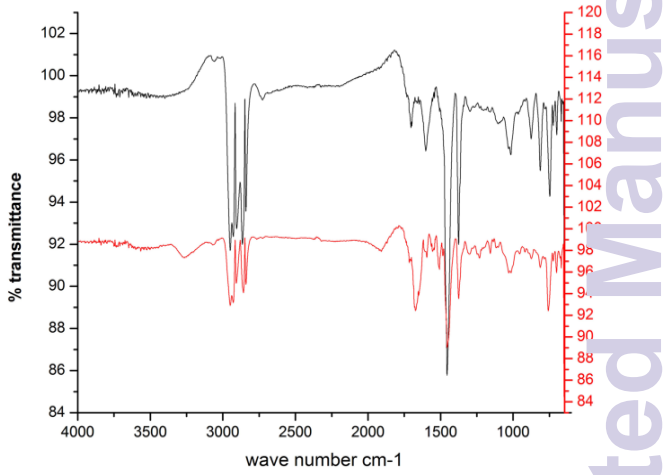


Fig. 2. FTIR spectra of DCM extracted portion of crude oil (▬) and the degraded crude oil (▬) during production of bioflocculant.

A number of bacteria have been reported to degrade alkanes and aromatics as the source of carbon and energy. At the cost of utilizing such nutrients of hydrophobic nature, bacteria often releases extracellular polymeric substance including surfactants and exopolysaccharides which help these microbes either to make the hydrophobic moiety of the substrate available to a hydrophilic one or by adhering on the hydrophobic surface and leading to formation of an emulsion [25]. Though surfactants are commonly known for its participation in availing the hydrophobic substrates for microbial utilization, but several investigations indicates the positive correlation between exo-polymer (such as bioflocculant) production and microbial degradation of hydrocarbons. Researchers hypothesized depending on their experiments on EPS producing bacteria that formation of oil aggregates is contributed by production of EPS, as well as to the fate of the oil by influencing the

dissolution, bioavailability and ultimate degradation of hydrocarbons by indigenous oil degrading communities. In particular, polycyclic aromatic hydrocarbons (PAHs) are poorly soluble and generally less amenable to biodegradation compared to their aliphatic counterparts. To circumvent limitations in hydrocarbon bioavailability, some microorganisms produce exopolysaccharides as a mechanism to increase the bioavailability of these compounds for biodegradation [27]. During utilization or degradation of petroleum based compounds by microbes, substrates are often converted into alcoholic, ester or acid derivatives. The way of bacterial action in degrading petroleum hydrocarbon and the bioflocculant production in this course which was later applied in heavy metal removal is presented here within a schematic diagram as Figure 3.

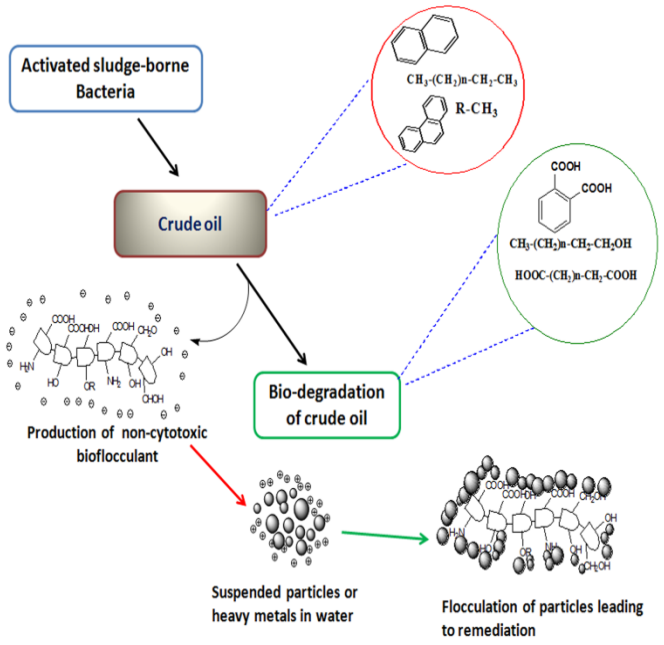


Fig. 3. Schematic presentation showing bacterial action for bioconversion of petroleum hydrocarbons through degradation along with production of active biopolymers.

In microbial actions, biodegradation of aliphatics is initiated by the oxidation of a terminal methyl group to a primary alcohol, which gets further oxidized to the corresponding aldehyde, and finally to the fatty acid. However, in some cases, both ends of the alkane molecule may be involved in the oxidation process thereby producing ω-hydroxy fatty acids, which further gets converted to dicarboxylic acid by β-oxidation[28]. Sub-terminal oxidation of n-alkanes has also been reported earlier[29]. The product generates a secondary alcohol which is converted to the corresponding ketone, and then oxidized by Baeyer–Villiger monoxygenase to an ester. The ester is hydrolyzed by an esterase, generating an alcohol and a fatty acid. Both terminal and sub-terminal oxidation can co-exist in action by some microorganisms. In the present study, the degradation of crude oil was around 77% when bioflocculating activity was at its maximum. The GC/MS analysis of the control crude oil sample generated the identity of 4 major polycyclic aromatic hydrocarbons (PAH, naphthalene, indene, phenanthrene and fluorene) along with benzene and 29 aliphatic hydrocarbons (C_{11} to C_{37}) that include pristine (C_{19}) and phytane (C_{20}). The test crude oil sample also showed the presence of two PAHs (naphthalene and fluorene), some benzene derivatives and 23 aliphatic hydrocarbons (C_{17} to C_{37}) along with pristane and phytane, and 15 prominent degradation intermediates forming different ester and acids (phthalic acid ester, hexadecanoic acid ester, etc.). The control and the test samples had 26 constituents in common out of the identified 49 constituents including degraded and no degraded intermediates. The sharing of these components are tabulated and shown in a Venn diagram with a comparative GC/MS chromatograph (Fig. 4a, 4b and 4c).

(a)

Major hydrocarbons and derivatives of control and test (degraded crude oil) with serial numbers

1. Undecane	11.Pristane	21.Hexacosane	31.Hexatriacontane	41. Phthalic acid, ester
2. Napthalene, 1,6 dimethyl	12.Octadecane	22.Heptacosane	32.Heptatriacontane	42. 9-Octadecenoic acid, methyl ester
3. Indene	13.Phytane	23.Octacosane	33.Phenanthrene	43. Heptadecanoic acid, 9-methyl ester
4.9H-Fluorene	14.Nonadecane	24. Nonacosane	34.Benzene	44. 6-Aminohexanoic acid, 2-[(5-fluorenyl) methoxycarbonylamino]-
5.Dodecane	15.Eicosane	25.Tricontane	35.5,8-Tridecandione	45. 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
6.Tridecane	16.Heneicosane	26.Hentriacontane	36.Pentadecane, 2,6,10-trimethyl-ester	46. Cyclohexane carboxylic acid, 4-propyl
7.Tetradecane	17.Docosane	27.Dotriacontane	37.1,2-Dihydroxynaphthalene	47. cis-11,14-Eicosadienoic acid
8.Pentadecane	18.Tricosane	28.Tritriacontane	38. 3,6-Undecandione	48. Heneicosanic acid, methyl ester
9.Hexadecane	19.Tetracosane	29.Tetratriacontane	39.Octacosanoic acid, methyl ester	49. Dodecane, 2,6,10-trimethyl-
10.Heptadecane	20.Pentacosane	30.Pentatriacontane	40.Hexadecanoic acid, methyl ester	

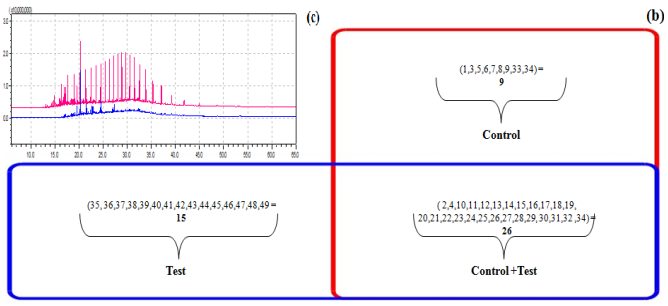


Fig. 4. (a) List of compounds detected through GC/MS analysis of control and test crude oil after microbial degradation **(b)** Venn diagram showing sharing of the components between control as box (—) and test as box (—).**(c)** GC chromatogram of the DCM extracted portion of control crude oil as line (—) and the degraded crude oil as line (—).

3.4 Chemical characterization, morphology and element composition of bioflocculant

Bioflocculants have been reported as consisting of proteins, glycoproteins, polysaccharides, lipids and glycolipids. In case of a bioflocculant produced by *P. aeruginosa* strain, chemical analysis of the biopolymer revealed it to be a sugar protein derivative, composed of protein and carbohydrate including neutral sugar, uronic acid and amino sugar as the principal constituents [30]. The biochemical tests conducted on the purified bioflocculant sample obtained from our selected bacterium confirmed the presence of around 85.1 % total carbohydrate but a low amount of protein content of 8.2 % indicating the glycoprotien nature of the bioflocculant. The carbohydrate part consisted of neutral sugars, amino sugar and uronic acid in the ratio 80.5, 4.4 and 0.2 % respectively. Benedict's and DNS tests confirmed the presence of reducing sugars in the bioflocculant while Seliwanoff's reaction showed the absence of fructose. The presence of xylose and glucose were also recorded from ketose test, Orcinol test and Pyrocatechol reaction.

The SEM image (Fig. 5a) of the bioflocculant indicated a crystalline and irregular shaped morphology of the purified bioflocculant. The SEM-EDX analysis of the dried purified bioflocculant sample showed the presence of carbon, nitrogen, oxygen, sodium, phosphorus and chlorine in the proportions of 25.30, 13.91, 48.85, 10.22, 0.33 and 1.39 % in the scanned area (Fig. 5b and 5c). The abundance of carbon, nitrogen oxygen further proved the elemental characteristics of the bioflocculant as a carbohydrate-protein complex moiety.

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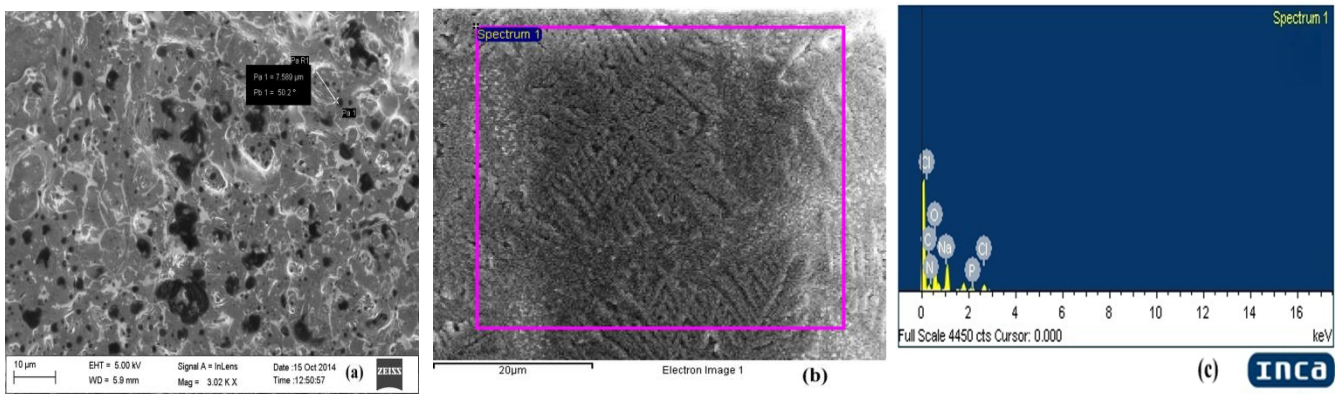

Fig. 5. (a) SEM image of the purified bioflocculant showing crystalline and irregular structure of it. **(b)** Bioflocculant image using EDX and **(c)** EDX spectra of the purified bioflocculant indicating presence of C, N, O, Na, P and Cl in the scanned area.

3.5 FTIR and LC/MS analysis of bioflocculant

The FTIR spectrum in the ATR mode of the purified bioflocculant has a broad absorption band at 3272.77 cm^{-1}, assigned to –OH or –NH vibrations. An asymmetrical band at 1633.65 cm^{-1} could be attributed to –NH or C=O stretching vibrations from $–NHCOCH_3$ groups and a weak band at 1403.51 cm^{-1}, to symmetrical and asymmetrical stretching of C=O groups of the carbohydrate in the bioflocculant. A small band around 2918.15 cm^{-1} points to C–H stretching vibrations of $–CH_2$ groups while strong bands at 1052.87 and 1064.86 cm^{-1} could be attributed to asymmetrical stretching of C–O–C ester linkage. The presence of β-glycosidic linkage between the sugar monomers is indicated by a small absorption band at 861.82 cm^{-1} (Fig. 6). The functional groups in the polymer molecule are important determinants for the flocculating activity. The -OH, -COOH, COO^- groups in the bioflocculant and H^+, OH^- groups on the surface of the suspended particles may form hydrogen bonds when the bioflocculant chains approach the surface of particles[31].

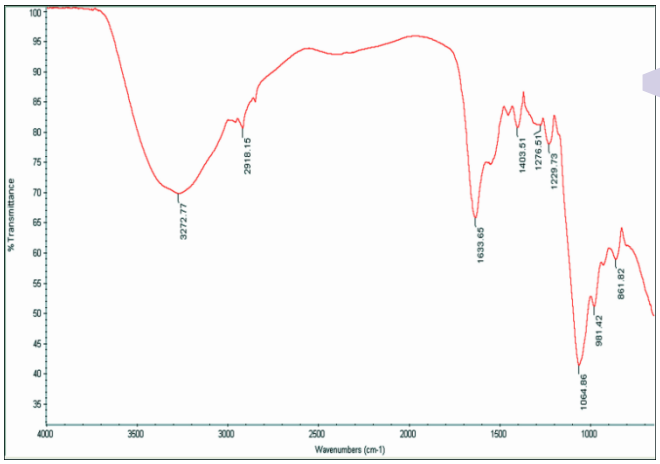

Fig. 6. FTIR-ATR spectra indicating bands for –OH, –NH and C–O–C in the bioflocculant content.

The anionic nature of the bioflocculant is established by detection of carboxyl, hydroxyl and amine groups in the FTIR spectra. These groups are likely to serve as the binding sites for divalent cations and suspended particles in a solution [32]. Ther

functional groups in the bioflocculant have their origin in the sugars and the proteins and could be the sites for the bioflocculant activity.

The LC/MS study of the purified bioflocculant had given the proper idea about the monomer constituents of exopolysaccharide composition. In the LC/MS data based investigation the characterization of exopolysaccharides was focused, as bioflocculants were investigated as none but a composition of extracellular or surface polysaccharides in a combination with outer-membrane proteins or lipid moieties. The exopolysaccharides play a principal role in the formation of cell aggregates, initiation of flocculation and similar processes. This property is vital for wastewater treatment and particle aggregation [33]. Hino et al. [7] first investigated the EPS-producing bacteria that could utilize petroleum hydrocarbons as the sole carbon source for cell growth. They noted that exopolysaccharides play central roles in the formation of biofilms, and coined a term "slime" where it was signified that the "slime-producing bacteria" could be an utilizer of petroleum hydrocarbons. These biofilms are also known as a mass of extracellular components often bearing cell-adhesion or attaching property against suspended particles.

Precisely, the constituents of bacterial exopolysaccharides often shares in the compositions of bioflocculants. Bacterial EPS were reported as a broad range of non-volatile sugar compositions such as glucose, rhamnose, galactose, mannose, xylose, n-acetyl galactosamine, n-acetyl fucosamine, n-acetyl glucosamine, mannuronic acid etc [34]. The LC/MS analysis for the detection of the sugar monomer composition of the bacterial bioflocculant revealed the existence of glucose (Glu), xylose (Xyl) and n-acetyl hexosamine (HexNac) in the EPS portion through ESI mass spectrum[35].

LC/MS-ESI is a suitable interface for MS of polar and thermally labile compounds which includes the detection of non-volatile sugars, proteins etc. In the present case, Na^+ and H^+ ion adducts have been identified for the sugar monomers of the sample depending on fragmentation and elution time. Sodium adduct ions are formed for glucose as $(Glu+Na)^+$(*m/z* 203.1) and $(Glu+Glu+Na)^+$(*m/z* 383.1). The *m/z* of 244.2 represents n-acetyl hexosamine forming sodium adduct $(HexNac + Na)^+$. The presence of xylose could be predicted from the formation of both proton and sodium adducts with *m/z* of 151.4 $(Xyl+H)^+$ and 325.0 (Xyl+Xyl+Na) respectively (Fig. 7a and 7b).

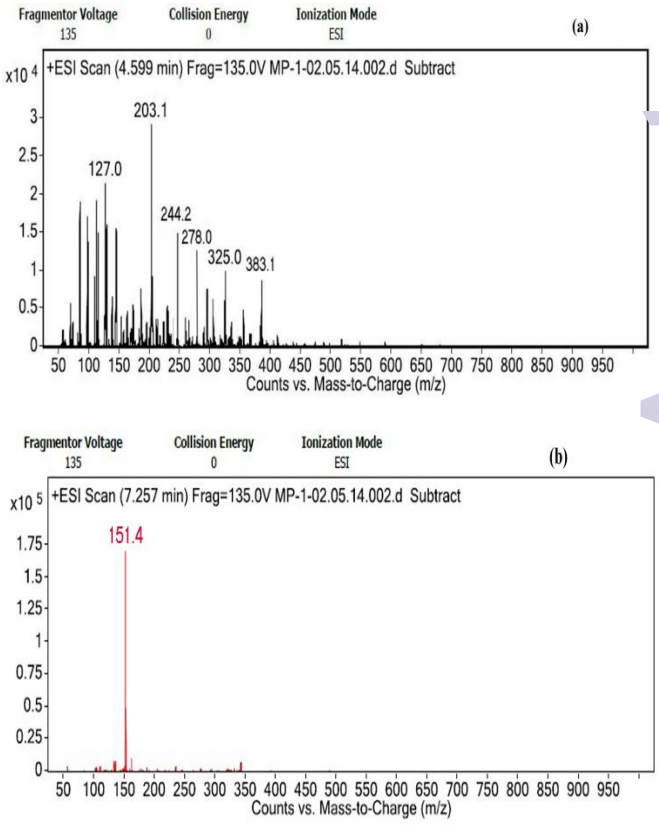

Fig. 7. Positive ion mode ESI-MS spectra recorded from LC/MS analysis of the hydrolyzed bioflocculant sample showing **(a)**N adduct ions of *m/z* 203.1$(Glu+Na)^+$,383.1$(Glu+Glu+Na)^+$, 244.2$(HexNac+Na)^+$ and 325.0$(Xyl+Xyl+Na)^+$ and **(b)** H^+ adduct io of *m/z* 151.4 $(Xyl+H)^+$ respectively.

These results along with the functional groups deduced from FTIR analysis played a pivotal role in the prediction the bioflocculant as a polymer consisting of glucose, xylose and n-acetyl hexosamine with a small protein content.

3.6 Dose optimization of purified bioflocculant and cytotoxicity test

The purified bioflocculant was found valorised when optimized in dose, where it had shown a constant flocculating activity of 89.1 ± 0.08 % when 90 to 110 µg of it was employed in kaolin precipitation assay. Bacterial metabolites often cause harm to host leading to epidemic diseases along with neurotoxic and cytotoxic effects. However, in the present case, the bioflocculant was not seen to exhibit any such harmful activities. There are reports of the non-toxic effect of bioflocculant obtained from a *Klebsiella* species in mammals in terms of external clinical symptoms [36, 37]. Interestingly, *Pseudomonas aeruginosa* species are known as notorious producers of toxic substances like exotoxins, but utilization of such extracellularly produced flocculants need not necessarily establish them as hazardous biological materials. The application of the bioflocculant obtained from the selected strain in this work showed no toxic effect on mouse fibroblast L292 cell line and produced a bench-mark as a non-cytotoxic glycoprotein-like polymer which could be used as a probable biological material, including biological interfaces. The cells of this mouse fibroblastic cell-line were found viable through the MTT dye conversion assay ranging from 95.1 to 84.2% when 10 to 150 µg of the purified bioflocculant were subjected to the micro-titer plate wells (Fig. 8). This proves the probable utility of this bioflocculant encompassing safety standards on animals and humans and therefore may be considered as a 'novel compound'.

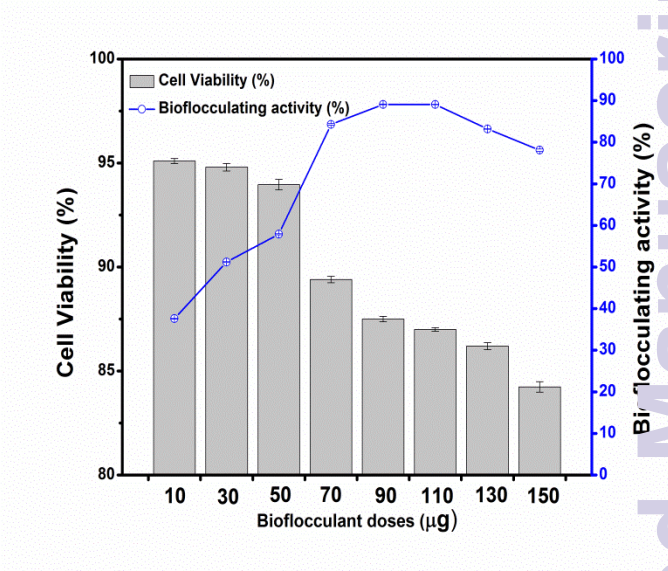

Fig. 8. Cytotoxicity of different doses of bioflocculant upon treating L292 cell line in terms of percentage of cell viability along with the differences in flocculating activity with the advancement of bioflocculant doses.

3.7 Application of bioflocculant for heavy metal removal

Bioflocculants, like extracellular polysaccharides often play an important role in controlling heavy metal pollution in the sewage treatment process and they are recommended as surface-active agents for the removal of heavy metals. Bacterial species from genus *Pseudomonas*, *Bacillus*, *Herbaspirillium*, *Paenibacillus*, etc. are always being mentioned as good bioflocculant producers and their application in heavy metal removal has been studied. Due to extensive capacity of bioflocculants for metals, they are recommended as surface-active agents for the removal of heavy metals. Appropriate physical and chemical properties, availability of appropriate binding sites and the tertiary structure of the bioflocculant may all contribute to metal-binding interactions. Th

efficiency of bioflocculation depends on the conformation of the polymer with adsorbed ions [38]. In this investigation, biochemical tests, FT-IR and LC/MS analyses have established the structure of the bioflocculant as consisting of sugar-protein, with –OH, and –C–O–C linkages which could be the possible sites for metal binding and chelation through chemical interactions or siderophore expression. These groups, together with the amino group, could serve as a binding site for metal ions which enhance flocculating activity of the bioflocculant by bridging between it and the suspended particles in solution, and are likely to be the preferred groups for the process of adsorption[39].Explaining the flocculation mechanism, Wu and Ye suggest that the cations (from $CaCl_2$) are effectors in the action of bioflocculant polymer by neutralizing and stabilizing the residual negative charge of functional groups by forming bridges between particles[3]. The DCB (Divalent Cation Bridge) theory, the most convenient one in explaining the mechanism of flocculation, states that divalent cations bridge negatively charged functional groups within the EPS and this bridging helps to aggregate and stabilize the matrix of biopolymer and microbes and therefore promote bioflocculation [40]. In general, bioflocculants have been found to have a net negative charge and the cation source ($CaCl_2$ in the present case), by giving a positive charge to the heavy metals opens the provision for the bioflocculant polymer to undergo ion interactions leading to formation of the floc. The presence of acid moiety of polysaccharide which was found through biochemical characterization of the present bioflocculant could also assist in metal uptake. Carboxylic groups present in exopolysaccharides work as a non-specific ion exchange material, which may convey chelating property. Protein constituents of a glycoprotein-like flocculant containing multiple carboxyl groups are also important in the process of flocculation with heavy metals. The efficiency to reduce the surface charge density by adsorption of the bioflocculant and the particles such as the heavy metals becomes crucial[30].

In the present study, when the raw culture supernatant was applied as bioflocculant, it was found to be removing the heavy metals Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+} by 72.22 ± 0.22, 55.42 ± 0.31, , 50.10± 0.13, 36.19 ± 0.08, 33.68 ± 0.16 % respectively. Compared to this, the purified bioflocculant (with optimum dose) was found capable of removing Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+} by 76.14 ± 0.11 , 62.69 ± 0.48, 53.22 ± 0.04, 47.64 ± 0.47 and 40.58 ± 0.28 % respectively. This signifies the efficiency of this bacterial bioflocculant in removing the chosen heavy metals in a pattern of Ni^{2+}> Zn^{2+}> Cd^{2+}> Cu^{2+}> Pb^{2+} in both the cases of supernatant and the purified component (Fig. 9).

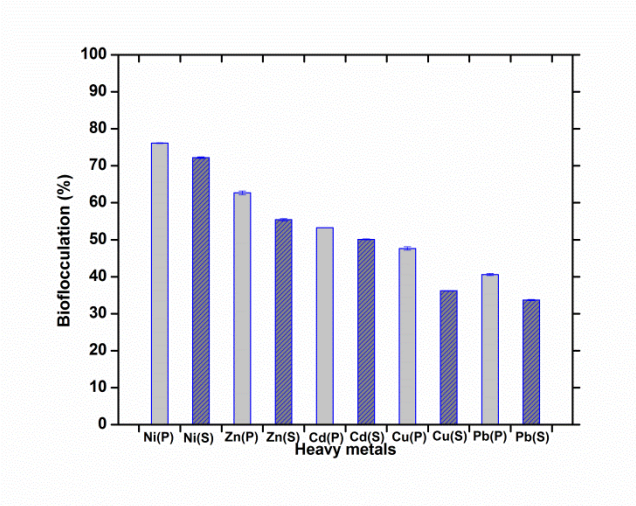

Fig. 9. Bioflocculation of heavy metals by treatment of purified bioflocculant (P) and the raw culture supernatant (S). The error bars represent the standard deviation of the data.

The pH of the samples remained unaffected by the addition of the polymeric bioflocculant in its optimum dose and the highe activity of the bioflocculant was found around the pH 7 of the sample solution. Heavy metal removal capacity of a bioflocculant in

an aqueous system is reported to be lower at low and high pH values of the system; at low pH, a high concentration of protons competes for the anionic sites on the polymer preventing the removal of the divalent cations. Thus, divalent cation binding is low. As the pH increases to its optimum value, which differ from one metal ion to another, the adsorbing surface is saturated with negative charges, resulted in increased efficiency to bind and adsorb metal cations. At pH higher than the optimum value, hydroxo species of the metals can be formed that do not bind to the adsorption sites on the surface of the adsorbent [41]. The stability of pH obtained in the present study could be advantageous in removing other heavy metals and pollutants too from various waste water streams by bioflocculants in required doses.

Conclusions

The present work has identified a bacterium and further enumerated an efficient bioflocculant production system which has been optimized to an extent whereby the microorganism that thrives on petroleum crude oil and known for its hazardous effects towards the environment could be utilized for enormous benefit in the study of producing effective microbial biopolymers in the course of utilizing such a source of energy. The bioflocculant obtained from these experiments has an excellent flocculating capacity of 86.2 ± 0.51% which could significantly remove 5 different heavy metals from aqueous solutions by a pattern of Ni^{2+}> Zn^{2+}> Cd^{2+}> Cu^{2+}> Pb^{2+}, Ni^{2+} at highest by 76.14%. Experiments in support of characterizing this biopolymer, concludes the nature of it as a glycoprotein-like substance including glucose, xylose and n-acetyl hexosamine as the constituents of the exopolysaccharide part of the bioflocculant that produced during utilization of hydrocarbons of crude petroleum oil by the selected bacterium. The bioflocculant was found to be sufficiently non-cytotoxic to the mammalian cell line even after application of it in the optimum dose necessary for showing the highest flocculating activity. This study is likely to lead to a system of symbiosis reflecting the microbial degradation- flocculation phenomena as a mean to treat industrial effluents and the trial for production and characterization of more effective microbial bioflocculants may help other researchers to grab a thread leading to the idea in employing microbial degradation and flocculation mechanism to treat various natural resources contaminated with industrial and anthropogenic activities.

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