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Isolation, Screening and molecular characterization of bio surfactant producing microbes from hydrocarbon polluted soil

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***Abstract*---**Biosurfactants are ampliphlic compounds which can reduce the surface and interfacial tension between the compounds and have multiuses in various industries and agriculture. The isolated lipopeptides and lipoprotein producing microbes are useful in removal of heavy metals from industrial effluents using bioremediation, pesticides and hydrocarbon contaminated sites. Lipopeptides and lipoproteins used as bio-control agent to protect plant against different diseases, leads in increases crop yield. The objective was to isolate potential bio surfactant producing microbes from hydrocarbons contaminated soil. Initially 21 microbes were isolated amidst them ten bio surfactant producers were screened based on the surface tension, emulsification index, drop collapse method and oil displacement method. The isolate KLEF21 was the most successful bio surfactant producer as it showed the minimum surface tension (32.12 dyne/cm) with an emulsification index of 68.97% and oil displacement activity 3.5 mm. The microbe was identified based on the partial sequence of

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16S rRNA and it belongs to *Alcaligenes* and was indicated as *Alcaligenes sp.*KLEF21.The stain was also competent of employ polycyclic aromatic hydrocarbon composed of three fused benzene rings as the highest microbes biomass was produced by this strain in polycyclic aromatic hydrocarbon alter MSM media compared to other isolates. This indicated that identified stain *Alcaligenes sp.*KLEF21 can be have capacity to degrade the hydrocarbons contaminated soil or water that could be useful in conservation of natural resources.

Keywords*---Alcaligenes sp.,*** Bio surfactant, surface tension, emulsification index, drop collapse method, and oil displacement method.

Introduction

Biosurfactants are surface active compounds which can produce by various microorganisms[1]. Surfactants are very enchanting surface tension and emulsification index[2]. They are classified either on the basis of molecular weight or their origin[3]. They comprised lipopeptides, glycolipids, phospholipids, lipopolysacchrides, neutral lipids and polysaccharides-protein complex[4]. Biosurfactants produced by yeast, fungi and bacteria but the most of surfactants produced from bacteria. *Alcaligenes sp.*produced by utilizing water immiscible and water soluble substrates. From the literature most of the bio surfactants belongs to the *Pseudomonas, Bacillus, Corynebacterium* sp*, Acinetobacter* sp., *Archromobacter sp, Falvobacterium sp.* and Proteobacteria [5].

Now a day's biosurfactants are more useful in many fields of industries such as food, pharmacy, design of washing agents, house hold purposes, petroleum industry and environmental protection and agriculture[6]. *Alcaligenes sp.*produced by microbes is useful in bioremediation of heavy metals from industrial effluents, pesticides and hydrocarbon contaminated soils and water. They are also used as biocontrol agent to protect plant against various diseases indicates in higher yield in crops and harvesting [7]. *Alcaligenes sp.* functional groups they have much more potential to be used in agriculture i.e. in soil management and plant protection strategies to ensure better place for sustainable food production, bioremediation and cosmetics. Given their properties and applications, studies on the properties of surfactants the search for *Alcaligenes sp.* producing bacteria is yet an interesting area of research because of the diversity of these fascinating molecules and their application in broad spectrum application in agriculture.

The present research was accompanied to isolate biosurfactant producing microbes from hydrocarbon contaminated soil. It was estimated that the hydrocarbon contaminated soil may supply accommodation for biosurfactant producing microbes, which not only lower the surface tension and demonstrated useful in bioremediation of hydrocarbons contaminated soils. The objectives of the present research were the (i) Isolation of biosurfactant producing microbes based on their capability of reducing in surface tension and higher emulsification index. (ii) Estimation of growth of selected isolate on carcinogenic reform media as possible indicator for bioremediation of hydrocarbon contaminated soils.

Materials and Methods

Collection of soil samples

The petroleum by products polluted soil samples were collected near sixth battalion petrol bunk in Vaddeswaram (32° 12′ 24.31″ N, 72° 56′ 37.9″ E), Tadepalli (mandal), Guntur (district), Andhrapradesh using procedure described by Bodour and Miller-Maier (2000)[8]. A total of fifteen humus soil samples were collected. Based on the sampling location (Petrol Bunk, Vaddeswaram), these samples were indicated as KLEF. Every illustration was put into 250 mL sterile conical flasks. The conical flasks were covered with sterile cotton so as to stop any contamination. The samples were transported to the laboratory in an ice cubes for isolation of microorganisms having capability of producing bio surfactants.

Isolation of Biosurfactant producing microorganisms

3ml of cooking oil as carbon source, 5gm of contaminated soil sample was inoculated in the production media containing (g/L): $MgSO_4$, 1 g; $NaNO_3$, 1.5 g; $CaCl_2$, 0.002 g; $(NH_4)_2SO_4$, 1.5 g; $FeSO_4$, 1.5 g; and KH_2PO_4, 1 g; dissolved in 1liter of distilled water. The initial pH of the medium was adjusted to seven. All fermentations were carried out at 30°C-35°C in shaker flask held on rotary platform shaker at 120 rpm. The composition of 100 mL of MSM medium was prepared in 250 mL conical flask given in Table.2.

Table 1: Composition of Mineral Salt media (MSM)

Components	Weight (in gm) / liter of water
KH_2PO_4	1
$MgSO_4$	0.5
$FeSO_4$	0.01
$NaNO_3$	1.5
$CaCl_2$	0.002
$(NH_4)_2SO_4$	1.5

1 ml of inoculum was obtained under aseptic condition and was serially diluted in distilled water up to 5 concentrations. Using a sterile loop these samples were streaked on nutrient agar plates. These plates were incubated at 37°C for 24 hours. After then 1ml of incubated culture was streaked on the Petri plates. The samples then were serially diluted up to 10^{-6} dilution. 1 ml of 10^{-6} time dilution was transferred to nutrient agar for spread culture. The plate was inverted and incubated at 30°C, for 72 hours. After incubation, morphologically ten distinct colonies were selected for further studies.

Screening of Bio surfactants and Bioemulsifierproducing Strain
Qualitative estimation of Bio surfactants

Without cell filtrate arranged by centrifugation is indispensable for biosurfactant purging and recovery. Hence, centrifugation of culture media was done at 12000 g for 30 min and cell free supernatant was prepared. For fundamental screening of

biosurfactant conveying tiny creatures, abstract tests were performed using drop breakdown test as portrayed by[9]. This test was done in the polystyrene top of a 96-microwell (12.7 cm×8.5 cm) plate (Biolog Inc., Harward, CA, USA). Before performing test, each cover was washed with bubbling water, ethanol, and refined water on numerous occasions and subsequently dried. By then, every small scale all around was covered with slight 2 µL layer of oil. The wells were covered with 96-well micro plate cover. These mechanical gatherings were left for 24 h to ensure a uniform oil covering. Five µL of aliquot with seven-day improved social orders were moved to the prepared oil-all around covered regions using a micro syringe. Each time the needle was washed on different occasions with water and thereafter with $(CH_3)_2CO$ before the development of every model. The drop size was seen 1 min later with the guide of enhancing glass. The result was seen as sure for biosurfactant creation when the drop distance across was in any occasion 1 mm greater than that conveyed with deionised water[10]. Each test was acted in five replications.

Quantitative measurement of biosurfactant production

Surface Tension Reduction: Decline in surface pressing factor was used as one system. For this, the cell free supernatant after centrifugation at 8000 rpm was used further for assessing surface pressing factor using The Fisher Surface Tensiometer, Model 20 (Fisher Intelligent USA). Control was set where no inoculation was done. All assessments were done at room tempreture after carefully dunking the platinum ring in the lifestyle plan for few second to accomplish balance. From the outset, for change of instrument, the surface pressing factor of unadulterated water was done. The strategy was reiterated triple and surface pressing factor of the model was conveyed as ordinary of three replications. The going with formula was used to choose the abatement in surface pressing factor (dyne/cm):

Surface tension reduction
= Recorded surface tension in the media (before inoculation)–
Recorded surface tension after inoculation (culture supernatant).

Emulsification index: The emulsification record (E_{24}) gives a speedy and reliable proportion of the measure of biosurfactant. The E_{24} was settled as delineated by[10-12]. Two millilitres (2 mL) of lamp oil were added to a similar measure of sans cell stock. The blend was vortexed at a rapid for 2 min. After 24 h, the stature of the steady emulsion layer was estimated. E_{24} record, characterized as the level of the tallness of emulsified layer partitioned by the all out stature of the fluid section[13], was resolved. Right now, dodecyl sulphate (SDS) and water were utilized as positive and negative controls, respectively:

$$E_{24} = \frac{\text{Height of the emulsification layer}}{\text{Total Height}}$$

Oil-spreading Technique**:** This is phenomenal contrasted with different strategies used as a piece of recognizing the closeness of biosurfactant (BS) creators[14-15].Twenty microliters (20 µl) of grungy oil was added to 40 mL of refined water (DW) in a Petri plate. To oil-secured water surface, 10 µl culture supernatant was

incorporated. An area enveloped by an emulsified brilliance was seen as positive for BS creation[16-17]. The width of the cleared zone on oil surface was imagined and evaluated after 30sec.Uncovered this viewed emulsified crown compares with surfactant activity and is known as expulsion activity [18].

Hydrocarbon degradation of biosurfactant using Biomass determination

To choose hydrocarbon spoiling capacity, separates were become on MSM modified with phenanthrene (1%). Biomass appraisal was done by taking model from the lifestyle stock after 7 days. The model was centrifuged at 12,000 rpm for 30 min at 4^{o}C. The cell pellets were molded which were then washed with 1% (w/v) NaCl course of action. The dry weight was evaluated (g/L) by drying the cell pellets in a hot air grill at 60-70^{o}C. The drying was continued until a predictable dry cell weight was cultivated.

Identification of biosurfactant microbes through 16S rRNA

The character of the chose detach was affirmed dependent on 16S rRNA quality succession investigation. Genomic DNA was secluded from the bacterial example utilizing Chromous bacterial genomic DNA segregation pack. The general groundworks of 16S rDNA sections, 27F and 1492R, were utilized to intensify the 16S rDNA. The arrangements of preliminaries were as per the following: (27F) AGAGTTTGATCMTGGCTCAG and (1492R) TACGGYTACCTTGTTACGACTT (Bioserve Biotechnology, Hyderabad). A phylogenetic tree was developed utilizing halfway 16S rRNA quality arrangements of the separate and different successions, firmly related with the reference strain, acquired from NCBI database. Clustal Omega was utilized for different succession arrangement of groupings. Neighbor joining tree was developed with complete cancellation utilizing bootstrapping at 10,000 bootstraps preliminaries with Kimura-2 parameter utilizing MEGA 6.0 programming[19]. The confine GSR21 was at long last recognized as *Alcaligenes sp.* The arrangement of the 16S rRNA quality of the strain GSR21 is accessible in NCBI under the GenBank increase number JQ746488.

Statistical Analysis

The results were compared by the one-way analysis of variance (one-way ANOVA) and multiple range tests to find the differences between the measurement means at 5 % (0.05) significance level using Mat lab R2013a (version 8.1.0.604).

Results and Discussions

Isolation and Screening of Biosurfactant Producing microbes

Out of 21 disconnects screened, just *Alcaligenes sp.*KLEF21 showed a positive outcome in the drop-crumple test. The other six disengages (*Achromobacter denitrificans* KLEF01, *Achromobacter pulmonis* KLEF02, *Achromobacter sp.* FBHYA2 KLEF03, *Bordetella petrii* KLEF04, *Alcalisenes sp.* BZC5 KLEF05 and *Achromobacter xylos* KLEF06) indicated positive outcome in the microplate investigation while eight disconnects (*Achromobacter denitrificans* KLEF07, *Achromobacter pulmonis* KLEF08, *Achromobacter sp.* FBHYA2 KLEF09, *Bordetella*

petrii KLEF10, *Alcaligenes sp.* BZC5 KLEF11, *Achromobacter xylosoxidans* KLEF12, *Achromobacter sp.* A3 KLEF13 and *Achromobacter insuavis* KLEF14) were recognized positive by the oil-spreading procedure. The other seven disengages (Achromobacter sp. FBHYA2 KLEF15, Achromobacter anxifer KLEF16, Bordetella petrii KLEF17, Alcaligenes sp. BZC5 KLEF18, Achromobacter xylos KLEF19, Achromobacter denitrificans KLEF20, Achromobacter insuavis KLEF22) indicated positive outcomes in the frothing action while seven bacterial disconnects (*Achromobacter denitrificans* KLEF23, *Achromobacter pulmonis* KLEF24, *Achromobacter sp.* FBHYA2 KLEF25, *Alcaligenes sp.* BZC5 KLEF26, *Betaproteobacteria* bacterium KLEF27, *A. xylosoxidans quality* KLEF28 and *Achromobacter xylos* KLEF29) were distinguished positive by the tilting glass procedure as appeared in (Table 2).

TABLE 2: Screening of potential biosurfactant production using Surface tension (dyne/cm), Emulsification index (E_{24}%) and Oil spreading test

Bacterial isolates	Surface Tension(Dyne/cm)	Emulsification index (E_{24} %)	Oil spreading test (mm)
Achromobacter denitrificans KLEF01	61±0.32	13.1±2.0	**+**
Achromobacter pulmonis KLEF02	67.6±0.05	43.5±1.0	+
Achromobacter sp. FBHYA2 KLEF03	60.25± 0.06	28.1±2.0	+
Achromobacter sp. XRF-1 KLEF04	55.1 ± 1.48	29.6±0.8	+
Achromobacter ruhlandii KLEF05	56.4 ± 1.09	39.8±0.9	-
Alcaligenes sp. KLEF21	**32.12 ± 0.25**	**68.97±0.5**	**++++**
Achromobacter anxifer KLEF06	62.7 ± 0.74	35.7±2.2	-
Bordetella petrii KLEF07	61.4 ± 3.80	31.9±2.8	-
Bacterium strain TLSY-1 KLEF08	65.0 ± 1.99	30.6±3.1	+
Alcaligenes sp. BZC5 KLEF09	60.8 ± 0.71	32.3±2.6	-
Betaproteobacteria bacterium KLEF10	63.1 ± 7.08	0	+
Achromobacter xylosoxidans gene KLEF11	59.5 ± 0.09	0	-
Achromobacter xylos KLEF12	47.8±0.09	28.7±00	-
Achromobacter sp. Ir-12.2 gene KLEF13	68.7 ± 1.84	0	**+**
Achromobacter sp. Ir-1 gene KLEF14	68.3 ± 0.85	0	-
Achromobacter sp. A3 KLEF15	69.6 ± 4.24	37.8±1.5	-
Achromobacter sp. BV11 KLEF16	69.2 ± 0.64	0	+
Achromobacter denitrificans KLEF17	69.8 ± 0.21	32.9±2.8	-
Achromobacter insuavis KLEF18	65.9 ± 1.06	0	-
Achromobacter sp. CanL-53 KLEF19	57.6 ± 0.64	33.7±3.2	+
Alcaligenes sp. TG19 KLEF20	58.8±0.05	40.9±2.9	-
Distilled water	79.1 ± 0.14	0	-
MSM + 1% (v/v) Vegetable oil	59.6 ± 0.07	0	-
1% (w/v) SDS	41.0 ± 0.00	53.95±2.4	-

These outcomes proposed that the oil-spreading procedure is more delicate than alternate techniques for biosurfactant location in the supernatant from a culture medium. As indicated by[20-21], the drop-crumple strategy isn't as delicate as the oil-spreading method in distinguishing low levels of biosurfactant generation. So also, microplate investigation was not able recognize the nearness of surfactant at low levels.

Quantitative examination including emulsification file (%EI_{24}) and surface strain estimation was observed to be a more dependable strategy for measurement of the dissolvable biosurfactant in the medium. A segregate was chosen as a biosurfactant-maker on the off chance that it lessened the surface pressure beneath 40 dynes/cm[21] as well as kept up no less than half of the first emulsion volume 24 h after arrangement of emulsification[21]. ***Alcaligenes sp.* KLEF21** showed positive outcomes in every single subjective test and in the quantitative assessment created a higher lessening in surface pressure (32.12±0.25 dynes/cm) and a higher level of emulsification at 24 h (68.97±0.5) than did the positive control SDS with 41.0 dynes/cm and a %EI_{24} of 54%.

Segregates *Achromobacter pulmonis* GSMSR2B, *Bordetella petrii* GSMSR8B and *Alcaligenes sp.* BZC5 GSMSR10B despite the fact that did not create biosurfactant, are bioemulsifier maker on the grounds that their emulsification list was the most noteworthy among others. This investigation demonstrated that quantitative examinations were more dependable for discovery of the nearness of biosurfactant in the medium by bacterial separates.

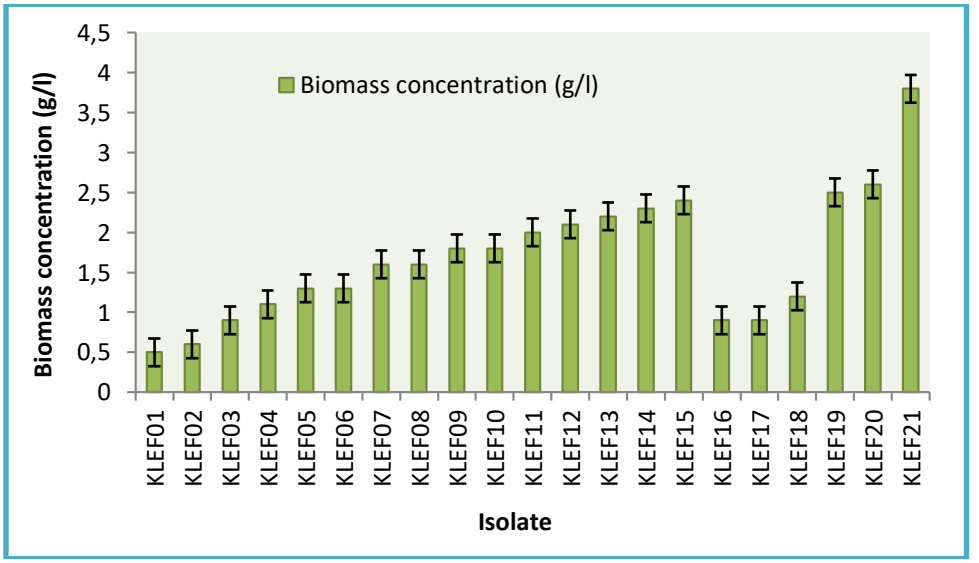

Figure 1: Biomass estimation of bacterial isolate cultivated in vegetable oil amended mineral salt medium

This strategy was performed to test capacity of bacterial disengages to develop on PHE revised MSM. Biomass fixation in the scope of 0.5-3.8 g/L was noticed for the chose disengages. Most extreme biomass (3.8 g/L) was seen on account of bacterial confine KLEF21 following 7 days hatching at 35°C (Fig.1), while least (0.5 g/L) was noticed for bacterial detach KLEF01.

The phylogenetic investigation in light of the 16S rRNA quality of the grouping created from the seclude ordered the separate as *Alcaligenes* strain KLEF21 (Fig.2) the succession has been stored under the increase number, JQ746488.1. The separate *Alcaligenes sp.* KLEF21 utilized as a part of this investigation indicated comparability with the accompanying Gene bank segregates: *Alcaligenes*

sp. KLEF21 JQ746488, 98 %; *Anchromobacter anxif* KX400775, 97 %; *Bacterium* strain KX881913, 97 %; *Betaproteobacteria* KT903074, 97% *Bordetella petris* KX016589, 98%. The creation of biosurfactant by *Alcaligenes sp.* KLEF21 has been accounted for despite the fact that not generally. This work has, along these lines, additionally approved the generation of biosurfactant from *Alcaligenes sp.* KLEF21. The wellbeing part of the segregate utilized as a part of this examination was mulled over.

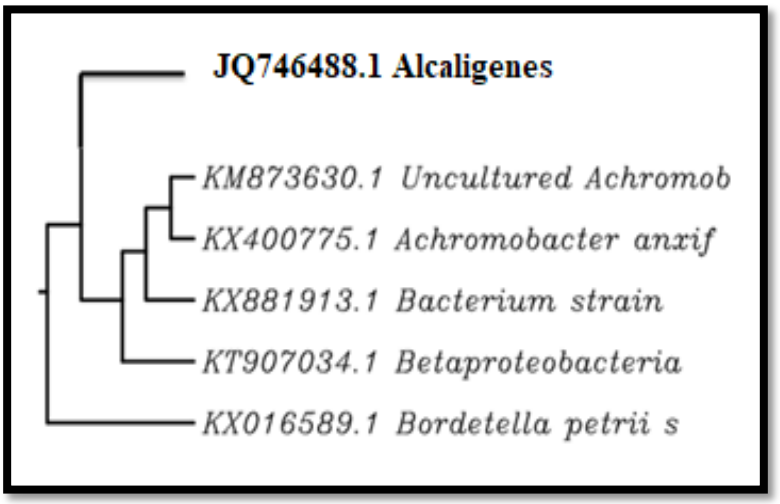

Figure.2: Neighbour-joining phylogenetic tree of isolate KLEF21 made by MEGA 6.0. Bootstrap values of >50% (based on 100 replicates) are given in the nodes of the tree. Nucleotide substitution mode used jukes and cantor

In the current examination, a few bacterial separates were gotten from hydrocarbons dirtied soil and slop tests defiled with raw petroleum. These bacterial secludes had a variable potential to deliver biosurfactant and corrupt phenanthrene. One of the bacterial disengage KLEF21 showed high potential to deliver biosurfactant just as to debase PHE. These outcomes suggested that unrefined petroleum polluted destinations contain microbes fit for delivering biosurfactant that could successfully bring down the surface strain and improve emulsification file. In this manner, biosurfactants work with biodegradation measure. That is the reason in this examination biodegradation of PHE was considerably more noteworthy within the sight of biosurfactant than that saw without biosurfactant. Besides, cell biomass was likewise more noteworthy on account of bacterial disengage that had higher potential for creating biosurfactant. This bacterial separate (KLEF21) was recognized as *Alcaligenes sp.* beforehand, none of the examinations announced the capability of *Alcaligenes sp.* to create biosurfactant and debase PHE; be that as it may, *Pseudomonas, Klebsiella* and *Bacillus spp.*, which showed shut family members with *Alcaligenes sp.* are equipped for delivering biosurfactants that have been accounted for by analysts. The closeness of *Alcaligenes sp.* with *Pseudomonas, Klebsiella* and *Bacillus species* demonstrates that they chose segregate has huge hereditary potential to create biosurfactant and could be utilized for bioremediation.

Conclusion

Eleven out of 21 isolates showed positive in qualitative test (oil spreading method). However, best isolate based on surface tension reduction capability and emulsification index was found to be KLEF21 which was identified as *Alcaligenes sp.* KLEF21. The selected strain showed more than 98% similarity index with *Alcaligenes sp.* KLEF21.The biosurfactant producing strain not only exhibited excellent capability of reduction in surface tension (32.12 dyne/cm) but also showed higher emulsification index (68.97%). The results obtained suggest the possible use of *Alcaligenes sp.* KLEF21 in bioremediation of crude oil and many other hydrocarbon contaminated sites.

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