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Pseudomonas aeruginosa involves biosurfactants in Hydrocarbon degradation of oil contaminated water

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ABSTRACT

Aim: The ability of *Pseudomonas aeruginosa* strains of hydrocarbon degradation by producing biosurfactant has been investigated. **Methods:** The water samples were collected from reservoir receiving soybean oil waste and detected for the presence of *P. aeruginosa* by 16S rRNA having potential of biosurfactant production when tested by oil spreading and emulsification assays. These isolates also recorded for the hydrocarbon degradation when tested by the COD reduction. **Results and Discussion:** The water bodies receiving heavy load of soybean oil was found to be positive for the several strains of *P. aeruginosa* capable of degrading hydrocarbon due to production of biosurfactant with different inherent capabilities. **Conclusion:** Isolated *P. aeruginosa* strains were found to be capable of degrading hydrocarbon due to overproduction of biosurfactant when tested in lab conditions.

KEYWORDS: *Pseudomonas aeruginosa;* Biosurfactant; 16S rRNA, Hydrocarbon degradation

1. INTRODUCTION

The ability of the *P. aeruginosa* in hydrocarbon degradation by biosurfactant production has been investigated in great detail by number of scientists earlier. In reports where change in the level of mono- and dirhamnolipids with respect to pH and nitrogen percent produced by *P. aeruginosa* has been reported where medium pH set at 7 produced maximum yield and pH below 7.3 reduces mono- and dirhamnolipids yield from 45 to 24.[1] According to Patowary R *et al* (2016) use of paneer whey; waste material released from the food industry has been used for the rhamnolipid biosurfactants production originated from *P. aeruginosa*. The strain SR17 of *P. aeruginosa* capable of reducing surface tension of the medium from 52 to 26.6mN/m and total biosurfactant yield reaches to 4.8 g/l from 2.7 g/l since the addition of 2% glucose and mineral salts.[2] In another study addition of glycerol supplemented production of monorhamnolipids at minimal salt medium with pH 6 and 1% glycerol (v/v) and 2% glucose (w/v) at 37 °C and at 180 rpm, and reached 2.164 g/L after 54 hours (0.04g/Lh) in presence of *P. aeruginosa*.[3] Pretreatment of the olive oil mill (OMW) waste with enzymatic hydrolysis proved beneficial for the increased production of the biosufactant produced by co-cultures of *P. aeruginosa* and *Bacilllus subtilis* and that yields to 29.5 mg/l and 13.7 mg/l rhamnolipids and surfactants, respectively.[4] With the ability to produce biosurfactant *P. aeruginosa* also found to be capable of degrading 99% of biphenyl within 72 h when supplied as the sole source of carbon. After degradation several metabolites such as 2-Hydroxy-6-oxo-6-phenylhexa-2, 4-dienoate has been detected.[5]

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2. MATERIALS AND METHODS:

2.1. Isolation of Pseudomonas aeruginosa

In an order to sample out the hydrocarbon degrading and biosurfactant producing *P. aeruginosa* strains, sampling of waste water released from the oil refinery industry from Betul city (India) was done and 1 ml sample was then added in 100 ml of 0.1X M9 minimal medium supplemented with 0.1 ml of soybean oil as the only carbon source. Inoculated medium was kept incubating at 28°C in a rotatory shaker and checked daily of the turbidity as an indicator of growth. In an incubating medium, 20 ml of minimal medium and soybean oil was added regularly at the end of the week. By the end of the month about one ml of sample was withdrawn from each flask and diluted by adding it in 9:1 ratio with sterile distilled water. Diluted sample was further spread plated on 0.1X M9 minimal media plates added along with waste water as a sole carbon source. Plates were incubated at 28°C and colonies were counted after 48 hours which

has represented the capability to utilize hydrocarbons available. The only plates showed excellent growth were preferred having diverse colonies. Individual colony was screened for fluorescent *P. aeruginosa* isolated and sub cultured on nutrient agar plates to purify.

2.2. Molecular Identification of Pseudomonas aeruginosa

In the process first protocol involved as the isolation of genomic DNA as per Purohit *et al*., 2003[6], followed by targeting 16S rRNA genes in the genomes of the bacteria and sequencing. To carry out PCR, reaction was set at 35 cycles having thermal conditions as: Denaturation at 94°C for 1 minute, Annealing at 55°C for 1 minute, Extension at 72°C for 1 minute. The PCR mix start was prepared as given below: autoclaved double distilled water: 31 µl; 10X PCR buffer (contains 15 mM of $MgCl_2$): 5µl, dNTPs (200 micro moles): 2.5µl, Primer (25 Pico moles): 5µl (2.5µl for Reverse and 2.5µl for forward primers each), Taq Polymerase (3 units/µl): 1.5µl, Template DNA: 5µl. The universal primers set was used to amplify all the bacterial isolates as Forward primer: 5'-AGAGTTTGATCMTGGCTCAG-3' and Reverse primer: 5'- TACGGYTACCTTGTTACGACTT-3'. The positive amplicons were sequenced for the forward and reverse primers amplicons and aligned to obtained homology and phylogram created to finally confirm the *P. aeruginosa* species and sequences were submitted to NCBI for allotting accession number.

2.3. Oil Spreading Technique

In the first method of biosurfactant marker detection, ability of oil spread as an indicator in Petri plate was assayed for biosurfactant production by the *P. aeruginosa* as per Anandraj B. et al., (2010).[7]

2.4. Emulsification Activity Measurement

In a second test, emulsification index (E24) was measured as suggested by Ilori MO et al. (2005)[8] with the given formula used in calculation of Emulsification Index:

$$\text{Emulsification Index} = \frac{\text{Height of emulsion layer}}{\text{Height of total column}} \times 100$$

2.5. Extraction of Biosurfactants

After confirming the formation of biosurfactant by the *P. aeruginosa* strains, process of extraction was adopted in which 50 ml of minimal media was inoculated with the isolate and supplemented with one ml of soybean oil which was further used for the extraction as suggested by Samadi N et al., 2007.[9]

2.6. Purification of Biosurfactants

The biosurfactant formed in the last step was carefully purified with the help of micropipette and transferred in sterile centrifuge tubes. This sample was added with one ml of distilled water and thoroughly vortexed to ensure uniform mixing. The sample again centrifuged at 7000 rpm by maintaining 4°C for 30 minutes. The supernatant was discarded and the pellet was allowed to dry for 24 hours to obtain crude extract of biosurfactant.

2.7. Dry weight of Biosurfactants

Dry weight of the crude biosurfactant was measured as suggested by Anandaraj B et al. (2010)[7] with given formula

Dry weight of Biosurfactants (g)= Weight of watch glass after dry - Weight of the empty watch glass after dry

2.8. Degradation of specific hydrocarbons and growth analysis of selected bacterial cultures

In experimentation, 100 ml of 0.1X M9 medium was supplemented with sterilized 20% waste water effluent coming from soybean processing plant and kept in 250 ml as the only carbon source. The flasks were individually inoculated with potential *Ps. aeruginosa* strains with a CFU set at 2 x 10^8 per ml. In a control set, medium was kept uninoculated by keeping other things constant. The entire flasks were kept aerated with sterile air under controlled room temperature of 28°C and samples were withdrawn from zero minutes to next six days with 24 hrs of time interval in triplicates and results were statistically analyzed by ANOVA for any change in COD values.

2.9. C.O.D estimation

The Chemical Oxygen Demand (COD) was calculated by employing following formula.[10]

$$\text{COD as mg/lit} = \frac{\text{(A-B)x N x 8000}}{\text{Ml of Sample taken}}$$

Where,
A= ml of FAS required for blank
B= ml of FAS required for sample
N= normality of FAS i.e. 0.1N
8000 milliequivalent wt. of oxygen x 1000 ml / lit [10]

3. RESULTS

3.1. Isolation and Screening of Biosurfactant Producer

Based on the sites of sampling the count was recorded to be varying but all of those were the indicator of capable of growing with the given composition in the medium and especially found to be utilizing waste carbon (Table 1). These all isolates were further screened for the typical isolation of floureascent *P. aeruginosa* by transferring them on the selective medias such as Mac Conkey agar, King's B agar and Cetrimide Agar Medium and finally those isolates showcased ability to fluoresce under UV light were selected and analyzed biochemically as shown in Fig. 1.

Table 1: Colony Count in Soybean oil enriched M9 media inoculated by

	Industrial water		
	Site 1	Site2	Site3
Water	200 ±23	50 ±13	112 ±27

Mean of three replicates, ± Standard Error.

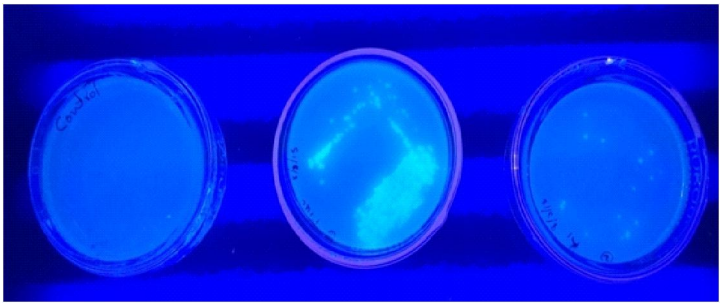

Fig. 1: The sample when inoculated on the Kings B medium developed colonies were exposed to UV light and with presence of fluorescent colonies, selected colonies were considered positive for Pseudomonas species. 1: Control plate; 2: Fluorescent colonies after subculture; 3: Isolated colonies showing fluorescence indicated Pseudomonas species.

3.2. Identification of Pseudomonas aeruginosa

Based on the promising features of *P. aeruginosa*, when they tested for the 16S rRNA gene sequencing, only three isolates named as NGB 2, 3, and 4 were identified as *P. aeruginosa.* The PCR amplicon of all three isolates were successfully amplified for the 16S rRNA gene and their sequences were submitted to NCBI/DDBJ/EMBL databases with the accession numbers as: NGB2:Ac. No. LC176070; NGB3: Ac. No. LC176071; NGB4: Ac. No. LC176072. Obtained homology was also highlighted as phylogram prepared along with five top scored sequences from the database which further confirmed the species level identification (Fig. 2 a/b/c).

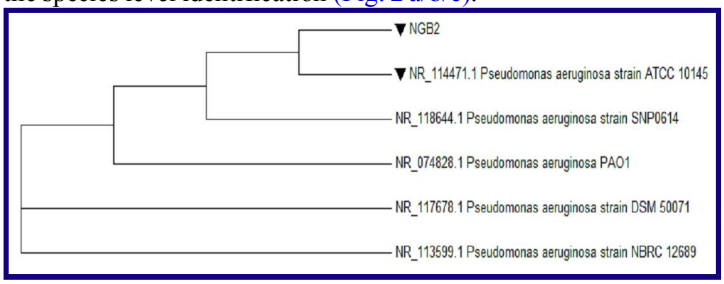

Fig. 2 a: *Pseudomonas* sp. Strain NGB2 showed homology with *P. aeruginosa* ATCC 10145

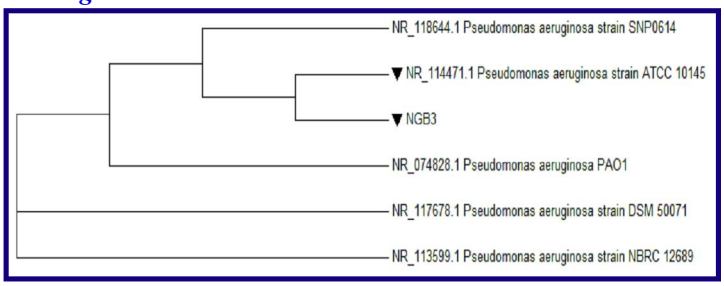

Fig. 2 b: *Pseudomonas* sp. Strain NGB3 showed homology with *P. aeruginosa* ATCC 10145

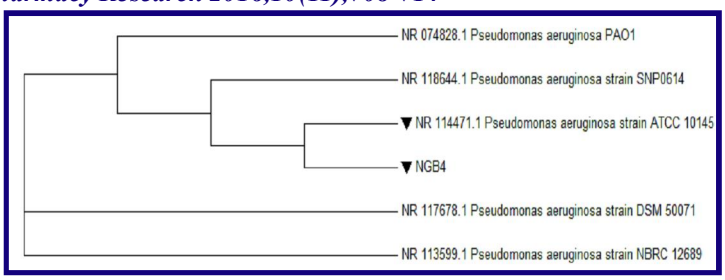

Fig. 2 c: *Pseudomonas* sp. Strain NGB4 showed homology with *P. aeruginosa* ATCC 10145

3.3. Oil Spreading Technique

To determine the capability of the three isolates for biosurfactant production, oil spread by the extracellular biosurfactant produced by isolates NGB 2, 3 and 4 in the oil was successfully tested. In which all three isolates found to be positive for the oil spreading capability as presented in Fig. 3. Further by measuring the formed clear zone it has been observed that the NGB3 proved to be better isolate with almost 7.9 mm of clear zone in the oil followed by NGB2 as 7.4 mm and 7.0 mm in NGB4.

Fig. 3: The spreading of extracellular-biosurfactant on oil surface layer by *P. aeruginosa NGB2*

3.4. Emulsification Activity Measurement

Active compounds produced in the form of biosurfactants was preliminary investigated with the capability of emulsification when the cell free extract of each isolate was checked for the formation of emulsification activity which was calculated by the given formula. Based on the calculation of time scale readings, it has been observed that in the early time of 2 hrs, the emulsification index was remained on higher side in all three isolates including the control and among them,emulsification percent recorded highest with isolate NGB4 (68%) followed by NGB3 (60%) and least by the isolate NGB2 (56%) at 2 hours of reaction of crude oil with cell free extract. All these values were close to the positive control (1% SDS) emulsification percent (59%) strongly suggested inherent emulsification capability imparted due to extracellular biosurfactant produced by the isolates (Fig.4). Further it has been observed as the time progressed, emulsification index showed regular reduction in the activity in a testing group as well as in control and finally become steady in the range of 40 - 44% (Table 2-5).

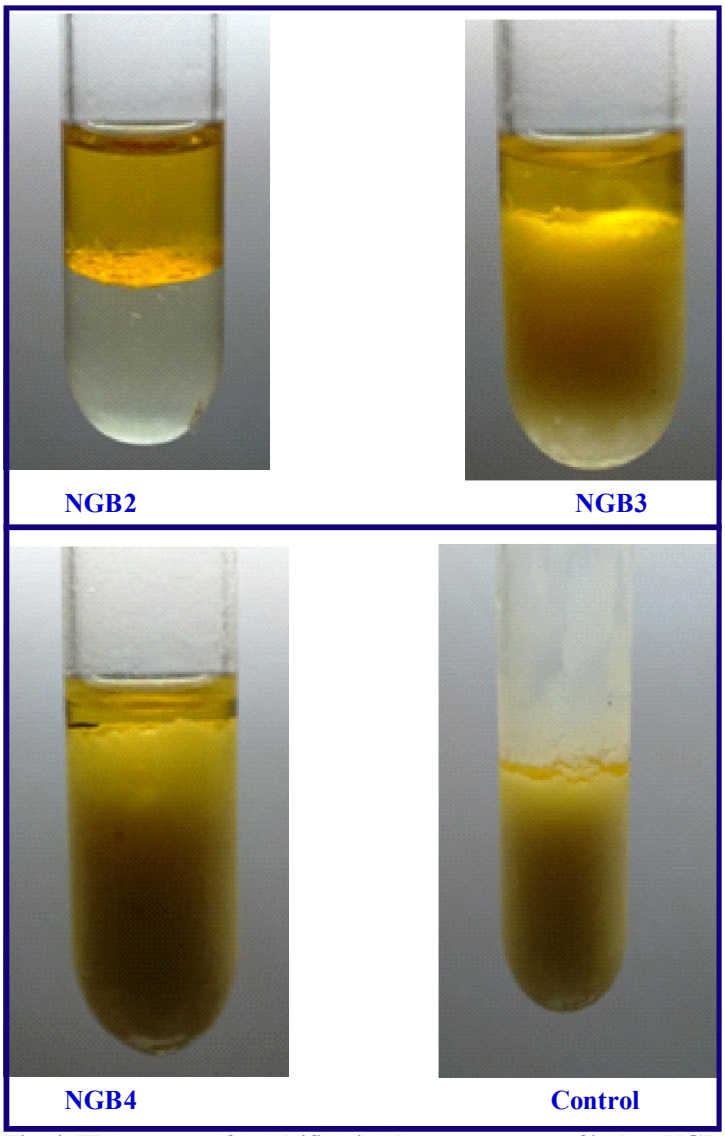

Fig. 4: The process of emulsification by supernatant of isolate NGB 2 , NGB3 and NGB 4 and Control (1% SDS)

Table 2: Emulsification index of NGB2 with Soybean oil

	Emulsion layer	Total liquid column	E_{24} Value	%
2 hrs	1.3	2.3	1.3/2.3*100	56
24	1.3	2.3	1.3/2.3*100	56
48	1.2	2.3	1.2/2.3*100	52
72	1.2	2.3	1.2/2.3*100	52
96	1.2	2.3	1.2/2.3*100	52
120	1	2.3	1.0/2.3*100	43

Table 3: Emulsification index of NGB3 with Soybean oil

	Emulsion layer	Total liquid column	E_{24} Value	%
2 hrs	1.4	2.3	1.4/2.3*100	60
24	1.3	2.3	1.3/2.3*100	56
48	1.1	2.4	1.1/2.4*100	46
72	1.1	2.4	1.1/2.4*100	46
96	1	2.4	1.0/2.4*100	45
120	1	2.5	1.0/2.5*100	40

Table 4: Emulsification index of NGB4 with Soybean oil

	Emulsion layer	Total liquid column	E_{24} Value	%
2 hrs	1.5	2.2	1.5/2.3*100	68
24	1.4	2.2	1.4/2.3*100	63
48	1.4	2.4	1.4/2.4*100	58
72	1.2	2.4	1.2/2.4*100	50
96	1	2.5	1.0/2.5*100	40
120	1	2.5	1.0/2.5*100	40

Table 5: Emulsification index of 1% SDSPAGE with Soybean oil

	Emulsion layer	Total liquid column	E_{24} Value	%
2 hrs	1.3	2.2	1.3/2.2*100	59
24	1.3	2.2	1.3/2.2*100	59
48	1.2	2.4	1.2/2.4*100	50
72	1.2	2.4	1.2/2.4*100	50
96	1.1	2.5	1.1/2.5*100	44
120	1.1	2.5	1.1/2.5*100	44

3.5. Dry Weight of Biosurfactants

Selected isolates *P. aeruginosa* NGB 2, 3 and 4 grown upto 168 hours were analyzed for the crude biosurfactant formation by weighing the content after complete drying. The results indicated that the initial culture growth produce a typical biosurfactant with concentration of 0.019-0.023 g/ml in a supernatant and with 48 hours, content increased almost to the double except in isolate NGB4 where in contrast reduction was observed for about half of the initial value. In the next 72 hours, significant increase in the crude biosurfactant content was recorded in NGB2 and 3 and thereafter the content dropped down till seventh day when the data recorded last. In comparison between isolates, NGB 3 produced highest 0.075±0.00 g/ml of biosurfactant compared to NGB2 with 0.064±0.00 g/ml and the least value recorded as 0.023±0.00 g/ml for NGB4 as shown in Table 6.

Table 6: Dry weight of crude biosurfactant (g/ml) recorded for 24 hours interval from supernatant of P. aeruginosa isolates (NGB 2/3/ 4)

Hours	NGB2	NGB3	NGB4
24	0.019±0.00	0.022±0.00	0.023±0.00
48	0.036±0.00	0.042±0.00	0.014±0.00
72	0.056±0.00	0.053±0.00	0.012±0.00
96	0.058±0.00	0.075±0.00	0.017±0.00
120	0.064±0.00	0.042±0.00	0.012±0.00
144	0.031±0.00	0.038±0.00	0.008±0.00
168	0.013±0.00	0.028±0.00	0.009±0.00

± = *standard deviation*

3.6. Degradation of specific hydrocarbons and growth analysis of selected bacterial cultures

By recording the capability of each *P. aeruginosa* isolate (NGB 2/3/ 4) for biosurfactant production the comparative performance of these

isolates was assessed based on the capability to utilize these biosurfactant with reference to degradation of soybean oil containing waste water collected from kosmi dam area.

Initially the ability of each isolate to utilize the only carbon source provided by the soybean oil containing waste water was assessed spectrophotometrically by recording the optical density of the growth and it has been observed that the cultural growth of all isolates remained on a slower side up to 48 hours post inoculation and thereafter significant rise of upto 72 hours was recorded indicated the utilization of the waste water by the isolates. After 72 hours of incubation the cell doubling reached steady state and a barely marginal rise in the growth rate was observed till 120 hours (Table 7).

Table 7: Change in the growth pattern of three *P. aeruginosa* in presence of soybean processing plant waste water as only carbon source in 0.1 X M9 media

Time in hours	Control	NGB2	NGB3	NGB4
24	0.203±0.01	0.353±0.00	0.95±0.01	0.55±0.00
48	0.211±0.00	0.415±0.00	0.821±0.00	0.761±0.00
72	0.271±0.00	1.492±0.00	1.404±0.00	1.563±0.00
96	0.253±0.00	1.541±0.00	1.589±0.00	1.646±0.00
120	0.22±0.00	1.637±0.00	1.675±0.00	1.787±0.00

± = standard deviation

Along with the growing ability of cultures comparative utilization of hydrocarbons was analyzed by recording the decreasing COD (mg/L) values with the increase in cell number and time of exposure in an incubating condition. Based on the COD values recorded after six days of incubation, isolate NGB4 reduced the COD at the maximum to (276 mg/L) followed by NGB2 (465 mg/L) and NGB3 (574 mg/L). COD removal by all the selected bacterial cultures was found to be significant (P<0.01) as compared to control (Table 8).

Table 8 : Comparative assessment of the COD values (mg/L) obtained for control and tested *P. aeruginosa* NGB 2/3/4 for soybean oil containing water of Kosmi Dam

Hrs	Control	SD	SE	NGB2	SD	SE	NGB3	SD	SE	NGB4	SD	SE
0	883	±3	1.9	881	±2	1.5	883	±1	0.9	883	±2	1.2
24	868	±4	2.3	786	±2	1.5	822	±2	1.2	727	±2	1.5
48	884	±2	1.3	725	±4	2.3	776	±2	1.5	656	±2	1.5
72	873	±2	1.2	578	±2	1.5	744	±2	1.7	438	±2	1.2
96	869	±2	1.5	536	±3	1.8	705	±3	1.8	416	±2	1.5
120	865	±2	1.5	496	±2	1.2	666	±2	1.5	309	±4	2.3
144	846	±1	0.9	465	±2	1.3	574	±2	1.5	276	±3	1.7

SD : standard deviation; SE : standard error ; COD values at mg/litre

Further the ability of isolates to reduce COD values has been statistically analyzed in terms of change in the COD with given time compared to control. As per analysis it has been observed that the culture NGB2 reduced the COD at the rate of 80 mg/L/day upto 48 hours and thereafter the reduction rate got lowered to 35 mg/L for next 48 hours and thereafter the rate decreased to 10 mg/L/day till data recorded for 144 hours

Similarly, culture NGB3 till 24 hours reduced the COD value upto 45 mg/L/day and then increased to 65mg/L/dayat 48 hours. From third day onwards the rate of COD reduction remained steady at about 35-40 mg/L/day till last recording. In culture NGB4 COD reduction recorded highest on first day with 140 mg/L/day and on 48 hours the rate reduced down to 90 mg/L/day. Interestingly on the 72 hours the COD reduction reached to maximum of 210 mg/L/day and then reduced to 120 mg/L/day on 120 hours. During investigation it has also been evident that after any high value reduction in COD value the next 24 hours were recorded with the lowest change in the COD and then again the increased COD reduction was evident as shown in table and all these values were statistically significant (P<0.001) when compared to control which has indicated the potential of all these bacteria to degrade the soybean contaminated water reaching to kosmi dam (Table 9). The ability of the culture NGB 2/3/4 to completely degrade waste water was evidenced on 144 hours when mineralization of waste water was and also the biosurfactant production in the form of froth was also recorded.

Table 9: Time lapsed change in the COD value in *P. aeruginosa* NGB2/3/4 compared to control

	Control vs NGB2		Control vs NGB3		Control vs NGB4		
Hours	Diff.	Sig.	Diff.	Sig.	Diff.	Sig.	P value
0	-1.3	ns	0	ns	0.67	ns	P > 0.05
24	-82	***	-45	***	-140	***	P<0.001
48	-160	***	-110	***	-230	***	P<0.001
72	-300	***	-130	***	-440	***	P<0.001
96	-330	***	-160	***	-450	***	P<0.001
120	-370	***	-200	***	-560	***	P<0.001
144	-380	***	-270	***	-570	***	P<0.001

*ns : non significant; * : significant ; Diff: Difference ;Sig: Significance*

4. DISCUSSION AND CONCLUSION

Betul city being agriculture rich area also witnessing ever increasing food based industrialization and by which life is suffering from the alarming hazards arising from water, soil and air pollution and till date merely any preventive measures have been taken by local authority to make it sustainable. As per preliminary report, pollution of the Betul rivers and reservoirs with the industrial effluents and local activities continuing as recorded by local researchers and confirmed the requirement of strategy designing of handling the pollution being transferred unattended in fresh water received by the city part.[11,12]

The samples collected from the affected areas often possess the microbial consortium which showcases the ability of degrading the hydrocarbon getting released in a continuous mode. In the present

study similar attempt was made where a well-known bacterium of family *Pseudomonas* called as *P. aeruginosa* was preferentially isolated as it has showcased the ability to degrade the hydrocarbon by producing high level of biosurfactants. In process to isolate the *P. aeruginosa* from the contaminated water and soil by involving standard dilution and medias like Mac conkey, King's B and Cetrimide agar with soybean oil, a potent population of the *P. aeruginosa* was isolated successfully. Earlier, it is known that *P. aeruginosa* plays a vital role in hydrocarbon degradation by producing the biosurfactants which are microbial secondary metabolites with capabilities to decrease the surface tension and have emulsifying capacity. Many strains of *P. aeruginosa* have been isolated from the sources where heavy load of hydrocarbon recorded and asks for the degradation at a faster rate than normal as evidenced in study of diesel oil contamination[13]; crude oil contamination[14]; long-chain n-alkanes and crude oil[15]; olive and fish oil factories[16] and palm oil mill effluent[17] in recent times.

16S rRNA gene sequencing was adopted to comprehensively detect the *P. aeruginosa* from the close species grown in selective media and this study selectively identified strains NGB2, 3, and 4 of *P. aeruginosa*. As it is known that 16S rRNA gene is a housekeeping genetic marker and occurs in almost all bacteria as a multi gene family, or operons.[18]

In first assay as oil spread method, highest clear zone was formed by the *P. aeruginosa*NGB3 followed by NGB2 and least by NGB4 ranging in 8 mm in diameter. In agreement with our study another work carried out in India with the biosurfactant producing bacteria which were able to displace oil to 5 mm. This showcased better spreading in spilled oil experiments by our strains as compared to zone of 5mm recorded earlier.[19]

In emulsification activity which is one of the recommended tests for biosurfactant has also showcased that three strains *P. aeruginosa* NGB2, NGB3and NGB4 capable of potent emulsification and expressed extracellular biosurfactant whose percent values recorded in decreasing order as 68% > 60%> and 56% for NGB 4, NGB3 and NGB2 respectively at 2 hours' time. Further in time course study, the emulsification index showed gradual decrease in percent of all strains and recorded to be 40-44% with overall 25% decrease. This decrease in emulsification index was also recorded with other biosurfactants of *P. fluorescens* when tested in different oil combinations by worker Peter JK and Singh DP (2014) while investigating the formation of rhamnolipid.[20]

In the current study *P. aeruginosa* NGB2, NGB3and NGB4 were allowed to form the biosurfactant and the dry weight (g) per ml was estimated in which it has been observed that the NGB3 produced maximum of dry weight biosurfactant (75 mg/ml) followed by NGB2 (64 mg/ml) and NGB 4 (23 mg/ml) and in comparison among strains significant difference in the amount was observed. In a similar study by using waste motor engine oil and peanut oil cake comparative change in the *P. aeruginosa* biosurfactant production was estimated. Results highlighted that the peanut oil cake showed higher biosurfactant of 4.37 mg/ml at 132 h of incubation.[21] The given value was many fold lower than the biosurfactant produced by our strain with waste water which has suggested the high impact of these strains in biosurfactant production.

As per growth kinetics study, all strains showed initial lag period of 48 hours once inoculated in the minimal medium added with waste water as carbon source and later on they entered into the log phase and almost double the bacterial number till next 48 hours. This transition is critical in the terms of utilization of the hydrocarbons available in waste water because as the cells reaching the log phase; they also started to reduce the COD values has showed direct relation between them. Among the strains rate of reduction in the COD values was also remain varied where highest reduction showcased by strain NGB4 (66%) followed by NGB2 (47%) and least by the NGB 3 (35%) put forward the capability of each strain under constant treatment conditions of shake flask culture. It also showcased that due to formation of the biosurfactants probably all strains showed the better ability to degrade hydrocarbon even in absence of any consortium of bacterial population. In a similar study it has been observed that by using the sequencing batch reactors added with *Pseudomonas aeruginosa* SP4 in mineral medium with palm oil at temperature of 37 degrees C, culture showed ability of reduce the chemical oxygen demand upto 90% and palm oil removal upto 97% due to biosurfactant production which is in agreement to our study also that *P. aeruginosa* once again showed the potential of better degradation due to biosurfactant production.[22]

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