Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 55 (1): 47–56 (2018)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Enhanced Production of Biosurfactant by *Pseudomonas aeruginosa* Isolate GW-7 under Nitrogen and Phosphate Limiting Conditions

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Abstract: A novel bacterial strain of *Pseudomonas aeruginosa* has been isolated and characterized by screening samples from formation water of local oil wells and other habitat, which overproduced potent biosurfactant under phosphate limiting condition within 36 hours of incubation. Biosurfactant producing bacteria were isolated from the oil, formation water and from the soil contaminated with oil from the local oil wells; strains were grown on different medias having different carbon sources. Selected 66% culture reduced surface tension lesser than 33 mN/m and interfacial tension lesser than 3 mN/m whereas 85% showed hemolytic activity. Pseudomonas spp. was most common while some *Micrococcus* and *Acinetobacter* were also identified. Most potent biosurfactant producers were *P. aeruginosa, P. fluorescens* and *Micrococcus spp. P. aeruginosa* strain GW-7 was selected for further studies. BH-mineral medium was best for biosurfactant production as it reduced surface tension to (32 mN/m), interfacial tension (2.5 mN/m).Other best medium was an inorganic phosphate limited medium containing proteose peptone/glucose/ ammonium salts, as its surface tension and interfacial tension reached to 30 mN/m and 2.5 mN/ m, respectively, after 36 hours. *P. aeruginosa* isolate GW-7 isolated from oil enriched soils from Gwadar coastal area produced potent biosurfactant (rhamnolipid) in large amount in reasonably short period of time (36 hours) under nitrogen and phosphate limiting conditions.

Keywords: Bacteria, biosurfactant production, characterization, nitrogen and phosphate limitation, culture media

1. INTRODUCTION

A wide and diverse variety of microorganisms produce surface-active compounds or biosurfactants (BS). These molecules have ability of reducing the surface tension of both aqueous solutions and hydrocarbon mixtures, as well as interfacial tension of the growth medium. They are composed of diverse chemical structures like lipopeptide, glycolipids, neutral lipids and fatty acids [1, 2]. They also increase the bioavailability of organic pollutants, including crude oil components and thus enhance biodegradation [3-6]. They are highly promising for use in remediation technologies as substitute to the synthetic surfactants because of their biodegradability, low toxicity and effective-ness at extreme temperature and pH [7, 8]. Ecologically agreeable and bio-degradable surfactants (ionic or non- ionic) for consistent environmental cleanup are really required. Currently biosurfactants are mostly used in studies on enhanced oil recovery and hydrocarbon bioremediation. Moreover biosurfactants also have prospective uses in agriculture, cosmetics, pharmaceuticals, detergents and paint industries [7, 3]. Commercially feasible BSs must be economically competitive, so development of worthy microbial BS producing cultures is essential [6]. Vital problems currently under exploration are development-isolation of BS producing microorganisms (consortia or isolate),

Received, November 2016; Accepted, March 2018
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refinement of their production capability by varying their incubation circumstances (temperature, time, nutrient), substrate type toward attaining a high production and fabrication of lipid mixtures having a striking /preferred configuration [9]. Enhanced rhamnolipid production is associated with depletion of nitrogen and a shift to nitrogen limited growth [10, 11]. Mulligan et al. [12], have shown a reduction in surface tension on phosphate exhaustion but concurrent nitrogen concentration was not reported. While the association of nitrogen exhaustion and rhamnolipid production is widely acknowledged. Phosphate limitation had opposite effects as it led to rhamnolipid production while reducing HSL synthesis and/or stability [13]. Clark et al. [14] mentioned that rhamnolipid accumulation under phosphate exhaustion and nitrogen excess suggests a non-specificity of limiting nutrient and that rhamnolipids will be synthesized provided carbon is in excess of metabolic capacity. The present study is aimed to optimize different conditions for maximum production of biosurfactant in least time and inexpensively by *P. aeruginosa* isolate GW-7.

2. MATERIALS AND METHODS

2.1 Sampling

Various samples used for this work included oil, soil and formation water from some local oil wells of Khyber Pakhtoonkhaw; Manzali, Chanda, Makori, Nashpa, Mela and oil enriched soils from Gwadar coastal area and Petroleum Refinery Limited (PRL), Karachi.

2.2 Enrichment on Different Growth Media

Samples from different ecosystems were enriched on five growth media with different carbon sources. The media used for enrichment of biosurfactant producing bacteria were BH [15], mineral medium, modified mineral medium for biosurfactant production with Makori crude oil as carbon source [16]. Nutrient broth (NB), marine broth (MB) and nutrient broth yeast extract (NBYE) were also used for comparison. Flasks were set on rotary shaker (100 rpm) at controlled temperature (300 ^{O}C) for 6 days of incubation.

2.3 Screening: Hemolytic Activity

Red blood cell lysis assay, a rapid screening method for surfactant production is based upon the ability of the surfactant to lyse red blood cells producing clear zone around colonies [17].

2.4 Growth of Mixed / Pure Cultures on Makori Crude Oil

Morphologically different types of bacterial isolates isolated from different ecosystems were grown 0.5% Makori crude oil as carbon source. For mixed culture studies the number of strains originally isolated from a particular ecosystem was inoculated with mixture of all these cultures in equal amounts. After six days of incubation, the level of growth, level of conversion of oil and emulsion stability was checked by visual observation [18].

2.5 Level of Growth

Growth was monitored by measuring the optical density at 660 nm by spectrophotometer [12]. Growth in terms of dry biomass was also determined by the method of Guerra-Santos et al. [19]. The turbidity produced as a result of bacterial growth was monitored visually at the end of incubation period and assigned (+,-, ++++) depending upon the density of turbidity in aqueous phase in the flask.

2.6 Surface Tension Measurement

Surface tension of cell free culture broth was measured by ring method using KRUSS Tensiometer K10T. Reduction in surface tension in comparison to control was taken as a confirmatory test for biosurfactant production by these isolates.

2.7 Level of Oil Conversion

The amount of oil separated out from the culture medium represent the oil not metabolized or transformed (Total−transformed) and assigned as A, B, C and D (100-25%).

2.8 Emulsion Stability

Different types of emulsions have been observed by these strains and categorized by I, II, & III, indicating the formation of stable, less stable and unstable emulsions, respectively.

2.9 Selection Criteria

Taking into account the above parameters, preliminary selection criteria were developed. The bacterial isolates with ++++/+++ level of growth, A/B level of oil conversion and I/II types of emulsion stability were selected for further studies.

2.10 Characterization of Biosurfactant Producing Bacteria

The potent biosurfactant producing bacteria selected on the basis of above parameters were identified up to species level. All the isolates were examined for oxidase reaction [20]; Gram's reaction, morphology, mobility and production of catalase [21]. Biochemical tests were performed with QTS-20 test kits. The schemes of Macfaddin, 1980 [22], were followed when grouping the organisms down to species level.

2.11 Interfacial Tension

Interfacial tension was measured by submerging the platinum ring in cell free culture broth and adding sufficient hexadecane so that did not break through the upper surface before the interfacial film is ruptured [23]. The reciprocal of critical micelle concentration (CMC-1), a direct measurement of surfactant concentration was determined by measuring surface tension of serially prepared dilutions of cell free culture broth.

2.12 Emulsion Index

Emulsifying activity was measured by adding 3ml of n-hexadecane to 3ml of spent culture medium and vortexing at high speed for 2 minutes. Emulsion index (E 24) was measured after 24 hours. The emulsion index (E 24) is the height of emulsion layer, divided by the total height and multiplied by 100 [24].

2.13 Growth of Biosurfactant Producing Bacteria on n-hexadecane

The selected strains with above mentioned inoculum size were grown on 0.65% (500 mg) n-hexadecane in BH-Mineral medium. The cell free culture broth was checked after every 24 hours for reduction in surface tension by KRUSS Tensiometer K10T using ring method.

2.14 Comparison of Biosurfactant Production by Different Bacteria grown on n-hexadecane and Glucose

The selected strains were compared on the basis of their potential for biosurfactant production when grown on glucose (500 mg carbon) and n-hexadecane (500 mg carbon) separately. After six days of incubation, supernatant of spent culture broth was checked for reduction in surface tension, interfacial tension and emulsion index.

2.15 Production of Biosurfactant by *P. aeruginosa* Isolate GW-7 in media with different carbon & nitrogen sources

P. aeruginosa isolate GW-7 isolated and characterized as most potent biosurfactant producing bacterium was grown on two types of media (minimal and complex), comprising of total five in number for evaluation of production of biosurfactants. Minimal BH-M, M2 and M3 [15, 16, 25], containing 1% hexadecane as carbon source were used. Complex media; Phosphate rich (PPGAS) [26], Phosphate sufficient Kay's minimal medium [27] and phosphate limited proteose / glucose/ammonium salt medium (PPGAS) [28].

2.16 Effect of Different Concentrations of Glucose on Growth & Biosurfactant Potential of *P. aeruginosa* Isolate GW-7.

Shake flask studies were conducted at 30 ^{0}C at 100 rpm on a rotary shaker along with their control. Medium was autoclaved at 121 ^{0}C and 15psi with 0.5% - 4% glucose (autoclaved separately). One hundred ml side arm flasks with 25 ml of each medium were also prepared for the measurement of growth by taking optical density at 660 nm. Residual glucose was analyzed at 550 nm by dinitrosalisylic acid (DNS) method [29].

2.17 Time course of Biosurfactant Production

After selecting the optimum substrate concentration, the isolate GW-7 was grown on phosphate deficient medium. Two set of flasks were prepared, one set labeled as "sampling" and other as "backup".

2.18 Extraction of Glycolipid from Cell Free Culture Broth and Hydrolysis of Glycolipid

pH of cell free culture broth was lowered to 2.0 by adding conc. H_2SO_4. The solvent mixture containing chloroform: methanol (2:1) was used in equal amount. Crude glycolipid was hydrolyzed by adding 1N HCl (5 ml: 50 mg lipid) and heating the reaction mixture at 90 ^{0}C for 3 hours. After cooling fatty acids were extracted by chloroform. Aqueous layer contains sugar portion of lipid which was extracted with hot ethanol.

2.19 Identification of Sugar Moiety by Paper Chromatography

Sugar was analyzed by paper chromatography using n-propanol: ethyl acetate: H_2O (7:1:2) solvent system.

2.20 Identification of Lipid Moiety using Gas Chromatography

Fatty acid portion was methylated by adding 2.0 mL of methanol & 0.5 mL of concentrated H_2SO_4 (4:1), heating reaction mixture at 100^{0}C for 5 minutes. Methyl esters were again dissolved in spectroscopic quality chloroform for running on Gas Chromatography (GC). The resulting methyl esters of the fatty acids were measured by GC by the method of Brandl et al.1988 and as studied systematically in recent times [30].

3. RESULTS AND DISCUSSION

3.1 Selection of Growth Medium

It was found that morphologically different bacteria enriched on various growth media were different. The minimum number of colonies detected on marine broth (MB). This medium contained 20gm/L of NaCl which might be inhibitory to these bacteria. Among the nutrient broth (NB) and nutrient broth yeast extract (NBYE), the later was found to be better as nutrient broth along with yeast extract is essential for some bacteria. Among BH and MMFB maximum numbers of colonies were enriched on BH mineral medium with Makori crude oil as carbon source. Finnerty & Singer [16] have used MMFB for the isolation of biosurfactant producing bacteria. But in this study BH mineral medium with Makori crude oil as carbon source was found better than MMFB. It might be due to different N-source (NH_4NO_3) in BH medium while MMFB contained $(NH_4)_2SO_4$. This is in confirmation with findings of Cooper et al. [31]. Most recently Eleftheria et al. [9] mentioned that minimal (ONR7a) medium with crude oil as a sole carbon source was the best for biosurfactant production. Therefore, BH mineral medium with Makori crude oil was selected for further studies.

3.2 Screening: Growth Studies of Mixed/Pure Culture on Makori Crude Oil

A significant variation among the various bacterial isolates to utilize crude oil as carbon source was noticed. However, some of the strains have ability to transform (metabolize) 50% of the crude oil which resulted in emulsification of oil in water. Among the total of 62 morphologically different isolates 10% formed type I (stable) emulsion with ++++ level of growth and "A" level of oil conversion (75-100%). Level of growth, level of conversion of oil and emulsion stability was found to be inter-correlated. The isolates (21) which fulfilled the said selection criteria (See Materials & Methods) correspond to 37% of the total tested and were used for further studies. The ability to emulsify oil in water is an important property of microbes that use hydrocarbon as sole source of carbon [23]. The biosurfactants produced from bacterial isolates of refinery wastewaters showed good emulsification activity against different hydrocarbon sources. It was observed that the refinery wastewaters are a suitable source for isolation of biosurfactant-producing bacteria, but are not a substrate for biosurfactant production [32].

3.3 Hemolytic Activity

When comparing the hemolytic activity with growth in oil medium, percent conversion of oil and emulsion stability did not correlate with hemolytic activity. Reduction of surface tension, interfacial tension of culture broth grown on either glucose or hexadecane could not reflect the same picture as that hemolytic activity. So from these studies it can be concluded that there is no positive correlation between hemolytic activity and biosurfactant

Table 1. Comparison of different bacterial strains (identification also given) total growth, surface tension, Interfacial tension (IFT) and emulsion index of culture broth after 6-days growth on glucose and n-hexadecane at 30^0 C and 100 rpm.

Strain No.	Growth on BH + oil	% Conversion	Emulsion Stability	Hemolytic Activity	Surface tension N/m		IFT N/m		Emulsion Index (E24)		Identification of bacterial isolates
					Glu.	Hexad	Glu.	Hexad	Glu.	Hexad	
4	+++	B	II	+	31.5	31.5	3.1	2.8	--	62	*P. aeruginosa*
7	++++	A	I	+	34	30.1	3.2	2.7	--	61	*P. aeruginosa*
9	++++	A	II	+	32.0	37	3.8	7.0	--	54	*P. aeruginosa*
10	+++	--	I	+	36.0	31.0	--	1.5	--	59	*Micrococcus*
12	++++	AB	II	+	52.2	40.6	25	12.8	--	66	*P. aeruginosa*
26	++++	A	I	+	51.7	30.1	--	2.2	--	65	*P. pseudomallei*
27	++++	A	I	+	37.0	32.3	--	2.1	--	59	*P. aeruginosa*
28	+++	A	II	+	55.5	30.7	--	2	--	64	*P. aeruginosa*
29	++++	A	II	+	35.6	33.5	4.2	3.5	--	62	*P. aeruginosa*
30	++	A	II	+	32.2	51.3	2.9	--	61	--	*P. aeruginosa*
32	+++	D	III	+	---	51.2	--	--	--	--	*P. pseudomallei*
35	++++	B	II	+	31.2	36.5	3.3	--	58	--	*P. pseudomallei*
42	++++	A	II	-	58.8	50.5	--	--	--	--	*Micrococcus*
44	++++	B	II	+	42.7	33.1	--	2.2	--	63	*P. aeruginosa*
45	++++	A	I	+	37.9	28.5	--	1.0	--	60	*P. fluorescens*
48	++++	B	II	+	46.3	33	20.3	2.4	--	64	*P. aeruginosa*
52	++++	B	I	+	45.2	32.7	12.8	2.3	--	56	*P. aeruginosa*
55	++	C	III	-	41.9	45.8	--	--	-	--	*Pseudomonas sp*
56	++++	A	II	-	53.6	49.6	25.7	25.1	--	--	*Acinetobacteria*
62	++++	A	I	+	50.9	30.2	8.9	2.3	--	63	*P. fluorescens*
69	++++	B	II	+	36.7	39.1	7.5	9.1	--	60	*P. pseudomallei*

Note: *BH: Bushnell and Haas Medium

+ --- ++++ = more + more growth

A:	75 – 100 %	I: Stable emulsion
B:	50 – 75 %	II: Less stable emulsion
C:	25 – 50 %	III: Unstable emulsion
D:	00 – 25 %	IV: No emulsion

production by these bacteria. Microorganisms which produce biosurfactant only during growth on alkane cannot be always detected by this method [33, 34].

3.4 Growth Studies of Selected Isolates on n-hexadecane

All 21 isolates selected on the basis of above selection criteria were categorized into five groups depending upon the time taken for reduction in surface tension of the n-hexadecane grown spent culture medium up to 33 mN/m. Group 1 is comprised of the isolates which reduce the surface tension of culture broth down to 33 mN/m as compared to control (65 mN/m) and distilled water (72 mN/m) within 48 hours of incubation. Similarly group 2, 3 and 5 bacteria reduce the surface tension up to 33 mN/m in 72, 96 and 120 hours of incubation. Reduction in ST, IFT has also been taken as criterion of biosurfactant production by other workers [3, 9, 31, 35, 36, 37]. Reduction in surface tension of spent culture medium has been correlated to the biosurfactant production by different workers [3, 9, 31, 35, 36, 37, 38, 39].

3.5 Comparison of Biosurfactant Production by Different Bacteria Grown on n-hexadecane / Glucose

The selected isolates were compared on the basis of their potential for biosurfactant production when grown on n-hexadecane and glucose on the same carbon basis. A good correlation among the visual observation of growth, emulsification, % oil conversion, hemolytic activity and tensiometric studies of the spent culture medium was perceived. Sixty six percent of the total bacterial isolates were found to be good biosurfactant producers, as they reduce the surface tension of spent culture medium up to 33 mN/m against control (65 mN/m) and interfacial tension from 42 mN/m to 3 mN/m, which are considered to be the confirmatory tests for

Table 2. Effect of different media containing different carbon and nitrogen sources on growth and biosurfactant potential of P. aeruginosa isolate GW-7.

Medium	Carbon Source	Nitrogen Source	Dry biomass (g/L)	CMC^{-1}	Interfacial Tension(IFT) (mN/m)	Emulsion Index (E24)	Surface Tension (mN/m)
BH	Hexadecane (1% v/v)	NH_4NO_3 (1 g/L)	1.87	22	2.1	73	30
M2	Hexadecane (1% v/v)	$(NH_4)_2SO4$ (2 g/L)	2.4	6	2	75	30
M3	Hexadecane (1% v/v)	$(NH_4)_2SO4$ (8 g/L)	2.77	1	2	65	53
PPGAS(PR)	Glucose (0.5%)	NH_4Cl (1.098 g/L)	2.3	2	5	60	50
PPGAS(PD)	Glucose (0.5%)	NH_4Cl (1.098 g/L)	0.5	15	1.5	76	32 (36 hrs)

Note: The incubation period was 72 hours.

biosurfactant production (Table 1). In present study *P. aeruginosa* was found as better biosurfactant producer when grown on either hydrocarbon or glucose as substrate. *P. fluorescens* (45 & 62) were found to be potent biosurfactant producer when grown on hydrocarbon substrate only. Both of these isolates substantially reduced the surface tension to 29.6 mN/m and interfacial tension (1.28 & 1.6 mN/m) of the spent culture medium. Similar results have also been reported by other workers [3, 9, 23, 31, 32, 34, 36, 37, 39, 40, 41].

Considerable variations have been noted as regards to the biosurfactant production by P. pseudomallei. One of the isolate identified as Micrococcus spp. has found to be the potent biosurfactant producer as indicated by the low surface tension (30.6 mN/m) and interfacial tension (1.6 mN/m) of spent growth medium. Many workers also reported Micrococcus spp. as potent biosurfactant producer [1, 38, 42, 43, 44, 45, 46].

3.6 Characterization of Biosurfactant Producing Bacteria

The potent biosurfactant producing bacteria were characterized for biochemical parameters down to the species level. It has been found that among the isolates from different ecosystem *Pseudomonas spp.* was most commonly found (Table 1). Among the same genus *P. fluorescens* and some of the *P. aeruginosa* were found to be potent biosurfactant producers. Some *Micrococcus spp*. was also found to be potent biosurfactant producers. Acinetobacter spp. was also isolated during this study but these were found to be non-biosurfactant producers. *P. aeruginosa* isolate GW-7 was selected for further studies.

3.7 Biosurfactant Production by *P. aeruginosa* Isolate GW-7 in Media with Different Carbon & Nitrogen Sources

Biomass increased gradually in all media but rapidly in PR and PD. Highest biomass (2.77 g/L) in case of M3 may be due to large amount of N_2 as it contained 8.0 g/L $(NH_4)_2SO_4$. The carbon source is utilized for the biomass production and not for biosurfactant production which are overproduced during N-limitation. Hence there is poor production of biosurfactant which is indicated by poor reduction in S.T (53 mN/m). Lowest biomass (0.5%) in case of PD is because PO_4 has become limited, so C-source could not be utilized for growth and metabolism is shifted towards biosurfactant (glycolipid) production. Hence reduction in S.T is reasonably good (30 mN/m) after 36 hours of incubation in case of PD medium. The reduction in surface tension was significant in both BH and M2 medium (30 mN/m) because in both cases N2 become limiting after 48 hours, hence the growth has stopped. The C-source is still available, so the metabolism is shifted towards glycolipid production which is well established phenomenon in oleagineos microbes (Table 2). Therefore it may be concluded that BH medium is better for higher yields with fewer nutrients but it takes longer time, i.e., 72 hours (Table 2). Instead the PD medium gives such

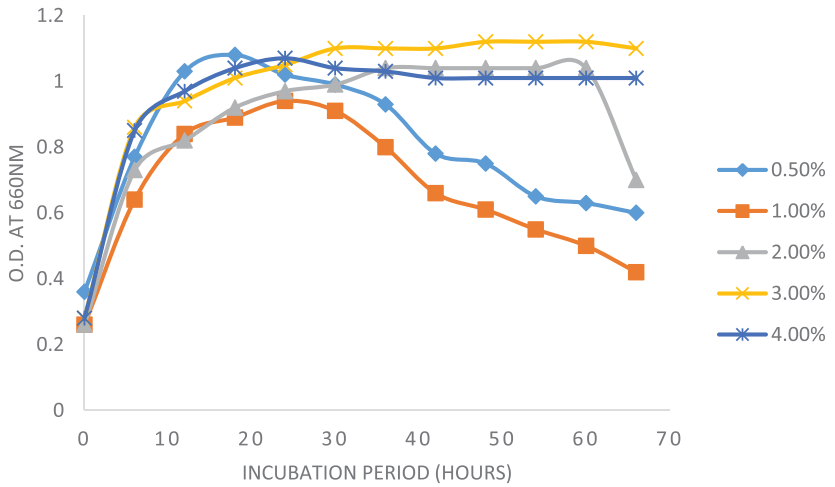

Fig. 1 Effect of glucose concentration on growth of P. aeruginosa strain GW-7. The culture were grown on 0.5-4% glucose in phosphate deficient medium in 250ml Erlenmeyer flask at shaker (100rpm) at 300C. The Growth was measured by taking optical density at 660nm.

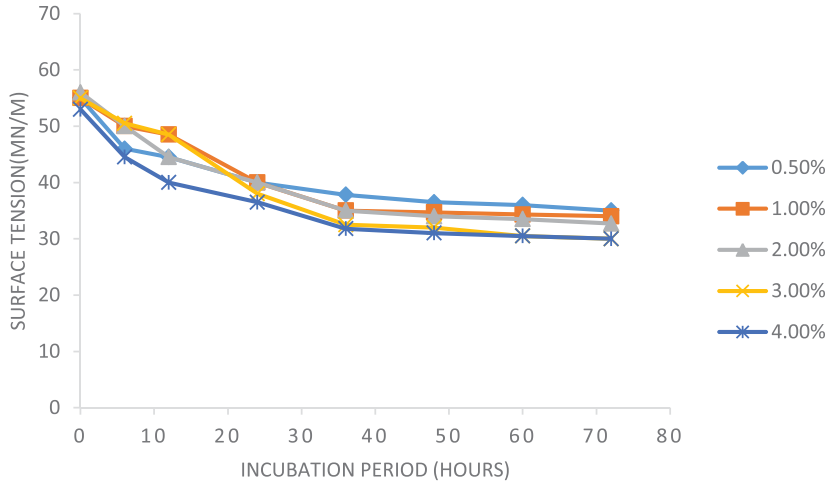

Fig. 2 Effect of glucose concentration on biosurfactant potential of P. aeruginosa strain GW-7. The culture were grown on 0.5-4% glucose in phosphate deficient medium in 250 ml Erlenmeyer flask at shaker (100 rpm) at 300C. The cell free culture broth was checked after every 24 hours for reduction in surface tension by KRUSS Tensiometer K10T using ring method.

reduction after 36 hours at 3% glucose. However the production of biosurfactant was dependent on type and quantity of nitrogen and phosphate.

Similar results have also been reported by Syldatk et al. [25] with MMFB, though growth behavior (O.D & dry biomass) was higher than BHM. But the surface tension (32 mN/m), interfacial tension (2.1 mN/m) and emulsifying properties (E 24=72) are comparable with the BHM, (32, 2.1 & 73, respectively). Whereas concentration of biosurfactant was 3 fold lower than the BHM. It may be due to higher concentration (8.0 gm/l) of phosphate in MMFB than (1.0 gm/L) in BHM. Sufficient supply of phosphate for this purpose was found inhibitory, as synthesis of surfactants under phosphate and nitrogen limitation is increased [12, 25].

Molligam et al. [12] reported emulsification ability of culture filtrate of P. aeruginosa ATCC 9027, as non-significant, when mixed with kerosene and water. Moreover, they reported quite lower IFT (1.3 mN/m against n-hexadecane as compare to 5.5 mN/m) in this study. Another event support this fact, that with the increase of glucose concentration in PPGAS medium. S.T, IFT decreased to 30 mN/m, 1.3 mN/m & concentration of biosurfactant

increased (CMC-1 = 18).

Increasing incubation period, in case of BHM, concentration of biosurfactant increased (CMC-1 11-22). It may be due to slow transformation of hexadecane into lipids in late stationary phase, under nitrogen and phosphate limiting conditions. Higher production of biosurfactant by P. aeruginosa grown under nutrient limiting conditions have already been reported [25]. Bazire et al. [13] reported that P. aeruginosa exhibited enhanced production of rhamnolipid biosurfactant under osmotic stress and phosphate limitation. A high yield of 4.261 g rhamnolipid/l production by P. aeruginosa ATCC 9027 under phosphate and nitrogen limitations is also reported by Clarke et al. [14].

3.8 Effect of Glucose Concentration on Growth and Biosurfactant Potential of *P. aeruginosa* Isolate GW-7

Production of biosurfactant and carbon source utilization (glucose 0.5-4% w/v) was also studied. Biosurfactant production was maximum on 3 & 4% glucose but carbon source utilization was only 52 and 57%. The growth was highest at 4% glucose (Fig. 1). The S. T (30 mN/m) (Fig. 2), IFT (1.5 mN/m), CMC^{-1} = (18), E24 = (76) and biosurfactant yield (0.4 g/L) were same on 3 & 4% glucose. Therefore 3% glucose was selected for further studies. Production of glycolipid increased gradually with time and reached at maximum at 96 hours (1.18 g/L). It is confirmed from results of paper chromatography that sugar moiety in rhamnolipid is rhamnose. The result of GC confirmed that the fatty acids of rhamnolipid are hexadecanoic acid. Rhamnolipids from Pseudomonas aeruginosa are commercialized by Jeneil Biosurfactant, USA, primarily as a fungicide for agricultural or an additive to stimulate bioremediation potential [3]. Recently, Eleftheria et al. [9] reported the production of rhamnolipid by *Pseudomonas* species.

4. CONCLUSIONS

The *P. aeruginosa* strain GW isolated from oil enriched soils from Gwadar coastal area produced potent biosurfactant in large amount in reasonably short period of time (36 hours) under nitrogen and phosphate limiting conditions. It is recommended that the study may be conducted on pilot scale in fermenter under these optimized conditions in order to utilize the process on industrial scale.

5. ACKNOWLEDGEMENTS

The author is thankful to the administration of Oil & Gas Development Corporation for providing the samples from oil wells.

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