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Production of bioemulsifier by *Acinetobacter* species isolated from healthy human skin

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Six *Acinetobacter* sp. isolated from healthy human skin were checked for the production of bioemulsifier. Optimization studies indicated that Luria Bertani broth *p*H 7 supplemented with calcium chloride (1%) was the optimum medium. Temperature at 37°C was optimum and inducer oils in the medium did not enhance bioemulsifier production. Partial purification of bioemulsifier and chemical analysis revealed that it is a proteoglycan with protein (53%), polysaccharide (43%) and lipid (2%). Maximum emulsification activity obtained was 400 EU/ml. Thin layer chromatography revealed the presence of mannose and rhamnose sugar and oleic and palmitic acids as parts of lipids. The yield obtained was 1.9 g / l. Reconstitution studies revealed that the protein and polysaccharide fractions together display 94.55% of emulsification activity. It was also noted that the bioemulsifier was stable for 72 hr at 37°C and displayed good cleaning property towards different oils. The partially purified bioemulsifier formed stable oil-in-water emulsions with plant oils.

Keywords: *Acinetobacter*, Bioemulsifier, Cleaning property, Emulsion, Human skin, Proteoglycan

Human skin is an organ of protection and an important component of first line of body defense. Though, many Gram-positive bacteria inhabit the skin, *Acinetobacter* is the only Gram-negative bacteria present on skin as a normal flora[1]. The fatty acids on skin are taken by microorganisms as a source of nutrients. Biosurfactants and bioemulsifiers are a structurally diverse group of surface active molecules synthesized by microbes. Microorganisms produce bioemulsifier / biosurfactant (BE/BS) for survival on hydrophobic substrates[2] and desorption from the hydrophobic substrate to increase the bioavailability of insoluble substrates[3]. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures which make them potential candidates for enhancing oil recovery[4]. Bioemulsifiers have gained increasing interest in recent years because of their lower toxicity, higher biodegradability, better environmental compatibility, high selectivity and specific activity at extreme temperatures, *p*H and salinity[5]. There are many reports on production of bioemulsifier by *Acinetobacter* strains from soil, marine water mud etc[6]. Bioemulsifier production by *Acinetobacter* strains from human skin has been reported earlier[7] and a patent has been filed[8]. Similarly, due to the interest on BS/BE production by different microbes, about 225 patents on BS/BE produced by diverse group of microbes are available[9]. BS/BE produced by varied microorganisms shows immense structural and functional diversity and consequently signifies the involvement of particular molecular machinery in their biosynthesis. Updated information on this topic has been documented[10]. The present study describes bioemulsifier production by *Acinetobacter* strains from human skin not exposed to modern antibiotics.

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Materials and Methods

Bacterial strains used — *Acinetobacter* strains (118) isolated from healthy human skin were used for the present study[11]. Out of these, six cultures (*A. haemolyticus* TA77, *A. haemolyticus* TA52, *A. haemolyticus* TA106, *A.* ungrouped TA30, *A. lwoffii* TA38 and *A.haemolyticus* TA74) showing lipase activity were further screened for bioemulsifier production. The strain which displayed maximum bioemulsification activity was used for further experiments. Control strains used were *Bacillus subtilis* MTCC 1427, *Bacillus subtilis* MTCC 2422 and *Pseudomonas aeruginosa* MTCC 2297 (IMTech, Chandigarh).

Bioemulsifier production — Luria Bertani broth (LB) (Tryptone-10 g, Yeast Extract-5g, Sodium chloride-10 g, Distilled water- 1000 ml, *p*H 7.2). Bioemulsifier production was carried out in 50 ml of LB in a 250 ml Erlenmeyer flask at 37°C at 150 rpm. 1% (v/v) inoculum was used. Emulsification assay was carried out after every 24 hr up to 72 hr using standard procedure[7].

Oils used as substrates — Almond oil, castor oil, olive oil, soybean oil and ground nut oil were used as substrates for emulsification. All oils used were of analytical grade and locally available.

Emulsification assay — Cell-free culture broth (3 ml) was mixed with 0.5 ml test oil, vortexed vigorously for 2 min and incubated at 37°C for 1 hr. Absorbance of the aqueous phase was then recorded at 400 nm using spectrophotometer (UV-1601 Shimadzu Corporation, Japan). Blank was prepared similarly with sterile production medium. An absorbance of 0.010 units at 400 nm multiplied by dilution factor, if any, was considered as one unit of emulsification activity per ml (EU/ml)[7].

Effect of physico-chemical factors on bioemulsifier production — Effect of temperature (20,28,37,40, 45°C) on bioemulsifier was studied by growing the test strain at the respective temperature in a shaking water bath at 150 rpm and then checking the culture supernatant for oil emulsification as per the method described above. Effect of *p*H (5,6,7,8,9) was also studied same way. The test strain was grown in LB supplemented with 1% and 2% calcium chloride and magnesium sulphate separately and effect of salts on bioemulsifier production was checked.

Effect of inducer oil on bioemulsifier production — *Acinetobacter* strain was grown in LB supplemented with 1% of test oils separately. The cell-free supernatants were assayed for emulsification of each test oil using emulsification assay.

Partial Purification of bioemulsifier — *A. lwoffii* TA38 which gave maximum emulsification activity in the initial screening experiment was used for purification. The cell-free supernatant obtained after 48 hr was centrifuged at 10,000 g for 15 min. It was then mixed with three volumes of chilled acetone and incubated at 4°C for 15 hr. The brown precipitate was collected by centrifugation at 10,000 g for 30 min and dissolved in 7 ml of sterile distilled water (*p*H-7). This solution was then dialyzed (Seamless cellulose tubing, width 40 mm, diameter 25 mm, retaining most proteins of molecular weight 12,000 or greater, Sigma Aldrich Chemie, Gmbh, Steinheim, Germany) extensively against sterile distilled water at 10°C for 48 hr. Distilled water in dialysis container was replaced every 10 hr. The dialysate obtained was frozen at -20°C and lyophilized. This bioemulsifier preparation was stored in airtight glass vials at room temperature (30°C)[7].

Chemical analysis of bioemulsifier — Protein content was analyzed using bovine serum albumin as standard[12]. Carbohydrates and lipids were also quantified[13,14]. Thin layer chromatography technique was used to know the type of sugar and lipid content of the bioemulsifier.

Detection of sugar — Sugar content was analyzed with butanol; ethanol: water (5:3:2) and developed by DPA reagent {Diphenyl amine reagent-Aniline: DPA: Acetone: Phosphoric acid (4:4:200:15)}.

Detection of lipid — The solvent system used for detection of lipid consisted of Benzene: Diethyl ether: Ethyl acetate: Acetic acid (80:10:10:1) and Hexane: Ethyl ether: formic acid (80:— 20:2). The spots were developed by iodine vapours.

Reconstitution of emulsification activity — On the basis of chemical composition of the partially purified emulsifier, protein and polysaccharide fractions were separated. Protein fraction of the purified bioemulsifier was prepared by hot phenol treatment. Extracellular protein was obtained by 60% ammonium sulphate precipitation of the cell-free culture broth. The polysaccharide fraction from the purified bioemulsifier was isolated by water extraction of phenol phase, while the capsular polysaccharide was obtained by acetone precipitation of homogenized culture supernatant[15]. These fractions were then checked for their ability to reconstitute the castor oil emulsification property, individually or in combination, according to the emulsification assay described above. It is important to note that *A. lwoffii* TA38 had capsule which was evident in the logarithmic phase but only small portion was visible at stationary phase. This could be due to the release of capsule into the medium.

Emulsion stability — Ability of the partially purified bioemulsifier to form stable oil-in–water emulsions was based on a modified version of the method by Cirigliano and Carman[16]. For this, a 2.5 ml aqueous solution of the purified emulsifier (0.02% w/v) was mixed with 0.4 ml of the test oil. The solution was allowed to stand for 10 min prior to measuring the turbidity of the bottom aqueous layer at

540 nm. The turbidity was measured up to 60 min after every 10 min. The log of these values was plotted against time. Slope of the curve generated was expressed as the decay constant K_d. This term describes the stability of the microemulsions formed[17]. Three systems were tried using partially purified bioemulsifier; almond oil/water/ bioemulsifier/butanol, olive oil/water/bioemulsifier/ butanol and castor oil/water /bioemulsifier/butanol. Decay plots were plotted using Log A_{540} Vs time and the slope of the line was considered as decay constant K_d which is a tool to know the stability of microemulsion. All values were derived as an average from triplicate experiments. Also, the vortexed mixture of test oil and emulsifier was incubated for 1 hr at 37°C. After incubation, absorbance was checked at 400 nm. Then one set was further incubated 37°C and other at 10°C.

Cleaning property of bioemulsifier — To check cleaning property of bioemulsifier, purified powder of bioemulsifier (10 µg) was dissolved in five ml of distilled water. About seven ml of glycerol, almond oil, groundnut oil, soybean oil and castor oil were added separately in clean glass test tubes. The tubes were then inverted and oils were removed in such a way that the portion of oil should stick to the walls of the tubes. 1 ml of bioemulsifier solution (10 µg/ml) was added drop by drop, very slowly in each tube by holding the tube in horizontal position. The inner surface of the tube was observed carefully for cleanliness[18].

Results

Bioemulsifier production by Acinetobacter from human skin — The time course of bioemulsifier is shown in Fig. 1. It was observed that maximum production occurred during mid stationary phase of growth. Out of 13 lipase producing *Acinetobacter* from human skin, six strains displaying good lipase activity were tested for bioemulsifier production. *A. lwoffii* TA 38 exhibited maximum bioemulsifier production *A. haemolyticus* TA 77 emulsified almond oil to the maximum extent whereas *A. lwoffii* TA 38 emulsified castor oil maximally. Fermentation was carried out up to 72 hr and maximum bioemulsifier production of 140 EU/ml was obtained in LB at *p*H 7 at 37°C at 150 rpm after 48 hr.

Effect of physicochemical factors on bioemulsifier production — In case of *A. lwoffii* TA 38, highest level of bioemulsifier was produced in LB supplemented with 1% $CaCl_2$ at *p*H 7.2 (Fig. 2) at 37°C (Fig. 3). Shaking at 150 rpm was favorable for bioemulsifier production. It was observed that at 50°C, more than 70% of bioemulsifier activity was inhibited. Addition of salts enhanced the emulsification activity (Table 1).

Substrate specificity of bioemulsifier — Among the six oils tested, castor oil was found to be emulsified the most by *Acinetobacter* ungrouped TA 30 and *A. lwoffii* TA 38 (Fig. 4). Soybean oil was least emulsified**.**

Effect of inducer oil on bioemulsifier production — It was observed that LB supplemented with different test oils as inducers did not show enhancement in the bioemulsifier production. However, salts were found to enhance bioemulsifier production in case of *A. lwoffii* TA 38. The results are presented in Table 2.

Partial purification and chemical analysis of bioemulsifier — The partially purified bioemulsifier from cell free supernatant of *A. lwoffii* TA 38 culture displayed 400 EU/ml. It was grown in presence of LB with 1% $CaCl_2$ at 37°C. The approximate yield of bioemulsifier was 1.9 g /l under the given set of conditions. Chemical analysis of this bioemulsifier revealed that protein (53 %) was the main constituent followed by polysaccharide (42 %). A minor fraction

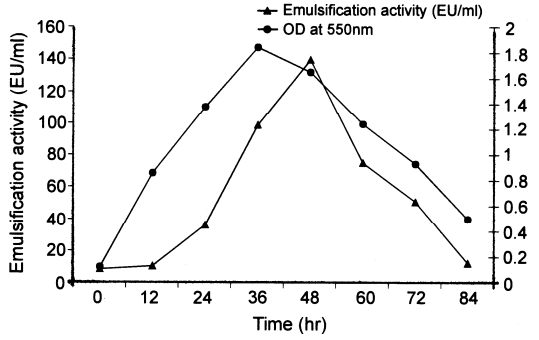

Fig. 1 — Time course of bioemulsifier production by *Acinetobacter lwoffii* TA38

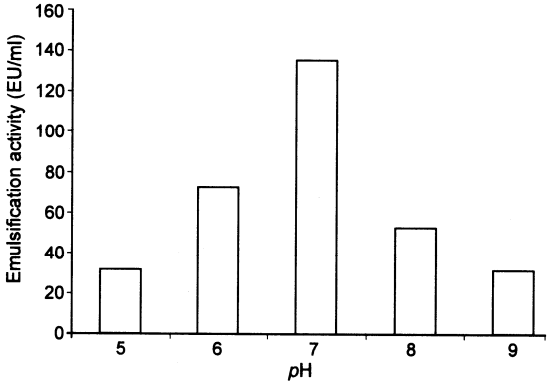

Fig. 2 — Effect of *p*H on bioemulsifier production by *Acinetobacter lwoffii* TA38

of lipid (2%) was also present in the bioemulsifier. Thin layer chromatography revealed the presence of rhamnose (R_f value-0.75) and mannose sugars (R_f value of 0.68) and palmitic and oleic acids (R_f value 0.7 and 0.65 respectively).

Reconstitution of bioemulsifier activity — Result of reconstitution study is shown in Table 3. Highest activity was observed when all the fractions were together. Protein fractions were more active as compared to the polysaccharide fractions.

Cleaning property of bioemulsifier — After the addition of purified bioemulsifier, the walls of the test tubes appeared clean. The partially purified bioemulsifier showed good cleaning property against different test such as almond oil, castor oil, soybean oil, ground nut oil and coconut oil. It suggests that the bioemulsifier could be used in oil recovery.

Stability of emulsions — It was found that the partially purified bioemulsifier could produce stable oil-in-water emulsions with vegetable oils such as almond oil, olive oil and castor oil. It is important property as microemulsions have many applications in different fields. The decay plots are shown in Fig. 5. The stability of emulsion was also confirmed by measuring the OD at 400 nm after 48 and 72 hr. The emulsions were stable after 72 hr at 37°C.

Discussion

In *Acinetobacter* species, production of extracellular bioemulsifiers is a widespread phenomenon[19]. About 16% of patents on bio-emulsifier production have been reported from *Acinetobacter* sp. alone[9]. Production of bioemulsifiers from *Acinetobacter* strains from human skin is already reported[7]. Due to the unique environment of human skin it is interesting to study the microorganisms on it and their products. However, after this study there is no report on the same topic. The present study aimed at bioemulsifier production by *Acinetobacter* strains from human skin of tribal population in India never exposed to modern antibiotics to compare with earlier report.

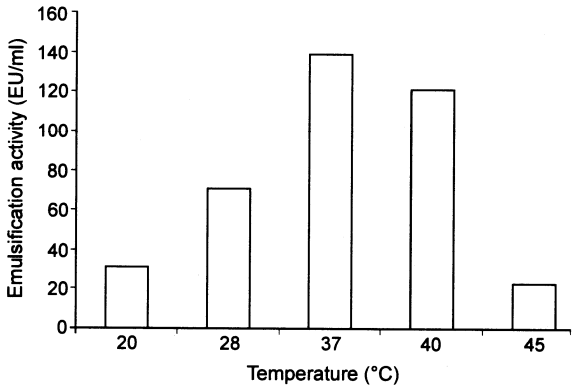

Fig. 3— Effect of temperature on bioemulsifier production by *Acinetobacter lwoffii* TA38

A. lwoffii TA 38 displayed maximum bioemulsifier production after 48 hr which is mid stationary growth phase of the organism. This observation is in accordance with the bioemulsifier from *A. junii* SC 14 from human skin[7] and emulsan production by *Arthrobacter* RAG-1[20]. Bioemulsifier production like any other chemical reaction is affected by a number of factors that either increase its productivity or inhibit it. Accordingly, an environmental factor like *p*H, salinity and temperature affect bioemulsifier production[21,22]. Optimum temperature for bioemulsifier production was 37°C which can be attributed to the habitat of the organism. This observation is also in agreement with the report on bioemulsifier production by *A. junii* (a human skin isolate) which displayed temperature optima as 37°C[7]. Maximum production of bioemulsifier was observed at *p*H 7 (135.1 EU/ml) though less activity was observed at *p*H 6, 8 and 9. This observation is contrary to that of alasan produced by *A. radioresistens* KA53 which is reported to show emulsification activity over a wide *p*H range of 3.3-9.2[23]. Similarly, maximum rhamnolipid production in *Pseudomonas* sp. is shown to occur at a *p*H range from 6 to 6-5 and decreases sharply above *p*H 7[24]. Biosurfactant production from *Pseudomonas* strains MEOR 171 and MEOR 172 is reported to be ineffective to temperature, *p*H and Ca, Mg

Table 1 — Effect of salts on bioemulsifier production by *Acinetobacter lwoffii* TA 38

Salt	Test oil	(EU/ml)*
	Almond oil	149
$CaCl_2$ (1%)	Castor oil	213.3
	Coconut oil	122.1
	Almond oil	152
$CaCl_2$ (2%)	Castor oil	150.5
	Coconut oil	107
	Almond oil	123
$MgSO_4$ (1%)	Castor oil	192.7
	Coconut oil	201.6
	Almond oil	153
$MgSO_4$ (2%)	Castor oil	205.7
	Coconut oil	153.3

*-An absorbance of 0.010 units at 400 nm multiplied by dilution factor, if any, was considered as one unit of emulsification activity per ml (EU/ml)

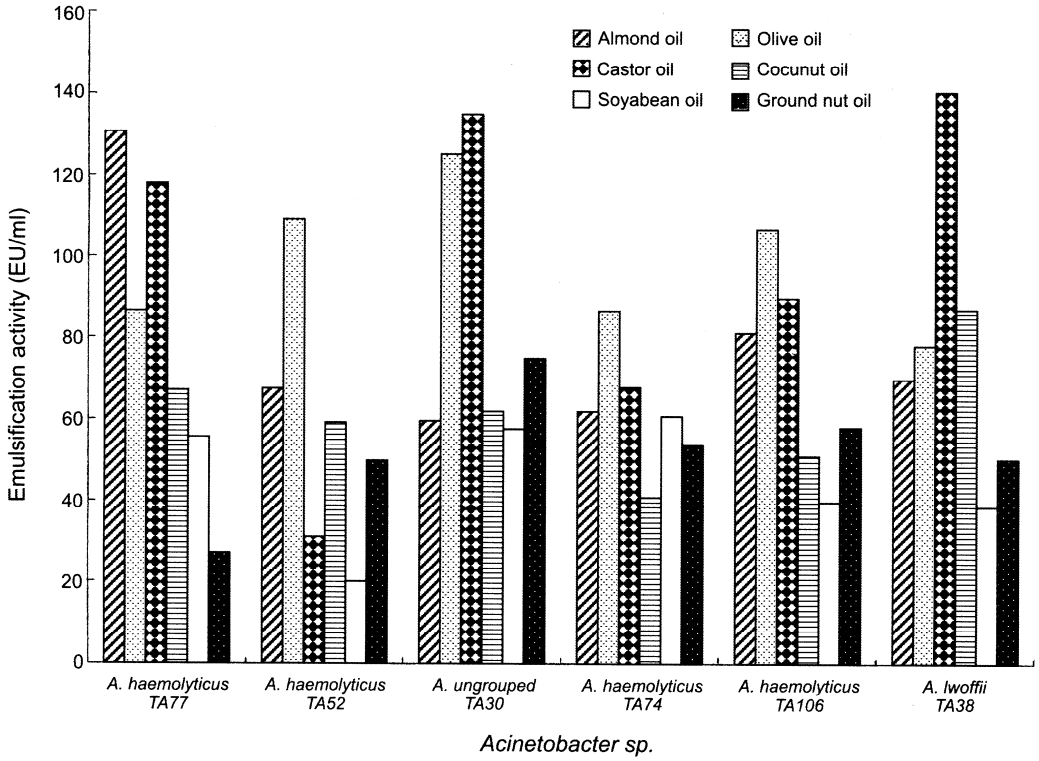

Fig. 4 — Emulsification of test oils by different *Acinetobacter* strains

Table 2 — Effect of inducer oils on bioemulsifier production by *Acinetobacter lwoffii* TA 38

Inducer Oil (1%)		Emulsification of (EU/ml)* Almond oil	Castor oil	Coconut oil	Soybean oil
Almond oil	T	69.7	79.2	57.2	45.1
	C	242.4	297.8	214.9	288
Castor oil	T	107.7	103.2	110.3	96.1
	C	231.6	249.2	262	215.9
Coconut oil	T	79.6	54.4	79.6	88.2
	C	228.4	253.6	217.9	210
Soybean oil	T	90.2	108	85.8	88.5
	C	245	234.3	244	218.4

*-An absorbance of 0.010 units at 400 nm multiplied by dilution factor, if any, was considered as one unit of emulsification activity per ml (EU/ml)

T- Test (*Acinetobacter lwoffii* TA 38) ; C- Control (*Bacillus subtilis* 1427)

Table 3—Reconstitution of emulsification activity by isolated fractions of the bioemulsifier produced by *Acinetobacter lwoffii* TA 38

Fraction	% activity	EU/ ml
Purified emulsifier	100	400
BEP*	70	280
EP[a]	68.37	273.5
BEPS[b]	63.8	255.2
EPS[c]	65.32	261.3
BEP +BEPS	73.85	295.4
BEP + EP	69.72	278.9
BEP + EPS	72.17	288.7
BEPS + EPS	63.45	253.8
BEPS + BEP + EP + EPS	94.55	389.2

BEP : Bioemulsifier protein; EP: Extracellular protein; BEPS : Bioemulsifier polysaccharide; EPS : Exopolysaccharide; *: Protein fraction of the purified bioemulsifier prepared by the hot phenol method ; [a] Extracellular protein obtained by 60% ammonium sulphate precipitation ; [b] Polysaccharide fraction from purified bioemulsifier isolated by water extraction of phenol ; [c] Capsular polysaccharide obtained by acetone precipitation

concentrations in the ranges found in many oil reservoirs[18]. It was observed that, addition of salts in the production medium enhanced the emulsification activity which is in agreement with alas an produced by *A. radioresistens* KA53[23]. Alasan production is stimulated by the addition of magnesium ions below and above optimum *p*H[23]. Similarly, biosurfactant produced by *Bacillus licheniformis* JF-2 is reported to show enhanced emulsifying activity in presence of calcium at a concentration of 25g/l[25]. On the contrary, bioemulsifier production by *Acinetobacter junii* SC 14 is not reported to be affected by the presence of salts[7].

The bioemulsifier produced in this study was found to be proteoglycan similar to that of *A. junii* SC14 reported earlier[7]. This observation is also in agreement with the bioemulsifier from *Yarrowia*

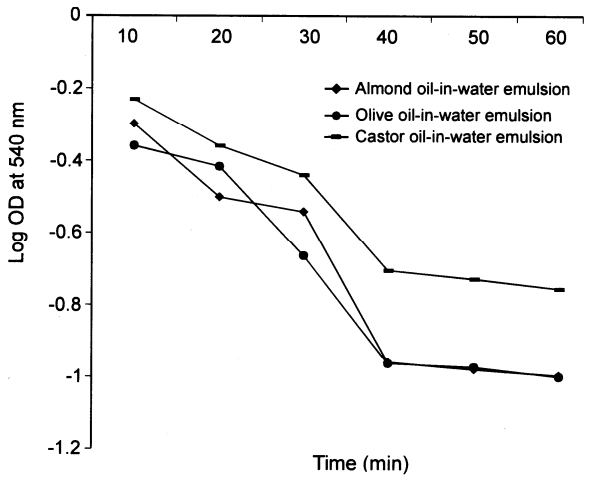

Fig. 5 — Decay plots of oil-in-water emulsions produced by partially purified bioemulsifier

lipolytica which is reported to consist of protein (47%), carbohydrate (45%) and lipids (5%)[26]. Similarly, bioemulsifier from *Penicillium* sp. is composed of lipids (67%), carbohydrates (11%) and protein (7%)[27]. Bioemulsifier from *Bacillus stearothermophilus* also found to contain protein (46%), carbohydrates (16%) and lipids (10%)[28]. A lipopolysaccharide bioemulsifier has also been reported by *Acinetobacter*[29]. Yield of bioemulsifier from *A. lwoffii* TA 38 was approximately 1.9 g/l which is less as compared to that produced by *A. junii* SC 14 reported to have a yield of 3.9 g/l[7], alasan which has a yield of 4.6 g/l[23] and a biosurfactant from *Pseudomonas fluorescens* having a yield of 2 g/l[30].

Reconstitution study revealed that all fractions together displayed maximum emulsification activity. It was also noted that proteins were more active. It has been demonstrated that protein content of the bioemulsifiers plays an important role in the emulsification activity[31]. This observation is in accordance with that of emulsan produced by *Acinetobacter venetianus* RAG-1. Emulsan, a complex of an acylated amino polysaccharide and proteins yielded a product named apoemulsan which had proteins. Apoemulsan retains more than 50% of the emulsifying activity[32,33]. During late logarithmic growth phase of *A.lwoffii* TA38, capsule was released into the medium contributing to the bioemulsification activity. This report is in accordance with *A. calcoaceticus* BD4, which produces a capsule which forms a complex with the proteins to give high emulsification activity after its release into the medium[34].

It is well established that large amount of two immiscible liquids (e.g. water and oil) can be brought into a single phase (macroscopically homogeneous but microscopically heterogeneous) by addition of surfactant or a surfactant mixture. This unique class of optically clear, thermodynamically stable and usually low viscous solutions are microemulsions. They can be prepared by controlled addition of lower alkanols like butanol, pentanol and hexanol to milky emulsions to give transparent solutions of either water-in-oil (w/o) or oil-in-water (o/w) type. The partially purified bioemulsifier formed stable oil-in water emulsions with vegetable oils. The K_d values for almond, olive and castor oil –in-water emulsions were -0.020, -0.00056 and -0.013 respectively. These values are higher than the reported K_d values of the cell free culture broth of *Pseudomonas aeruginosa* EBN-8 grown on different waste frying oils (WFO). Maximum stability has been shown by soybean WFO against n-hexadecane or paraffin oil[35]. Higher the K_d values, more stable are the emulsions formed. Liposan is also reported to stabilize a number of vegetable oils[17]. A glycoprotein bioemulsifier produced by marine *Antarctobacter* also exhibits the property of forming stable oil-in-water emulsions with different food oils[36]. Similarly, Yansan from *Yarrowia lipolytica* is also reported to be capable of stabilizing oil-in –water emulsions with several aliphatic and aromatic hydrocarbons[37]. The bioemulsifier from *Acinetobacter lwoffii* TA38 showed good cleaning property against various oils and also formed stable emulsions with plant oils which suggest its potential application. The present study demonstrated possible role of *Acinetobacter* strains from human skin in bioemulsifier production.

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