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Biosurfactant production by bacteria retrieved from hydrocarbon polluted environment

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Abstract
Bacterial isolates retrieved from stagnant water and hydrocarbon polluted soil using enrichment culture technique were studied for biosurfactant production. Sixteen isolates, showing blood agar haemolysis, were screened for biosurfactant production. Highest oil displacement was generated by isolate BK68 (0.0623 m) followed by 0.0248m, 0.0223m and 0.0206m by isolates BK23, BK34 and BK48, respectively. Highest E_{24} index was given by isolate BK66 (19.5%) followed by BK68, BK58 and BK23 (19.0%, each) after 4 days of incubation. Surface tension reduction equivalent to 0.071 Nm^{-1}, was shown by isolate BK68, followed by BK23 (0.037 Nm^{-1}) and BK66 (0.036 Nm^{-1}). Promising biosurfactant producers were subjected to identification to genus level. Gram positive and endospore forming isolates BK23, BK58 and BK68 were probably *Bacillus* spp, while catalase positive coccus bacteria BK66 could be classified as *Staphylococcus*. Gram negative, oxidase positive and non-glucose fermenting rods of isolates BK34 and BK48 were probably belong to genus *Pseudomonas*.

Keywords: Enrichment culture technique, Haemolysis, Emulsification index, Surface tension reduction, Oil displacement, Oil atomization

Introduction
Soil and water environments are frequently contaminated with oil hydrocarbons (OHC), polyaromatic hydrocarbons (PAH) and other hydrophobic substrates which often lead to severe environmental consequences. Microbial consortia display a wide array of metabolic mechanisms including production of emulsifiers and biosurfactants as one fairly effective strategy, for bioremediation of such pollutants (Parthipan *et al*. 2017) [15]. Biosurfactants are surface active metabolites containing hydrophobic and hydrophilic moieties that reduce surface and liquid-liquid or solid-liquid interfacial tensions (Shahaby *et al*. 2015) [20] and enhance solublization of hydrocarbons into water which eventually leads to better degradation of these pollutants. Besides, these surface active compounds are also having applications in enhanced oil recovery, food processing, pharmaceuticals *etc.* that can be exploited commercially (Santos *et al*. 2016) [18].

Many biosurfactant producing microorganisms, particularly bacteria and yeasts, are reported in literature such as *Pseudomonas* spp., *Bacillus* spp., *Lactococcus lactis*, *Lactobacillus* strains, *Streptococcus thermophilus*, *Nocardioides* spp., *Aeromonas* spp., *Serratia* spp., *Rhodococcus* strains and *Candida ingens* (Chen *et al*. 2007; Khopade *et al*. 2011; Rodrigues *et al* 2006; Sharma *et al*. 2019) [9, 13, 10, 21]. These are mainly abundant in soil or water samples contaminated with hydrophobic organic compounds like refinery wastes (Batista *et al*. 2006) [3], hydrocarbon polluted areas (Bento *et al*. 2004) [4] and marine environments (Antoniou *et al*. 2015) [1].

Currently, very few biosurfactants are commercially available e.g., surfactin, sophorolipids and rhamnolipids and the enormous market demands for surfactants are generally met by numerous synthetic mainly petroleum based, chemical surfactants which are usually non-degradable and toxic to the environment (Banat *et al*. 2000). Moreover, biosurfactants are more effective and versatile than many synthetic surfactants owing to their selective action, biodegradable nature and stability at high temperature, pH and salinity. The development of this line of research is of paramount importance, mainly in view of the present concern with protection of the environment.

Keeping in view, the commercial application of biosurfactants continuous efforts are required for unearthing superior biosurfactant producing microbial strains. The present investigation, therefore, was undertaken with the objectives of isolation, screening and identification of biosurfactant producing bacteria form hydrocarbon contaminated environment.

Materials and Methods

Collection of Water and Soil Samples

Water sample was collected in form of water surface microlayer (W_{SML}) by the glass plate method from multiple locations of a pond having stagnant water. A plexiglass plate was disinfected with 70% ethanol and immersed slowly in an upright position for 1 minute at water sampling site. The plate was then removed gently in the same position and allowed to drip for 30 seconds. The water adhering to both surfaces was wiped-off into a sterilized glass petri plate (0.15 m) by forcing the plate between two teflon wiper blades before collecting in a sterilized reagent bottle. In addition to this, total 5 soil samples were also collected in sterile plastic zipper bags from hydrocarbon contaminated sites of petrol pumps and automarket. All the samples were stored at room temperature until intended use.

Isolation of Biosurfactant Producing Bacteria

The water and soil samples were inoculated at the rate of 1% (v/v)/(w/v) for enrichment in mineral salt (MS) broths prepared by mixing, one liter of solution 'A' containing per liter, 2.5 g $NaNO_3$, 0.4 g $MgSO_4.7H_2O$, 1.0 g NaCl, 1.0 g KCl, 0.05 g $CaCl_2.2H_2O$ and 10.0 ml of 85% H_3PO_4 with 1.0 ml of solution 'B' containing per liter, 0. 5 g $FeSO_4.7H_2O$, 1.5 g $ZnSO_4.7H_2O$, 1.5 g $MnSO_4.2H_2O$, 0.15 g $CuSO_4.5H_2O$ and 0.1 g $NaMnO_4.2H_2O$ (pH-7.2, adjusted with KOH pellets). The MS broth was also supplemented with 1% (v/v) of either liquid paraffin, crude oil or commercial diesel in separate treatments before incubating at 120-130 rpm for one week. Sub-enrichment was repeated minimum three times by transferring 10 ml of enriched sample into fresh 90 ml MS broth of similar composition. Alternatively, samples without any enrichment were also used for isolation of bacteria. Isolation was done by streaking enriched broth on nutrient agar (NA) plates. Isolates were picked up after 24-48 h of incubation either depending upon difference in colony morphology or on the basis of appearance on different NA plates inoculated with variably enriched samples, purified by repeated streaking for 3-4 times and maintained on NA slants at 4ºC for further use.

Screening of Bacterial Isolates for Biosurfactant Production

All the isolates were first screened on the basis of blood agar haemolysis (Plaza *et al*. 2006) [16]. Bacterial isolates giving positive haemolytic test were screened on the basis of oil displacement technique (Kaur *et al*. 2017) [12] with a modification. The method was modified in this study, by using mobile oil to overlayer the water instead of almost colourless diesel or petrol so as to clearly visualize the oil displacement in a blackish red background. Other srcreening methods used for selection of biosurfactant producers were emulsification (E_{24}) index (Sarubbo 2006) [19], surface tension reduction (Suganya 2013) [22] and atomized oil assay (Burch *et al*. 2011) [7]. For all these methods, 24 h grown bacterial slant was transferred to 100 ml sterilized nutrient broth and incubated at 120-130 rpm. Ten milliliter of 15-16 h grown culture (of almost equal A_{540} nm) was transferred to 50 ml MS broth supplemented with 2% (w/v) glucose as carbon source and incubated at 160 rpm for 6 days. One flask of each culture was withdrawn after every 24 h and centrifuged at 6000 rpm for 15 minutes. The cell free supernatants were used in various screening methods for biosurfactant production and the results were compared with 0.2% SDS as positive control and distilled water as negative control. All the experiments were performed at 30±2ºC unless it is specified.

Identification of Promising Isolates

Promising isolates were identified upto genus level on the basis of morphological, cultural and biochemical characteristics (Bergey 1989) [6]. Cultural characteristics were studied by inoculating bacterial isolates on NA plates to record form, margin and elevation of colonies and single line streak on NA slant to observe their growth, pigmentation, opacity and form, for 24 h. Alternatively, nutrient broth was inoculated with selected isolates for observation of type and amount of growth under stationary condition. Morphological and biochemical characteristics were studied by negative, spore and Gram staining techniques as per standard methods. Catalase test was performed by adding 20 µL of 3% H_2O_2 to 24 h grown, broth of bacterial isolates taken on a clean slide where emission of effervescences was recorded as positive catalase test. Oxidase test was performed by adding few drops of 1% freshly prepared Wruster's reagent containing 1.0 g of N,N,N',N'-tetramethyl-p-phenylene diamine dihydrochloride in distilled water into 24 h old bacterial broth. Alternatively, 24 h grown bacterial isolates were streaked over the oxidase discs (Hi-media). Immediate appearance of deep blue to purple Colour was recorded as positive oxidase enzyme activity. Glucose fermentation was studied by inoculating 24 h old bacterial isolates into glucose fermentation broth of pH 6.8-7.0 containing per liter, 5.0 g peptone, 3.0 g beef extract, 10.0 g glucose and 1.0 ml of phenol red as indicator at 120 rpm for 24-48 h. Change in Colour of the broth to pale yellow was recorded as a positive test.

Results

Isolation of Biosurfactant Producing Bacteria

Spontaneous release and function of biosurfactants are often related to hydrocarbon uptake; therefore, hydrocarbon contaminated sites and other polluted areas are considered as most promising locations for isolation of these organisms (Bento *et al*. 2005) [4]. Total 78 bacterial isolates, including 16 from water surface microlayer (W_{SML}) of a polluted stagnant pond and 62 from hydrocarbon contaminated soil samples were retrieved. Total 63 isolates were selected from enriched treatments having crude oil, liquid paraffin and diesel as carbon sources while 15 isolates were from non-enriched soil samples in 'as-is' condition (Table 1).

Table 1: List of samples and their sampling sites

Samples and Location details	Enrichment details	No. of bacterial isolates	Total no. of isolates
Water Surface Microlayer (W_{SML}): Pond, CCS HAU, Hisar (Latitude 29º 08',41.2"N and longitude 27º 42',25.8"E)	W_{SML} + Crude oil	8 (BK1, BK2, BK3, BK4, BK5, BK6, BK7 and BK48)	16
	W_{SML} + Liquid paraffin	6 (BK8, BK9, BK10, BK11, BK12 and BK49)	
	W_{SML} + Diesel	2 (BK13 and BK14)	
Soil 1:Petrol pump, CCS HAU, Hisar (Latitude 29º 9',10.01"N and longitude 75º 41',51.64"E)	Soil 1 + Crude oil	5 (BK15, BK16, BK17, BK18 and BK19)	22
	Soil 1 + Liquid paraffin	5 (BK20, BK21, BK22, BK23 and BK50)	
	Soil 1 + Diesel	12 (BK24, BK25, BK26, BK27, BK28, BK29, BK30, BK31, BK32, BK33, BK34 and BK51)	
Soil 2: Petrol pump, Hisar (Latitude 29º 8',57.19"N and longitude 75º 43',18.19"E)	Soil 2 + Crude oil	3 (BK35, BK36, BK37)	8
	Soil 2+ Liquid paraffin	4 (BK38, BK39, BK40 and BK52)	
	Soil 2+ Diesel	1 (BK41)	
Soil 3: Automarket, Hisar: Location 1 (Latitude 29º 10',1.13"N and longitude 75º 43',37.32"E)	Soil 3-'As is'	5 (BK42, BK43, BK53, BK54 and BK55)	11
	Soil 3 + Crude oil	1 (BK56)	
	Soil 3 + Liquid paraffin	3 (BK57, BK58 and BK59)	
	Soil 3+ Diesel	2 (BK60 and BK61)	
Soil 4: Automarket, Hisar: Location 2 (Latitude 29º 10',7.92"N and longitude 75º 43',30.13"E)	Soil 4-'As is'	4 (BK62, BK63, BK64 and BK65)	11
	Soil 4 + Crude oil	3 (BK66, BK67 and BK68)	
	Soil 4 + Liquid paraffin	1 (BK69)	
	Soil 4 + Diesel	3 (BK70, BK71 and BK72)	
Soil 5: Automarket, Hisar: Location 3 (Latitude 29º 9',55.98"N and longitude 75º 43',39.25"E)	Soil 5-'As is'	6 (BK44, BK45, BK46, BK47, BK73 and BK74)	10
	Soil 5+ Crude oil	1 (BK75)	
	Soil 5+ Liquid paraffin	1 (BK76)	
	Soil 5 + Diesel	2 (BK77 and BK78)	
Total No. of bacterial isolates			78

Screening of Bacterial Isolates for Biosurfactant Production

Several protocols have been designed and listed in literature to demarcate biosurfactant producers in a general microbial population. Blood agar hemolysis is considered as simpler and preliminary method to testify biosurfactant production ability (Thavasi *et al*. 2011a) [24]. Total 16 isolates have shown blood agar hemolysis after 24-48 h of incubation (Fig. 1A). Five out of total 16 isolates were obtained from water surface microlayer while rest 11 bacterial isolates were retrieved originally from enriched soil samples. Since haemolytic activity could also be shown by several other metabolites; therefore, this method has to be supported by other available screening methods. Therefore, these 16 isolates were subjected to further screening using oil displacement technique, E_{24} index, surface tension reduction and atomized oil assay for stronger confirmation of biosurfactant production. Use of mobile oil instead of diesel during oil displacement technique clearly visualized oil displacement, in a coloured background (Fig. 1B). Bacterial isolates BK68, BK23, BK34 and BK48 were found to be giving significant displacement of oil equivalent to 0.0543, 0.0248, 0.0223 and 0.0206 m after 4 days of incubation. Isolate BK68 gave highest oil displacement, 0.0623 m after 5 days of incubation which was found comparable to positive control, 0.2% SDS (0.0660 m) as detailed in table 2.

Table 2: Screening of bacteria for biosurfactant production monitored using oil displacement and emulsification index

Bacterial isolates	Oil displacement (cm)						Emulsification (E_{24}) index (%)					
	Incubation period (days)						Incubation period (days)					
	1	2	3	4	5	6	1	2	3	4	5	6
BK3	0.50	0.83	1.03	1.13	1.16	1.16	5.0	5.0	7.5	10.0	12.5	12.5
BK5	0.91	1.03	1.15	1.25	1.25	1.24	10.0	12.5	14.0	17.0	17.5	17.5
BK13	0.73	1.20	1.18	1.26	1.26	1.25	12.5	12.5	12.5	12.5	15.0	14.0
BK16	0.46	0.81	0.91	1.23	1.22	1.22	5.0	5.0	7.5	9.0	9.0	9.0
BK23	0.81	1.41	1.91	2.48	2.45	2.44	7.5	13.0	17.5	19.0	19.0	18.0
BK34	1.36	1.25	1.51	2.23	2.21	2.20	9.0	12.5	17.5	18.0	18.0	18.0
BK35	1.21	1.23	1.31	1.51	1.50	1.49	7.5	10.0	12.5	14.0	14.0	14.0
BK38	1.35	1.43	1.48	1.51	2.36	2.36	10.0	10.0	10.0	14.0	14.0	12.5
BK41	0.73	1.00	1.18	1.20	1.21	1.21	12.5	12.5	14.0	14.0	15.0	15.0
BK48	1.90	1.90	2.00	2.06	2.06	2.06	10.0	12.5	15.0	15.0	15.0	14.0
BK49	0.86	1.00	1.23	1.28	1.28	1.27	5.0	7.5	10.0	12.5	12.5	12.5
BK50	1.18	1.20	1.26	1.26	1.25	1.24	7.5	12.5	15.0	15.0	15.0	15.0
BK51	1.00	1.16	1.20	1.63	1.53	1.53	7.5	10.0	15.0	17.5	17.5	16.0
BK58	1.25	1.25	1.28	1.36	1.36	1.36	15.0	15.0	17.5	19.0	19.0	19.0
BK66	0.70	0.93	1.06	1.18	1.18	1.17	15.0	15.0	17.0	19.5	19.5	19.5
BK68	1.43	2.53	3.83	5.43	6.23	6.23	10.0	14.0	17.5	19.0	20.0	20.0
0.2% SDS	6.60						37.5					
Distilled water	0.0						0.0					

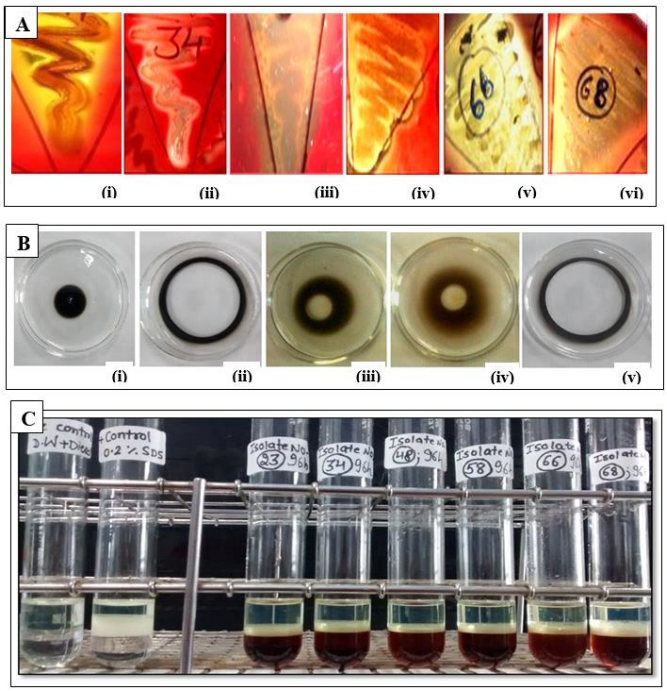

Fig 1: Bacterial isolates showing blood agar haemolysis (A): BK23 (i), BK34 (ii), BK48 (iii), BK58 (iv), BK66 (v) and BK68 (vi), oil displacement (B): Negative control (i), Positive control (ii), BK23 (iii), BK34 (iv) and BK68 (v), and emulsification index (C)

Isolate BK68 gave highest 20.0% E_{24} index on 5th day of incubation (Table 2 and Fig. 1B) followed by isolate BK66 (19.5%) and BK58, BK23 (19.0%, each) after 4 days of incubation. Oil atomization was studied using a fine spray of mineral oil which created light-diffractive halos around the equilibrated culture supernatant on LB agar plates. The radii of halos were measured from outer surface of culture supernatant up to halos formation. On 4th day of incubation isolate BK68 gave highest oil atomization up to 0.0043 m followed by isolates BK23 (0.0040 m), BK34 (0.0038 m) and BK66 (0.0035 m), however, the radii of halo further increased to 0.0050 m on 5th day of incubation in case of isolate BK68 (Fig. 2). Surface tension of a liquid reduces with increasing concentration of biosurfactant. Maximum reduction in surface tension, 0.071 Nm^{-1}, was observed in case of isolate BK68, followed by 0.037 and 0.036 Nm^{-1} by isolates BK23 and BK66, respectively as opposed to 0.091 Nm^{-1} shown by MS broth supplemented with 0.2% SDS as a positive control. In all the screening studies biosurfactant production was not increased beyond 5th day of incubation.

Isolates BK68, BK23 and BK34, clearly shines out as promising isolates by giving consistently encouraging results in all the screening methods. Isolates BK58 and BK66, however, did not gave reasonable performance in oil displacement test but have shown significant E_{24} index, surface tension reduction and oil atomization, therefore, these were also selected for further studies. Although, both the Isolates BK41 and BK48 have given comparable results in E_{24} index, surface tension measurement and oil atomization assay but isolate BK41 was found to be giving delayed result in comparison to BK48, in case of surface tension measurements and oil atomization assay. Additionally, BK48 indicated superior result in oil displacement (0.0206 m, after 4 days) against BK41, which was showing 0.0121 m oil displacement after an increased incubation of 24 h. Conclusively total six bacterial isolates *i.e.* BK23, BK34, BK48, BK58, BK66 and BK68 were considered as promising biosurfactant producers and were, therefore, selected for further studies. Production of biosurfactant by these promising bacteria was studied using an alternative carbon source, diesel. With diesel also, biosurfactant production measured in terms of E_{24} index, was found equivalent to 18.0% in case of culture supernatant of isolate BK68, after 5 days of incubation, followed by15.0% in isolates BK23 and BK34, 14.0% in isolates BK48 and BK58 and minimum 12.5% in isolate BK66.

Identification of Promising Isolates

All the six selected isolates were subjected to identification on the basis of determination of morphological, cultural and biochemical analysis. Colony appearances were found to be circular.

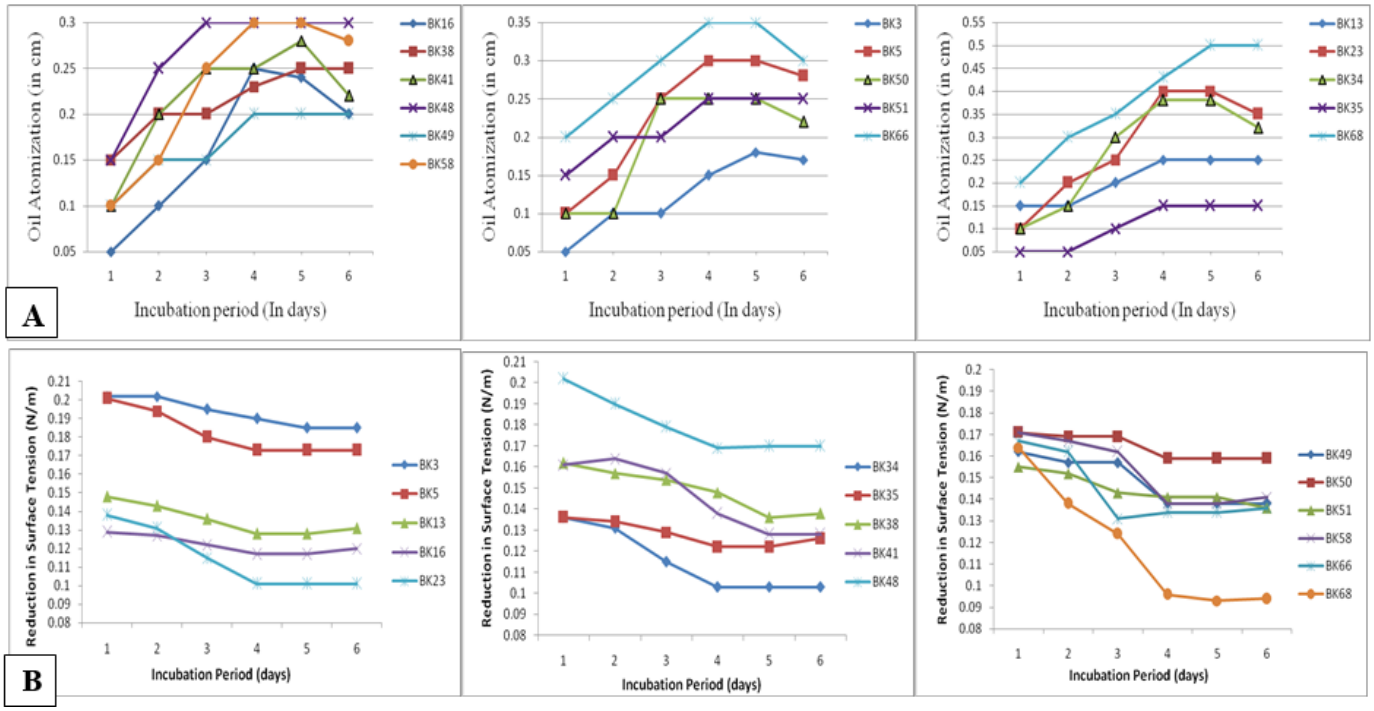

Fig 2: Screening of bacteria for biosurfactant production monitored in terms of oil atomization (A) and surface tension reduction (B)

except BK23 and BK68 which were having irregular colonies on NA plates. Margins appeared undulate in isolate BK68, serate in isolates BK23 and BK68 and entire in isolates BK34, BK48 and BK66. All the isolates were having flat elevations except BK66 which was having raised elevation. Growth of isolate BK34 was found scanty while all the other isolates were having moderate to abundant growth on NA slants. All the isolates were non-pigmented except BK66 which was having pink pigmentation. Single line streak on NA slants was found to be filiform except in case of isolates BK34 and BK58 which were having beaded and echinulate forms of growth, respectively. Growth of isolates in nutrient broth was found to be pellicle in isolates BK23, BK34 and BK48, sedimented in isolate BK58 and uniform type in isolate BK68 at 30+2ºC.

Morphologically all the selected isolates were found to be uniform rods except BK66 which was found to be a coccus, as observed by negative staining. Gram staining indicated isolates BK23, BK58, BK66 and BK68 as Gram positive while isolates BK34 and BK48 were found to be Gram negative (Fig.3A-F). Further, bacterial isolates BK23, BK58 and BK68 were observed as endospore formers while BK66 was found to be a non-spore former (Fig.3G-I). Conclusively, aerobic, Gram positive endospore forming bacterial isolates BK23, BK58 and BK68 were unspecified species belonging most probably to genus *Bacillus*.

Catalase test was examined to determine the presence of catalase activity. Emission of effervescences after addition of few drops of 3% H_2O_2 indicated BK66 as catalase positive. Since BK66 was a Gram positive coccus (in bunches), catalase positive, showing β-haemolysis and pink pigmentation, therefore, the isolate is expected to be of genus *Staphylococcus*.

To identify Gram negative bacterial isolates, oxidase test was conducted using Wruster's reagent. Isolate BK34 and BK48 gave deep blue colour within 5-10 seconds indicating positive reaction as compared with negative control (*Bacillus* sp.), which gave the deep blue colouration after 30 seconds. Alternatively, oxidase activity using discs further confirmed the results (Fig.3 J-K). Glucose fermentation broths were incubated at 30ºC for 48h. Moreover, both the isolates BK34 and BK48 gave a negative glucose fermentation test. In conclusion, Gram negative rods of bacterial isolates BK34 and BK48 giving negative oxidase and glucose fermentation test, may be identified as *Pseudomonas* spp. (Table 3).

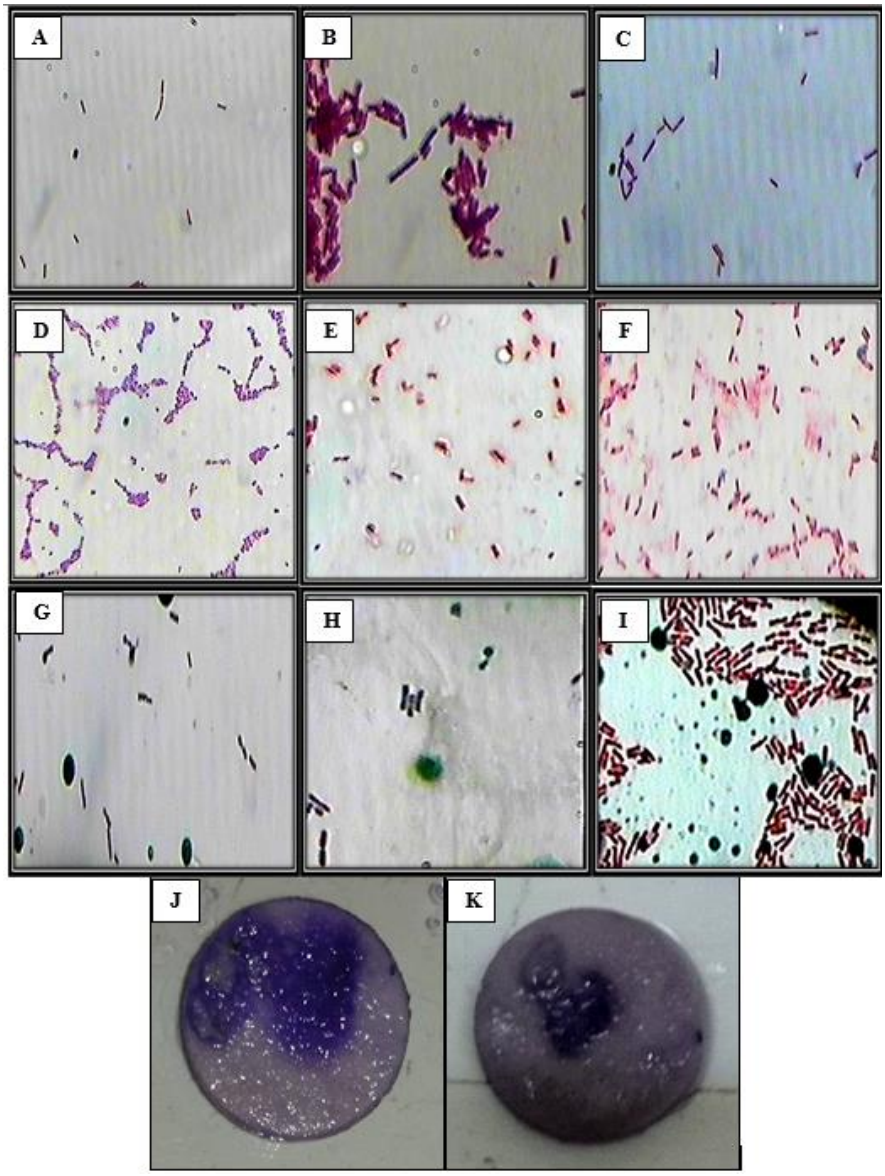

Fig 3: Positive Gram reaction shown by bacterial isolates BK23 (A); BK58 (B), BK68(C) and BK66(D); Negative Gram reaction shown by isolates BK34(E) and BK48 (F); Endospore formation in isolates BK23 (G), BK58 (H) and BK68 (I); and Positive oxidase test shown by isolate BK48 (J) and BK34(K)

Table 3: Biochemical parameters of biosurfactant producing bacterial isolates

Bacterial isolates	Colony appearance	Margin	Elevation	Growth (24h)	Pigmentation	Opacity	Form	Type and amount of growth	Shape	Gram Reaction	Spore Staining	Catalase Test	Blood Agar Haemolysis	Oxidase Test	Glucose Fermentation	Probable Genus
BK23	Irregular	Serate	Flat	Moderate	White	Opaque	Filliform	Pellicle and Moderate	rods	+ve	Endospore former	NA	B	NA	NA	*Bacillus* spp.
BK34	Circular	Entire	Flat	Scanty	White	Translucent	Beaded	Pellicle and Scanty	rods	-ve	NA	NA	A	+ve	-ve	*Pseudomonas* spp.
BK48	Circular	Entire	Flat	Moderate	White	Opaque	Filliform	Pellicle and Moderate	rods	-ve	NA	NA	A	+ve	-ve	*Pseudomonas* spp.
BK58	Circular	Serate	Flat	Abundant	White	Opaque	Echinulate	Sediment ed and Abundant	rods	+ve	Endospore former	NA	B	NA	NA	*Bacillus* spp.
BK66	Circular	Entire	Raised	Moderate	Pink	Transparent	Filliform	Uniform and Abundant	coccus in bunches	+ve	NA	+ve	B	NA	NA	*Staphylococcus* spp.
BK68	Irregular	Undulate	Flat	Moderate	White	Opaque	Filliform	Sediment ed and Moderate	rods	+ve	Endospore former	NA	B	NA	NA	*Bacillus* spp.

Discussion

Results of E_{24} index falls in a range of 9.0-20.0% however the isolates giving E_{24} above 18% were selected as promising isolates for further studies. *Bacillus* spp. and *Pseudomonas* spp. were reported to be giving E_{24} index with crude oil equivalent to 9.0% and 17.0%, respectively (Myla *et al*. 2010) [14]. Surface tension reduction, a confirmatory test for biosurfactant production, was measured by drop weight method using culture supernatant of all the 16 bacterial isolates. Seven out of the total 16 isolates which were tested positive for blood haemolysis test gave more than 0.030 Nm^{-1} reduction in surface tension of culture medium, while maximum reduction in surface tension was observed in case of isolate BK68 (0.071 Nm^{-1}). These values of surface tension reduction suggested significant biosurfactant production particularly in case of isolate BK68 which is further comparable to some of the reports available in literature giving maximum reduction in surface tension equivalent to 46.5mNm^{-1} (equal to 0.046 Nm^{-1}) by *Acinetobacter junii* (Bento *et al*. 2004) [5] and in the range of 28±1.03mNm^{-} to 51±1.36mNm^{-11} (equal to 0.028±0.00103 Nm^{-1} to 0.051±0.00136 Nm^{-1}) by *Bacillus amyloliquefaciens* (Thavasi *et al*. 2011) [23]. Atomized oil assay, in principle, is capable of identifying biosurfactant producing strains that would escape detection with most other methods. Halo radius in a range of 0.24-0.95 cm (equal to 0.0024-0.0095 m) has been reported during oil atomization assay (Burch *et al*. 2011) [7] by different bacterial strains was listed in some reports whereas maximum 0.0050 m oil atomization was resulted by isolate BK68 under non-optimized set of conditions in present investigation. Based upon screening results total six bacterial isolates BK23, BK34, BK48, BK58, BK66 and BK68 were considered as promising biosurfactant producers, which were further subjected to biosurfactant production using MS broth supplemented with 2% diesel as a carbon source. All the isolates were able to utilize diesel as carbon source producing 12.5-18.0% biosurfactant monitored in terms of E_{24} index. Biosurfactant production has been reported to the level of 9.0% by *Bacillus subtilis* and 17.0% by *Pseudomonas aeruginosa* as indicated by E_{24} index (Myla *et al*. 2010) [14]. Identification of isolates up to genus level could be achieved using morphological cultural and biochemical techniques. Wide varieties of biosurfactant producing bacteria are reported in literature, however, the most prevalently reported are *Pseudomonas* and *Bacillus* spp. (Chandankere *et al*. 2014; Gudina *et al*. 2015; Myla *et al*. 2010; Thavasi *et al*. 2011) [8, 10, 14, 23]. In present investigation, also isolates BK23, BK58 and BK68 were considered to be belonging to genus *Bacillus*, isolate BK66 was more likely to be a *Staphylococcus* sp. Isolates BK34 and BK48 being Gram negative, oxidase positive and non-glucose fermenting rods, could be identified as *Pseudomonas* spp.

Conclusion

The ability of bacterial isolates, specifically isolate BK68, to produce significant level of biosurfactant as indicated by multiple screening techniques, suggests their potential for exploitation at commercial level. However, fermentation production of these biodegradable and ecofriendly surfactants is limiting due to their increased cost of production at large scale. Therefore, to improve the overall economy of the process, optimization of physical and nutritional parameters along with utilization of cheaper raw material is strongly recommended.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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