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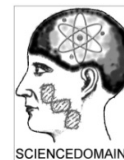

Evaluation of Different Screening Methods for Biosurfactant Producers Isolated from Contaminated Egyptian Samples Grown on Industrial Olive Oil Processing Waste

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NMS and HFM designed the study and wrote the protocol. Authors NMS, HFM and HIE managed the analyses of the study. Author NMS wrote the first draft of the manuscript. Author HIE performed the statistical analysis. Authors NMS and HFM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The present study aimed to screen biosurfactant producing microorganisms isolated from different Egyptian samples viz. soil sample contaminated with oil from fuel station, soil sample contaminated with kerosene from classic bread oven, samples from wall of drainage tube of kitchen and bathroom, also waste sample from gas cooktops of kitchen stove. All isolation samples were streaked on MSM medium supplemented with 1% olive oil processing waste as a sole carbon source to recover bacterial isolates with biosurfactant activity. Different screening methods e.g. Oil spreading assay, Emulsification index E24, Drop collapse test, Blue agar plate method (Cetyl trimethyl ammonium bromide-CTAB), Blood agar haemolysis, Reduction in SFT and Phenol

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sulfuric acid method were used to screen microbial biosurfactant producers. Fifty five bacterial isolates were obtained, consecutive screening was carried out between isolates to select the most promising biosurfactant producer. The selected isolate produced potential biosurfactant that belongs to glycolipid and identified by Biochemical and 16S rRNA analysis and was found belongs to *Pseudomonas aeruginosa* PAO1.

Conclusion: A combination of different methods is required for a successful screening, but it is recommend using both of drop collapse and CTAB tests, suggesting that strains highly active in one method were active in the other method. In addition, surface tension measurement and phenol sulfuric acid reaction is a must in case of the biosurfactant is of glycolipid type (rhamnolipid) to confirm the presence of anionic biosurfactant. In the present investigation using of efficient biosurfactant producer (*P. aeruginosa* PAO1) which prefer limited oxygen requirements (microaerobic) growing on low cost substrate (olive oil processing waste) is a privilege in the production cost.

Keywords: Biosurfactant; rhamnolipid; Pseudomonas aeruginosa PAO1; 16S rRNA analysis; screening assay methods.

1. INTRODUCTION

Biosurfactants are organic molecules that can be chemically and biologically produced [1]. Most of these surfactants are synthesized by living organisms [2].

Microbial Biosurfactants are mainly produced by aerobic microorganisms in aqueous media to assist in the growth of the microorganism by facilitating the translocation of insoluble substrates across cell membranes. The aqueous media must contain carbon source such as carbohydrates, hydrocarbons, fats, and oils [3].

Surfactants are widely used in almost all industries. As a result, every year millions of tons of surfactants (equivalent to billions of dollars) are commercialized in the world [4]. Surfactants are amphipathic molecules which reduce the surface tension between water and hydrocarbon interfaces. Most of the surfactants available are petroleum derivatives which are highly toxic and non-degradable [5].

In comparison with synthetic surfactants, biosurfactants have better surface activity, lower toxicity, they can bind heavy metals, have higher biodegradability, selectivity and biological activity, they are produced from renewable resources, can be produced through fermentation and can be reused by regeneration [6,7,8]. The other advantages of microbial surfactants are eco-friendly, high foaming ability and efficiency at extreme temperatures, pH and salt concentrations [9,10].

A number of microorganisms, such as filamentous fungi, yeasts, and bacteria, feed on immiscible substances in water, producing biosurfactants [11,12]. Biosurfactant produced on microbial cell surface or excreted extracellularly, and contain hydrophobic and hydrophilic moieties [13-15].

Biosurfactants are classified according to their chemical structure and microbial origin [16]. However, Rosenberg & Ron [17] suggested that, biosurfactants can be divided into low molecular mass molecules and the high molecular-mass polymers. In addition, Biosurfactants can classified into: Glycolipids, lipopeptides, phospholipids, surface active antibiotics, fatty acids, polymeric and particular biosurfactants [18].

Nowadays, biosurfactants are used in industries as a cosmetic [19], pharmaceutics, agriculture, petroleum [20] detergents in environmental protection and in management and enhanced crude oil recovery [21], emerged as potential agents in health care and food processing industries [22], possess several interesting properties of therapeutic and biomedical importance [23,24], and recently biosurfactants were found disrupting biofilm formation [25].

Grand View Research [26] reported that, the global biosurfactants market was 344,068.40 tons in 2013 and is expected to reach 461,991.67 tons by 2020, growing at a CAGR of 4.3% from 2014 to 2020.

Among bacteria, the genus *Pseudomonas* is known for its capacity to produce extensive quantities of glycolipids. The majority are of rhamnolipids (RLs) type currently are used from *P. aeruginosa* [27]. Recently, excellent potential

of rhamnolipid production for industrial scale from *Burkholderia thailandensis* E264 was elucidated [28].

Rhamnolipids has been extensively studied for exhibiting properties of great importance, their large-scale production based on renewable resources [29,30].

1.1 Aim of the Study

This work is aiming at isolation of biosurfactant producing microbes from contaminated Egyptian samples to control the problem of Olive oil processing waste from one hand and to produce a commercially significant biosurfactant from the other hand.

2. MATERIALS AND METHODS

2.1 Substrate Used

Olive oil processing waste from Nasef Factory (olive oil factory), Elbehira governorate, Egypt, was used as the sole carbon source during the isolation procedures.

2.2 Isolation Samples for Biosurfactant (BS) Producion

The samples collected from different locations such as, soil sample contaminated with oil from gas station, soil sample contaminated with kerosene from classic bread oven, samples from wall of drainage tube of kitchen and bathroom, also waste sample from gas cooktops of kitchen stove. All samples were collected in sterile polyethylene bags, transported to the laboratory aseptically and refrigerated.

2.3 Media Used

2.3.1 Nutrient agar medium [31]

Atlas [31] used in purification and maintenance of bacterial isolates. It prepared as ready-made manufacturer's direction (Micro media, Hungary). Only 28.0 g of commercially formulated nutrient agar was dissolved in 1.0 litre of distilled water prior to autoclaving at 121℃ for 15 minutes. This medium is used in isolation, purification and maintenance of bacterial isolates.

2.3.2 Minimal salts medium (MSM) [32]

This medium consists of (g/l):Na_2HPO_4, 2.2; KH_2PO_4, 1.4; $MgSO_4.7H_2O$, 0.6; $FeSO_4.7H_2O$, 0.01; NaCl, 0.05; $CaCl_2$, 0.02; yeast extract, 0.02; and 0.1 ml of trace element solution containing: (g/l): $ZnSO_4.7H_2O$, 2.32; MnSO4.4H_2O, 1.78; H_3BO_3, 0.56; $CuSO_4.5H_2O$, 1.0; $Na_2MoO_4.2H_2O$, 0.39; $CoCl_2.6H_2O$, 0.42; EDTA, 1.0; $NiCl_2.6H_2O$, 0.004; KI, 0.66 and pH of the medium was adjusted to 7.0 ± 0.2. Only, 1% Olive oil processing waste was added separately as the sole carbon source. Then sterilization was carried out at 1.5 psi (121°C) for 20 minutes. This medium is used for isolation, purification and maintenance of bacterial isolates.

2.3.3 Blood-agar [33]

Cheesbrough [33] is a qualitative assay to determine biosurfactant producers. Only those isolates which showed β-hemolysis were considered to be the potential biosurfactant producing microbes. To prepare blood agar medium, aseptically add 5.0% sterile sheep defibrinated blood to Nutrient Agar medium at 45-50℃, mix well and adjusted the pH at 6.8.

2.3.4 Medium for lipolytic assay activity [34]

The assay medium was composed of (g/l): Tributyrin, 2 ml; Gum Arabic, 4; Agar, 15; Phosphate buffer at pH 4.6, up to 1L.

2.4 Isolation of Biosurfactant Producing Bacteria

Isolation of biosurfactant producers was performed by streaking samples of isolation over sterile Minimal Salts Agar Medium (MSM) containing 1% of olive oil processing waste as the sole carbon source. Plates were incubated at 37℃ for 24-48 hrs. The bacterial colonies were detached and allowed to develop on the same isolation medium (MSM) and nutrient agar medium. Consecutive transfers and technical purification steps were carried out. All colonies of different forms and colours showing separate growth on media were picked up and subcultured on slants of the same media. Pure bacterial isolates were cryopreserved in nutrient medium supplemented with 15% glycerol at -80℃ [35].

2.5 Batch Fermentation of the Isolated Bacteria

Series of 100 ml conical flasks containing 24 ml of medium of MSM containing 1% olive processing waste as the sole carbon source, the pH was adjusted at 7.0±2 and autoclaving was carried out at 1.5 atm. (121℃) for 20 minutes. Each flask was inoculated with one of the

isolated biosurfactant producers (only 1 ml) and incubated in an incubator shaker at 200 rpm at 37℃ for two set of time, 24 & 48 h. Filtration was carried out, the cell free filtrate was used as a source of biosurfactant.

2.6 Screening of Bacterial Isolates for their BS Productivities

The cell free filtrate of the biosurfactant producers were subjected to different screening methods or may use the same isolate inoculum to test for biosurfactant production. The used methods were as follow:

2.6.1 Oil spreading assay technique

This method was employed to check the efficacy of the culture medium in displacing the oil layer as subscribed by Morikawa et al. [36], 1 ml of crude oil was added to the surface of 30 ml of distilled water in a Petri-dish to form a thin oil layer, 20 µl of culture supernatant was gently dropped on the centre of the oil layer, after one minute if the sample was +ve (containing biosurfactant), the oil is displaced and a clearing zone was measured.

2.6.2 Emulsification assay technique

The E24 of culture samples was determined as reported by Sarubbo [37] by adding 2.0 ml of kerosene and 2.0 ml of the cell-free broth in test tube, vortexed at high speed for 2.0 min and allowed to stand for 24 hrs. The E24 index is calculated by using the following equation:

$$\text{E24} = \frac{\text{Height of emulsion formed} \quad \text{x } 100}{Total\ heigh\ of\ soltion}$$

2.6.3 Drop collapse assay technique

It is a rapid and crude method to assess the surfactant activity according to Jain et al. [38]. In brief, about 10 µl of cell free broth was added in the center of an oil drop (20 µl of any oil) taken in a clean glass slide. The collapse of oil drop has been visualized and the less time taken indicates the higher activity of surfactant. Activity of microbial surfactant was compared with water and synthetic surfactant such as Tween 80.

2.6.4 Cetyl trimethyl ammonium bromide (CTAB) agar plate method

This method is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. It was developed by Siegmund & Wagner [39]. The isolated biosurfactant producers were cultivated on a mineral salts agar plate supplemented with CTAB (20 mg/100 ml, cationic surfactant), methylene blue (0.5 mg/100 ml, basic dye) and glucose (2% v/v). Microbes growing on the plate and form a dark blue halos are represented as glycolipid producers.

2.6.5 Hemolytic activity [40]

Isolates were screened on blood agar plates and incubated at 37℃ for 48 hrs. Hemolytic activity was detected as the presence of clear zone around bacterial isolates.

2.6.6 Surface tension (SFT) measurement

Measurement of SFT using a tensiometer is one of the common methods to screen BS producing organisms as reported by Pornsunthorntawee et al. [41]. The cell free filtrate of biosurfactant producers was measured by using a Du Nouy ring type tensiometer (Model: Manual Kruss Tensiometer k6). The results were expressed in dynes/cm. If BS is present in the cell free supernatant, the reading on vernier will decrease than that for distilled water as well as the used medium.

2.6.7 Lipolytic activity

The lipase assay medium was prepared and the cell free filtrate of the biosurfactant producer was used as a source of lipase enzyme as elucidated by [34]. Lipolytic activity was detected by clearing zones around the hole in comparison to the turbid background of the assay plates.

2.6.8 Phenol sulfuric acid reaction

The presence of carbohydrate groups in the biosurfactant molecule was assayed using the method of Dubois et al. [42]. A volume of 0.5 ml of culture supernatant was mixed with 0.5 ml of 5% phenol solution and 2.5 ml of sulfuric acid, and incubated for 15 min before measuring absorbance at 490 nm.

2.7 Selection of the Most Potent Biosurfactant Producer

All the isolated bacteria were screened for its ability to produce biosurfactnts by the previously mentioned methods to select the most promising biosurfactant producer.

2.8 Bacterial Identification by Biochemical and 16S rRNA Sequencing Technique

The most promising biosurfactant producer was identified based on its morphological and biochemical characteristics as Bergey's Manual of Determinative Bacteriology [43]. Gene sequencing 16S rRNA gene region was amplified with the universal primers. For setting up PCR, the following reaction mixtures were added into the PCR tube. The reaction mixtures were 5 µl of template,

> Primers: 1 µl of Forward primer- 27F (5' AGAGTTTGATCCTGGCTCAG 3'),
> 1 µl of Reverse primer- 1492R (5' TACCTTGTTACGACTT 3').

6 µl of assay buffer, 2 µl of Taq DNA polymerase and 5 µl of dNTP mix (Applied in Sigma Scientific Services Co. Cairo, Egypt). The amplification was carried out in a thermal cycler for 40 cycles using the following reaction conditions, denaturation of DNA at 95℃ for 10 minute, annealing at 65℃ for 1 minute and extension at 72℃ for 1 minute and 30 second. The amplified PCR product was mixed with 5 µl of gel loading buffer. 1.5% agarose gel was casted. The samples were loaded along with 5 µl of 3000 pb DNA ladder (Gene Ruler™ 100 pb plus DNA ladder) as a molecular marker. The gel was run and examined on UV transilluminator to visualize the bands. PCR products were purified by using GeneJET™ PCR Purification Kit (Thermo K0701). And it was sequenced with use ABI 3730xl DNA sequencer. Nucleotide sequence accession number and BLAST analysis of the nucleotide sequence 16S rRNA gene region data was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogentic tree, primer pairs were designed from NCBI database search tool.

2.9 Statistical Analysis

All analyses were performed in triplicates and expressed as mean ± standard deviation (SD). Excel was used for the statistical evaluation and graphical representations of the present study andall results were carried out by ANOVA, one way by Minitab (Version 11) software.

3. RESULTS AND DISCUSSION

Sampling and isolation of bacteria are the basis for screening of biosurfactant producers. Oil and hydrocarbon polluted environments are the most promising for isolation of biosurfactant producers, but many strains have been isolated from undisturbed different sites [44].

One hundred thirty oil-degrading isolates from hydrocarbon-polluted environments was reported using enrichment techniques [45]. Also, 40 bacterial strains were isolated from waste water of a petrochemical plant located in southern Taiwan according to their ability to reduce surface tension and to emulsify diesel and kerosene [46]. In addition, twenty seven bacterial isolates were isolated from twenty four crude oil contaminated soils located in repairing cars stations and petroleum refining companies [47]. Twelve bacteria and six fungi were isolated from different polluted sites in Al Madina Al Munawarah, KSA and tested their biosurfactant productivities by different conventional techniques [48]. Moreover, eighty-two bacteria were isolated from 90 samples of oil contaminated areas in Bangkok and vicinity using oil drop collapse technique [49]. Also, forty five oil-contaminated soil samples were collected from fuel station in Hilla city-Iraq, ten bacterial isolates with biosurfactant activity was recovered [50].

In the present investigation, 55 bacteria were isolated from different polluted samples as mentioned earlier. The industrial waste of olive oil processing was used as the sole carbon source in MSM agar medium, the incubation was carried out at 37℃ for 24 hrs. Many reports regarding the production of biosurfactants from different substrates, low-cost raw materials or even generated waste. But olive oil processing waste or its waste water has also been reported [51-54]. Recently, the effectiveness of hydrolysis pretreatment of olive mill (OMW) waste before use in biosurfactant production was demonstrated by *P. aeruginosa* and *B. subtilis* [55]. Raw materials account for 10–30% of the total production cost in most biotechnological processes [56,57]. So, use low-cost raw materials for the production of biosurfactants is desirable to reduce the cost. In addition, the use of low-cost raw materials for economical production of higher yields of biosurfactant by two oil-degrading strains of *P. aeruginosa* [45]. As well as, a strain of *P. aeruginosa* was isolated that can produce rhamnolipid from substrates such as n-hexadecane, paraffinic oil, babassu oil and glycerol [58].

In the present study, first screening for the 55 microbial isolates was carried out by oil

spreading assay technique, only 12 bacterial isolates B1, B2, B6, B7, B12, B13, B14, B15, B16, B17, B18 and B55 were selected as the most potent microbial isolates (Table 1 and Fig. 1). Isolates B55 and B13 gave the highest BS productivity with 14.50±0.5 and 14.25±0.25 cm, respectively. The present result represented a very promising compared to that present in the literatures.

Rismani et al. [59] reported that, the area of clearly formed oil displacement circle was 7.0 cm diameter as the activity of biosurfactants. While, El-Sheshtawy & Doheim [60] indicated that biosurfactant produced by *Pseudomonas aeruginosa* was highly positive and given 8.0 cm of oil displacement test. The oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms; a larger diameter represents a higher activity of the testing solution [48,61-63].

The most promising 12 BS producers were rescreened by emulsification assay technique. Isolate B13 followed by B55 exhibited the highest emulsification capacity, they gave 66.8±0.2 and 65.1±0.1 0f E24%, respectively (Table 2 and Fig. 2).

Table 1. Screening of biosurfactant productivity of bacterial isolates in terms of mean diameter of clearing zone (MDCZ) by oil spreading assay technique

Isolate code	MDCZ (cm)	Isolate code	MDCZ (cm)	Isolate code	MDCZ (cm)
B1	9.75±0.25	B20	0.0±0.0	B39	0.0±0.0
B2	10.50±0.5	B21	0.50±0.0	B40	0.0±0.0
B3	1.25±0.25	B22	0.0±0.0	B41	0.0±0.0
B4	0.54±0.035	B23	2.00±0.0	B42	0.0±0.0
B5	0.50±0.0	B24	1.60±0.1	B43	0.0±0.0
B6	12.25±0.75	B25	0.0±0.0	B44	0.0±0.0
B7	6.50±0.5	B26	1.95±0.05	B45	0.0±0.0
B8	1.50±0.0	B27	1.55±0.05	B46	0.0±0.0
B9	1.75±0.25	B28	0.0±0.0	B47	0.0±0.0
B10	0.54±0.35	B29	1.25±0.25	B48	0.0±0.0
B11	0.0±0.0	B30	0.0±0.0	B49	0.75±0.0
B12	8.25±0.25	B31	0.0±0.0	B50	0.45±0.05
B13	**14.25±0.25**	B32	0.40 ±0.0	B51	0.15± 0.05
B14	7.25±0.25	B33	1.50 ±0.0	B52	6.50±0.0
B15	10.75±0.75	B34	0.0±0.0	B53	2.00 ±0.0
B16	7.75±0.25	B35	0.0±0.0	B54	6.50±0.0
B17	13.25±0.25	B36	0.0±0.0	**B55**	**14.5±0.5**
B18	10.50±0.5	B37	1.25±0.25		
B19	0.0±0.0	B38	0.0±0.0		

Where: MDCZ means mean diameter of clearing zone. There is a statistically significant difference (P = 0.019)

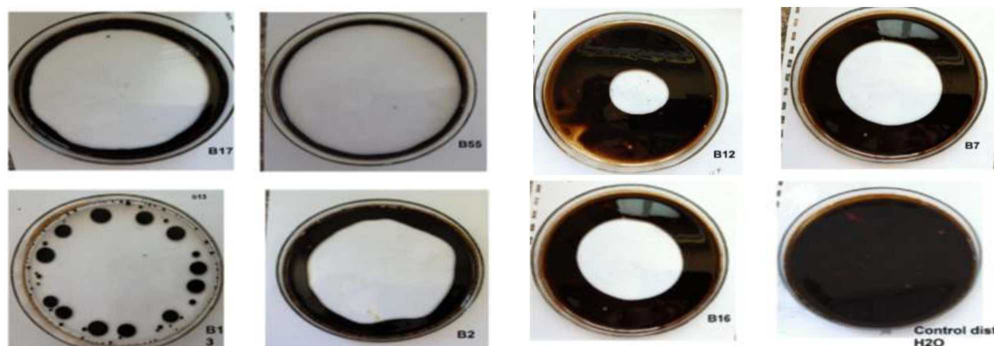


Fig. 1. Pictures of tests conducted by oil spreading assay technique showing results of the most promising biosurfactant producers, the changes seen in the oil present in the systems, compared to the control without changes

Table 2. Screening of biosurfactant productivity of bacterial isolates in terms of E24 (%) by emulsification assay technique to evaluate the performance of BS producers

Isolate code	E24 (%)
B1	35.75±0.75
B2	60.05±0.05
B6	59.5±0.5
B7	52.5±0.5
B12	0.0±0.0
B13	**66.8±0.2**
B14	60.1±0.1
B15	50.05±0.05
B16	56.15±0.15
B17	60.2±0.1
B18	49.7±0.3
B55	**65.1±0.1**

There is a statistically significant difference (P = 0.020)

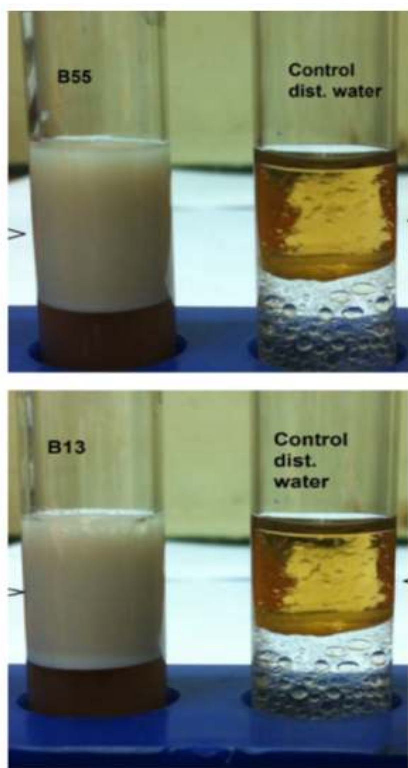


Fig. 2. Illustration of the emulsification assay technique of bacterial isolate B13 and B55, in which one can see the emulsion compared to the control without emulsion

Sarubbo [37] concluded that, the emulsification index (E24) provides a rapid and reliable measure of the quantity of biosurfactant. This method has advantages including simplicity, low cost, quick implementation and use of relatively common equipment that is accessible in almost every microbiological lab. El-Sheshtawy [21] investigated that, the foaming height due to biosurfactant production by *B. subtilis* DSM 15029 was obtained at 51%, while, El-Sheshtawy & Doheim [60] reported for *P. aeruginosa* to be 70%. Also, the lowering of the surface tension of the medium by 45% with an E24 value of 54% for *Pseudomonas aeruginosa* F23 was observed [64]. In addition, emulsification efficiency (E24) was reported after 24 hrs of 64% against kerosene oil by *Enterococcus faecium* [65]. Several studies focused on using emulsifying technique as recorded by many authers [66-68]. From the 12 selected bacterial isolates obtained from the first screening only seven bacterial isolates were selected from the second screening for further investigations.

Bacterial isolates B2, B6, B13, B14, B16, B17 and B55 which exhibited the highest BS productivity in the first and second screening were subjected for drop collapse technique. All bacterial isolates were found miscible with oil as in the case with synthetic surfactant (tween 80) and compered with water which is completely immiscible with oil. Isolates B13 and B55 showed the highest surface activity as recorded in Table 3 and Fig. 3.

The use of the drop collapse method was suggested as a sensible and easy to perform method which requires a small volume (5-10 µl) of culture broth to test the surfactant property [38]. In addition, it can be performed in Microplates [69], the positive drop collapse assay also revealed about the extracellular production of the biosurfactant and its surface active nature [70]. This assay has been applied several times for screening purposes [40,71].

Table 3. Screening of biosurfactant productivity of the most promising bacterial isolates by drop collapse assay technique

Isolate code	Drop collapse
Control (dist.H_2O)	-
Control tween80	+
B2	+
B6	+
B13	**++**
B14	+
B16	+
B17	+
B55	++

(-) negative result, (+) represent biosurfactant

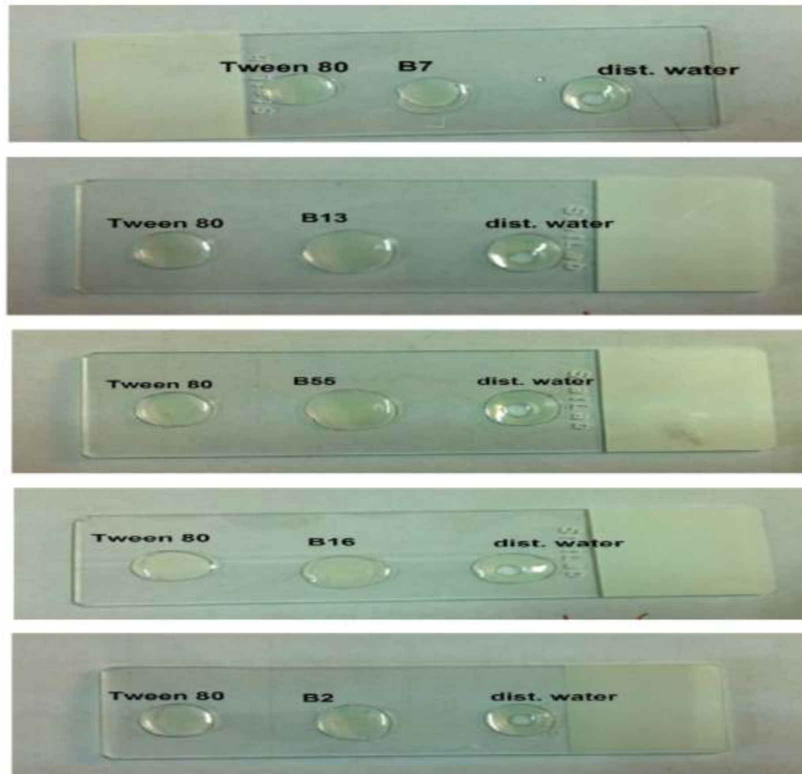


Fig. 3. Illustration of test conducted by Drop collapse technique showing highly active biosurfactant producers (B2, B7, B13, B16 & B55), the changes seen in the oil present in the systems, compared to the control (water) without any change and synthetic surfactant (Tween 80) which highly miscible with oil

Table 4. Screening of biosurfactant productivity of the most promising bacterial isolates by CTAB agar plate assay technique

Isolate code	Dark blue zone around colony
Control	-
B2	+
B6	+
B13	++
B14	+
B16	+
B17	+
B55	++

(-) negative result, (+) represent (1-6mm) and (++) blue zone range from (6-9 m)

In the present investigation, isolates B2, B6, B13, B14, B16, B17 and B55 were subjected for CTAB agar plate method, isolates B13 and B55 exhibited the highest BS productivity which confirmed the presence of anionic biosurfactant as shown in Table 4 above and Fig 4. It has been declared that, CTAB agar plate method is developed for the detection of extracellular rhamnolipids and other anionic glycolipids [39,72]. Also, [73] used CTAB technique for screening BS producing isolates and they isolated eleven different bacteria.

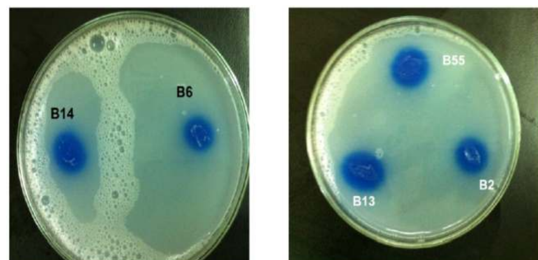


Fig. 4. CTAB assay technique for the most promising isolates; dark blue zone was formed around bacterial growth

In the present investigation, the most promising four isolates B2, B13, B17 and B55 were tested for blood haemolysis. Isolate B13 and B55 showed a complete hydrolysis of blood (beta

hemolysis) organisms as tabulated in Table 5 and illustrated in Fig. 5. This is based on the fact that surfactants interact strongly with cellular membranes and proteins. Exotoxins called hemolysins because lysis of the red blood cells [74]. Blood agar lysis was used to screen for biosurfactant production. This method was recommended as a preliminary screening method [75]. Haemolytic activity appears to be a good screening criterion in the search for biosurfactant-producing bacteria [76]. In addition, the hemolytic assay was a simple, fast and low-cost method for the screening of biosurfactant producers on solid medium. Many researchers have used this technique to screen for biosurfactant production by new isolates [77-79]. Many records on screening of biosurfactant producing organisms using blood haemolysis test [80-84].

Table 5. Screening of biosurfactant productivity of the four selected most potent bacterial isolates by blood haemolysis test

Isolate code	Blood hemolysis
B2	-ve
B13	β- heamolysis
B17	-ve
B55	β- heamolysis

Fig. 5. Illustration for complete haemolysis of blood by isolate B13

The most promising four isolates were investigated their lipolytic activity. Data provided in Table 6 indicated that, only isolate B13 has a lipolytic activity. Fig. 6 showed lipase clearing zone produced by the filtrate of isolate B13 in contrast with the turbid background of the assay plates (Table 6 and Fig. 6). Also, Sidkey & Al Hadry [48] reported that, *Bacillus cereus*, B7 which produced a lipopeptide biosurfactant has a lipolytic activity with 14.7 mm of clearing zone.

Tensiometeric technique is a quantitative assay, bacterial isolate, B13 reduce surface tension up to 28.23 mN/m followed by isolate B55 which gave 28.83 with 45 and 43.85% of reduction, respectively. B13 exhibited the highest BS productivity among the all tested isolates under study (Table 7). The criterion used for selecting biosurfactant producers is the ability to reduce the surface tension below 40 mN.m [85]. As well as, biosurfactants produced by *P. aeruginosa* strains were found to reduce the surface tension of distilled water from 72 to 30 mN/m [86]. It was stated that, a good surfactant can lower the surface tension of water from 72 to 35 mN/m [87]. Moreover, the production of biosurfactants by *Pseudomonas aeruginosa* MTCC7814 lowered the surface tension to 34 mN/m [88]. Moreover, biosurfactant isolated from *Enterococcus faecium* MRTL9 reduced surface tension from 72.0 to 40.2 mN m−1 [65]. Also, *P. aeruginosa* J4 has the ability to reduce surface tension up to 30 mN/m [46].

Table 6. Screening of biosurfactant productivity of the four most potent biosurfactant producers by lipolytic activity technique

Isolate code	Lipolytic activity
B2	-
B13	+
B17	-
B55	-

Fig. 6. Illustration for lipolytic activity of isolates B13 and B55, only isolate B13 exhibited clear zone in contrast with the turbid background

The present investigation showed that, isolates B13 and B55 have direct correlation between drop collapse, oil spreading, emulsification stability and surface tension assays and this is in complete accordance with Mounira & Abdelhadi [89] who found that, strains highly active in any one of these methods were active in other three methods.

Table 7. Screening of the most promising four bacterial isolates by tensiometeric technique in terms of reduction in surface tension

Isolate code	Reduction in surface tension (mN/m)	Percentage of reduction in surface tension (%)
dist.H_2O	72±0.288	-
Control(medium)	51.33±0.66	-
B2	34.5±0.288	32.78
B13	**28.23**±0.145	**45.00**
B17	30.33±0.166	40.91
B55	28.83±0.166	43.85

The results has statistically significant difference ($P = 0.005$)

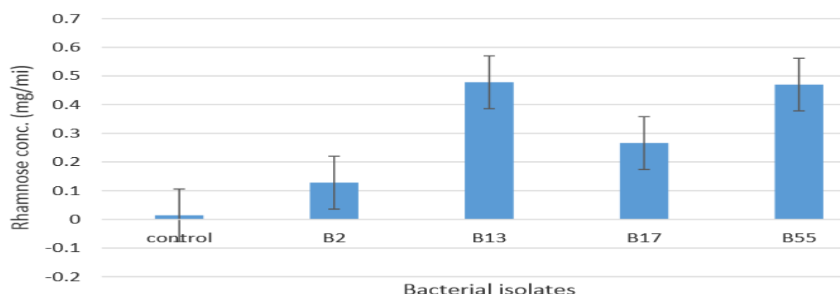
The four most promising bacterial isolates B2, B13, B17 and B55 were rescreened using phenol sulfuric acid reaction. Isolate B13 followed by B55 exhibited the highest BS productivity (Fig. 7) indicating that the produced biosurfactants contain carbohydrate group and belong to glycolipid type and may be rhamnolipid as reported by Vandana & Peter [79]. Also, if rhamnose test is positive the separated biosurfactant is of glycolipid type [32]. In addition, +ve rhamnose test indicating biosurfactant could be of rhamnolipid type [90].

In the present investigation, many methods were used for isolation and screening of biosurfactant producers, some are qualitative and the others are quantitative methods. As every method has its advantages and disadvantages, a combination of different methods is required for a successful screening as reported by many authers [38,39,48,72,81,89].

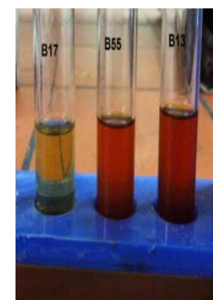
From the results in Table 8 the qualitative methods (drop collapse test, cetyl trimethyl ammonium bromide test, haemolytic assay and lipolytic assay test) were sensitive easy to use, but it can be advisable to be used only during the stage of isolation of microbial biosurfactant producers because more than one method should be included to identify potential biosurfactant producers [48,81]. In addition, it is recommended to use both of drop collapse test and CTAB test because most of the rhamnolipid producers gave +ve results with these tests, suggesting that strains highly active in one method were active in the other method. The CTAB method is a confirmatory one for the presence of anionic biosurfactant and is advisable to be used during the stage of isolation [39,72,73].

On the other hand, the quantitative methods (oil spreading assay, emulsification assay technique, reduction in surface tension, and rhamnose test) can be used during both the stage of screening of the biosurfactant producers and selection of the most promising one [38,48,72,81,89], because they are more reliable, accurate to decide which isolate can be chosen for further investigations, but two methods must be selected one of them is surface tension measurement and the other is phenol sulfuric acid reaction in case of the biosurfactant is of glycolipid type (rhamnolipid) to confirm the presence of anionic biosurfactant.

By comparing the results of all bacterial isolates in relation to all above screening assay techniques, bacterial isolate B13 followed by isolate B55 were exhibited the highest productivity among all the tested isolates under study. So, Isolate B13 was selected as the most potent microbial isolate for biosurfactant production and was subjected for further investigations.



(a)



(b)

Fig. 7. Rhamnose test for selected biosurfactant producers (a); high optical density at 490 nm (b)

There is a statistically significant difference ($P = 0.009$)

Table 8. A summary of the four most promising biosurfactant producers using different evaluation techniques

Isolate code	OSA (cm)	E-24 (%)	DCT	CTAB	HA	RST (%)	LA	RT (mg/ml)
B2	10.5±0.5	60.05±0.05	+	+	-	32.78	-	0.128±0.006
B13	14.25±0.25	**66.8±0.2**	**++**	**++**	**+**	**45.00**	**+**	**0.478±0.006**
B17	13.25±0.25	60.2±0.1	+	+	-	40.91	-	0.266±0.018
B55	**14.5±0.5**	65.1±0.1	**++**	**++**	**+**	43.85	-	0.469±0.001

Where: OSA, Oil Spreading Assay; E24, emulsification assay; DCT, Drop Collapse Test , CTAP, Cetyl trimethyl ammonium bromide test; HA, Haemolytic assay; RST, Reduction in Surface Tension, LA, Lipolytic assay; RT, Rhamnose Test (Phenol sulfuric acid reaction)

Table 9. Morphologial and some biochemical characterization of isolate B13

Characteristics	Isolate B13
Gram stain	Negative
Shape	Rods
Motility	+
Sporulation	-
Pyocyanin production	+
Growth at:	
30℃	+
37℃	+
40℃	+
50℃	-
55℃	-
65℃	-
Biochemical reactions	
Catalase test	+
Starch hydrolysis	-
Gelatinase enzyme	+
Indole	-
Citrate utilization	+
Nitrate to nitrite	+
Haemolytic activity	+

(-) negative result, (+) positive result

Bacterial isolate B13 was subjected for characterization on the basis of microbiological, physiological and biochemical tests and was studied according to Microbiological Methods 6th [43] and Bergey's Manual of Determinative Bacteriology [91].

Data represented in Table 9 indicated that, isolate B13 is belonging to Gram -ve bacteria, motile, rod in shape, catalase, gelatinase and citrate utilization are positive. The isolate B13 has β-haemolytic activity. Also, it reduce nitrate to nitrite and does not have amylase and not react with indole. In addition, the organism can grow at moderate temperatures viz. 30, 37 and 40℃, but it can't grow at higher temperatures viz. 50, 55 and 65℃.

In view of all the previously mentioned characteristics and according to the previous international keys, this isolate was suggested to be belongs to *Pseudomonas aeruginosa.* So isolate B13 gave the name and code number *Pseudomonas aeruginosa*-B13.

Hence to identify and confirm the *Pseudomonas aeruginosa*-B13 at molecular level, 16S rRNA gene region was amplified and sequenced. Genomic DNA was extracted from *Pseudomonas aeruginosa* by the standard method. The sequence of the 16S rRNA gene of the *Pseudomonas aeruginosa*-B13 was analyzed using the advanced BLAST search program at the NCBI website: http://www.ncbi.nlm.nih.gov/BLAST/ The BLAST analysis of 16S rRNA gene sequences of the selected strains showed alignments of these sequences with reported 16S rRNA genes in the gene bank. The nucleotide sequence was deposited to the gene bank and gene bank accession number for B13 isolate was obtained.

The highest similarities found with different species of the genus *Pseudomonas* are summarized in Table 10. On the basis of Phylogenetic data obtained the isolate B13 showed maximum similarity 97% with complete sequence with *Pseudomonas aeruginosa* PAO1 (Fig. 8) with accession number NR 074828.1, PCR amplification of 16S rRNA gene region by using universal primer of *Pseudomonas aeruginosa* PAO1, was provided in Fig. 9. Phylogenetic tree generated by NCBI tool proves that this organism genetically related with other organisms (Fig. 10). The 16S rRNA nucleotide sequences of the isolate was aligned with homologous regions from various *Pseudomonas* bacteria, and the phylogenetic tree was constructed by neighbor-joining method [92].

In view of all the previously mentioned characteristics and according to the international keys this isolate B13 was belongs to *Pseudomonas aeruginosa* PAO1.

Table 10. Gene bank accession numbers along with the alignments of sequences obtained with reported 16S rRNA gene sequences in the gene bank and highest similarity with different *Pseudomonas* species (16 strains)

Description	Max score	Total score	Query cover (%)	E value	Ident (%)	Accession (NR)
Pseudomonas aeruginosa strain SNP0614 16S rRNA gene, partial sequence	1642	1642	95	0.0	97	118644.1
Pseudomonas aeruginosa PAO1 strain 16S rRNA, complete sequence	1642	1642	95	0.0	97	074828.1
Pseudomonas aeruginosa strain DSM 50071 16S rRNA gene, partial sequence	1642	1642	95	0.0	97	117678.1
Pseudomonas aeruginosa strain NBRC 12689 16S rRNA gene, partial sequence	1642	1642	95	0.0	97	113599.1
Pseudomonas aeruginosa strain ATCC 10145 16S rRNA gene, partial sequence	1642	1642	95	0.0	97	114471.1
Pseudomonas resinovorans NBRC 106553 strain 16S rRNA gene, complete sequence	1587	1587	95	0.0	96	103921.1
Pseudomonas quezennel strain RA26 16S rRNA gene, complete sequence	1565	1565	95	0.0	96	114957.1
Pseudomonas otitidis strain MCC 10330 16S rRNA gene, complete sequence	1565	1565	95	0.0	96	043289.1
Pseudomonas aeruginosa strain DSM 50071 16S rRNA gene, complete sequence	1563	1563	95	0.0	96	026078.1
Pseudomonas resinovorans strain ATCC 14235 16S rRNA gene, complete sequence	1559	1559	95	0.0	96	112062.1
Pseudomonas stutzeri A1501 16S rRNA gene, complete sequence	1498	1498	95	0.0	94	074829.1
Pseudomonas indica strain NBRC 103045 16S rRNA gene, partial sequence	1498	1498	95	0.0	94	114196.1
Pseudomonas stutzeri strain ATCC17588 16S rRNA gene, partial sequence	1498	1498	95	0.0	94	041715.1
Pseudomonas stutzeri strain DSM 5190 16S rRNA gene, partial sequence	1498	1498	95	0.0	94	114751.1
Pseudomonas stutzeri strain CCUG 11256 16S rRNA gene, complete sequence	1498	1498	95	0.0	94	118798.1
Pseudomonas stutzeri strain NBRC 14165 16S rRNA gene, partial sequence	1495	1495	95	0.0	94	113652.1

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Query  49    CCT-GGAATCTGCCTGGNNAAGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATAC  107
             ||| |||||||||||||      |||||||||||||||||||||||||||||||||||||
Sbjct  117   CCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATAC  176

Query  108   GTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGG  167
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  177   GTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGG  236

Query  168   ATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGG  227
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Sbjct  237   ATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGG  296

Query  228   ATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG  287
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Sbjct  297   ATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG  356

Query  288   AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC  347
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Sbjct  357   AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC  416

Query  348   GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGA  407
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Sbjct  417   GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGA  476

Query  408   CGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG  467
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Sbjct  597   TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGT  656

Query  588   AGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAG  647
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Sbjct  657   AGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAG  716

Query  648   TGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAA  707
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Sbjct  717   TGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAA  776

Query  708   CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCT  767
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Query  768   TGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAG  827
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Sbjct  837   TGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAG  896

Query  828   GTTAAAACTCAAATGAATTGACGGGGNCCGCACAAGCGGTGGAGCATGTGGNTTAATTC  887
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Sbjct  897   GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC  956

Query  888   GAAGCAACGCNNAAGAACCTTACCNGGNNNTGANATGCTGAGAACTTTNCAGAGATGGAT  947
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Sbjct  957   GAAGCAACGCG-AAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGAT  1015

Query  948   TGGNGNCTTCGGGAANTnnnaannCAGGTGCTGCATGGNTGTCNNCAGCTC  998
             ||| | ||||||||| |   |   |||||||||||||| ||||  ||||||
Sbjct  1016  TGGTGCCTTCGGGAACTCAGACA-CAGGTGCTGCATGGCTGTCGTCAGCTC  1065
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Fig. 8. ***P. aeruginosa*** **PAO1 strain PAO1 16S ribosomal RNA, complete sequence**

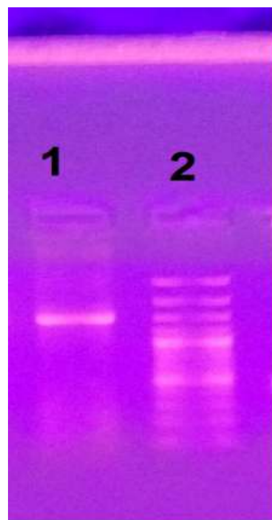

Fig. 9. Amplified fragment of 16S rRNA gene of *Pseudomonas aeruginosa* PAO1-B13. Lane (1) is 16S rRNA gene of *Pseudomonas aeruginosa* PAO1, B13 size is 1500 bp and lane (2) is DNA ladder the size is 3000 bp

Sabra *et al.* [93] reported that, *Pseudomonas aeruginosa* PAO1 was previously classified as 'non-mucoid' formed a clear polysaccharide capsule on the cell surface under oxidative-stress conditions and released a high amount of proteins into the culture broth. *P. aeruginosa* PAO1 prefers microaerobic conditions and form a polysaccharide capsule on the cell surface.

There are several reports of pathogenic bacteria that were found to produce biosurfactants [94]. Their exact role is not clear - may be they assist the colonization of host tissues or participate in increasing the bioavailability and degradation of hydrophobic organic contaminants by the host bacteria [95,96].

It is well known that, the opportunistic pathogen *Pseudomonas aeruginosa* can produced rhamnolipd biosurfactant and are mainly produced using shake flask, batch, fed-batch or continuous systems [97-100]. Similarly, Rashedi et al. [101] isolated a strain of *Pseudomonas aeruginosa* MM1011 from oil and has the capacity to produce rhamnolipid-type biosurfactants from substrates such as gasoline, paraffin oil, whey and glycerol. However, *Pseudomonas aeruginosa* A41 strain isolated from seawater in the gulf of Thailand, had the ability to grow on olive oil, palm oil and coconut oil and produce rhamnolipid [102].

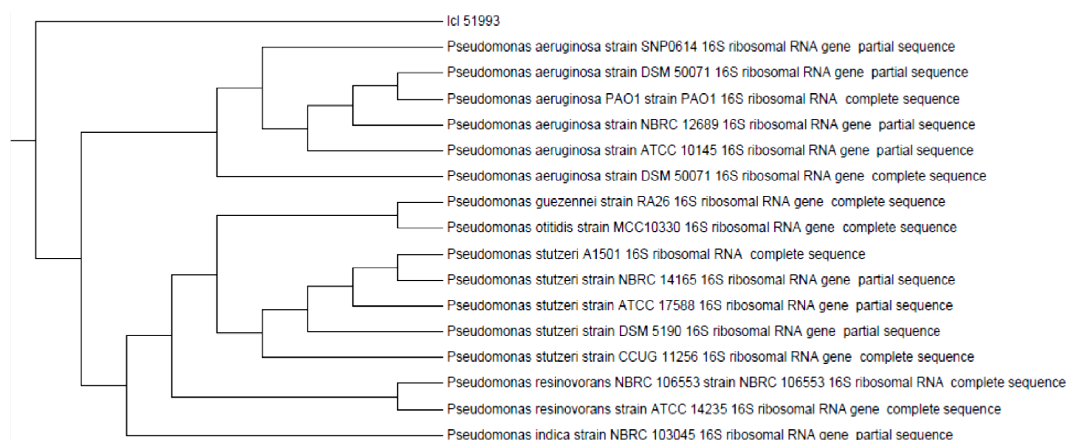

Fig. 10. Phylogenetic Tree of *Pseudomonas* strains including the present most promising *P. aeruginosa* PAO1, B13 isolate with complete sequence

4. CONCLUSION

Economical production represented the major corner stone in produced biosurfactant, as in the case with most bioprocesses. Moreover, the amount and type of a raw material can contribute considerably to the production cost; so, using of efficient biosurfactant producer (*P. aeruginosa* PAO1) which prefer limited oxygen requirements (microaerobic) growing on low cost substrate (olive oil processing waste) is a privilege in the production cost. So a subsequent study is recommended to optimize the BS production and evaluate its cost. A combination of different methods is required for a successful screening, but it is recommended to use both of drop collapse test and CTAB test, suggesting that strains highly active in one method were active in the other method. In addition, surface tension measurement and phenol sulfuric acid reaction is a must in case of the biosurfactant is of glycolipid type (rhamnolipid) to confirm the presence of anionic biosurfactant.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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