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Sequencing of three genes from Pseudomonas aeruginosa responsible for rhl production and application in pharmaceutical and bioremediation fields

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Abstract

Pseudomonas aeruginosa PAO1-B13 isolated from Cooktops of kitchen stove can produce amphipathic compounds, which allows it to perform some surface activities and are widely named rhamnolipid biosurfactant. Here, we report promising antimicrobial activity of rhamnolipid produced by the above strain against some tested fungi and bacteria in addition to antitumor activity against both Breast and Colon Carcinoma cell lines. Also, Rhamnolipid exhibited a high recovery of heavy metals from suspensions in the field of bioremediation. The cluster genes encoding for biosurfactant production (rhlB, rhlC and rhlI) were isolated and sequenced successfully. For these promising properties, efforts have been made for maximizing the production of rhamnolipid for commercial use through optimization of various nutritional and environmental factors. The maximum production rate was achieved at 37 ˚C, pH 6.8, incubation period for one week at 150 rpm, only one carbon source; sunflower oil; inoculum size, 4.84 x10^6 CFU; amino acid, Dl-valin; KCl, 500 ppm; vitamins, none. CH_3Cl:CH_3OH was used to extract Rhamnolipid in a 2:1 ratio. Here, we recommend further investigation for this promising strain for large-scale use in the field of pharmaceutical and bioremediation.

Keywords: ***Pseudomonas aeruginosa* PAO1-B13, Rhamnolipid, Genes Sequencing, Antimicrobial, Bioremediation.**

1. Introduction

Surfactants are essential components of daily life. They lower the system's free energy by absorbing large molecules with higher energy [1]. Sustainable technologies have prompted the advancement of the quest for biodegradable natural substances to remediate hydrocarbon-contaminated sites [2], which led to the discovery of biosurfactants from natural origin. A wide range of these surfactants is of biological origins, such as glycolipids [3]. Rhamnolipid shows impressive results as a therapeutic and pharmaceutical agent due to its low toxicity and also antimicrobial activity against bacteria such as *Staphylococcus aureus* and *Bacillus cereus* [4]. As a bioactive constituent, it is found to be effective in some skin refurbishing and injury healings making it beneficial in cosmetics [5].

As rhamnolipids are surface-active agents, that additionally led to their extensive use in detergent ingredients and laundry products [6]. Rhamnolipids also have a deep impact on agriculture, as they help to increase soil quality through soil remediation and removing plant pathogens. Also, they showed considerable contribution in improving the nutrients and fertilizers uptake and also as biopesticides [7]. It's worth mentioning that microbial biosurfactants are more favorable to use than plant surfactants for their multifunction, large-scale capacity, and rapid production. Also, plant surfactants are costly to produce in the industry [8]. Surfactants from microbes are complicated structures made up of a hydrophilic and hydrophobic moiety with a large variety of chemical structures [9,10]. Because of their configuration diversity, biodegradability, stability under extreme environments, and low toxicity, microbial surfactants can be substitutes to chemical surfactants [11]. The most famous biosurfactants are glycolipids. Mannosyleryth ritol lipids, trehalose lipids, sophorolipids, (MELs), and rhamnolipid (RLs) are the most extensively studied glycolipids to date [12,13]. Mono- and di-rhamnolipids are distinguished by the number of rhamnose moieties. In *P. aeruginosa*, the length of the fatty acid alkyl chain ranges from C8 to C14 [14]. Two -hydroxy fatty acids with C10 chains are found in the majority of well-known organisms [15]. As

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many as two unsaturated C-C bonds can be found in alkyl chains [16]. Five enzymes are used to make the rhamnose moiety from glucose-6-phosphate [17]. Fatty acid de novo synthesis produces hydroxy fatty acids [18]. The rhamnose synthesis pathway provides building blocks for the lipopolysaccharides found in these organisms' cell membranes [19]. Two rhamnosyltransferase enzymes generate mono- and di-rhamnolipids. (Fig 1) [20]. RhlAB (rhamnosyltransferase 1) catalyzes the synthesis of mono-rhamnolipids, which is followed by the transfer of dTDP-L-rhamnose to -hydroxydecanoyl—hydroxydecanoate by a glycosyltransferase enzyme. rhlA encodes a protein with a molecular mass of 32.5kDa and consists of 295 amino acids [21]. Even though RhlA is involved in the synthesis of rhamnolipids, its exact role is unknown. [22] RhlA is also needed for the maintenance of RhlB in the cell membrane, according to the findings. RhIA protein can also be involved in the biosynthesis of 3-hydroxyacyl-ACP precursors in polyhydroxyalkanoic acid biosynthesis, according to [23]. RhlB (47 kDa), on the other hand, is thought to be the catalytic subunit of rhamnosyltransferase and comprises two domains that are thought to anchor RhlB in the inner cytoplasmic membrane [22]. Rhamnolipid development by *P. aeruginosa* is generally restricted to high bacterial cell concentrations grown under phosphate [24] or nitrogen [25] limitation. A quorum-sensing system has been shown to control mono-rhamnolipid [26-29]. The rhl locus of *P. aeruginosa* includes the a quorum-sensing device rhlR-r hlI. [27] and rhlAB, which encodes rhamnosyltransferase 1 [22]. All gene promotors are working in the same direction, with only rhlAB being transcribed independently [22, 25].

The concentration of N-butyryl homoserine lactone synthesized by RhlI reaches a point where it binds to the RhlR (transcriptional activator) at high bacterial cell density [29]. The protein complex then binds to a consensus sequence in front of rhlAB, improving their expression and contributing to rhamnolipid biosynthesis [25]. Rhamnosyl transferase 2 is thought to convert mono-rhamnolipid and dTDP-L-Rhamnose into di-rhamnolipid (L-Rha-L-Rha—hydroxydecanoyl—hydroxydecanoate). In *P. aeruginosa*, the rhlC gene produces rhamnosyltransferase 2, which is required for the structure of di-rhamnolipid parts [21]. So the aim of this study is to optimizing rhamnolipid production and sequencing of genes responsible for rhamnolipid production and studying sum applications for the produced rhamnolipid.

2. Experimental

2.1. Inoculum Preparation and Production Medium

P. aeruginosa strain (PAO1-B13) partially characterized in Sidkey *et al*. (2016) [30] was used in this study. Rhamnolipid was produced on Bushnell and Hass (BHM) medium [31]. Synthesis of rhamnolipids was detected by measuring the reduction of the surface tension using a manual tensiometer (Krüss 6) also by the concentration of rhamnolipid with phenol-sulfuric acid reaction (Rhamnose test) [32].

2.2. Optimizing Parameters for Rhamnolipid Production

The incubation period [32], temperature, pH, inoculum size [33], shaking conditions, different types of oils, the concentration of sunflower oil, nitrogen source [31], amino acids, metallic ions, vitamins, and growth promoters have been tested to maximize the rhamnolipid biosurfactant out again by *P. aeruginosa* PAO1-B13 in three replicates. 1 % olive oil was supplemented to the production medium as the only carbon source.

2.3 Molecular Detection and Identification of rhl Genes

2.3.1. Genomic DNA extraction and amplification of rhl genes.

Gene Jet genomic DNA purification kit was used to extract genomic DNA. Kit (Thermo K0721) (BIO101, Carlsbad, CA, USA). This study used specially designed oligonucleotide primers for PCR amplification and sequencing of the partial length of rhl genes (rhlB, rhlC, and rhlI), as defined in Zhu *et al*.(2004) & Bazire *et al*. (2005) [34,35], see table (1). Maxima Hot Start PCR Master Mix was used for the PCR reactions (Thermo K1051). Initial denaturation at 95°C for 1 minute, 30 cycles of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for two minutes, and final extra extension at 72°C for 10 minutes comprised a PCR loop suitable for amplifying all rhl genes. GeneJETTM PCR Purification Kit was used to gel-purify the PCR products. (Thermo K0701), (The PCR instrument is ARKTIK thermal cycler (Thermo). Gel electrophoresis was used to visualize the products [36]. The PCR product was allowed to wash up using Gene JET™ PCR Purification Kit (fermentas).

2.3.2. DNA sequencing of the isolated RhI genes

The PCR product was sequenced using an ABI 3730 xl DNA sequencer at GATC German Company using the following primers; rhlB2-F 5′ CATTTCCTCGACCTGGAGTC 3′ rhlI2-F - 5′

TGCCGTTCATCCTCCTTTAG 3′ and rhlC4-F-5′AACTGGCGGCGGCGTTTCC 3′. The Big Dye terminator-ready sequencing kit was used to conduct the DNA sequencing reactions. The reaction mixture contained 8.0 l of the readymade reaction mix, 1.0 g of DNA, and 3.2 pmole of forwarding primer in a total volume of 20 l. 95°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes were the cycle sequences, which were replicated 25 times with rapid thermal cycling. The nucleotide sequence was automatically detected on the ABI 3730xl DNA sequencer by using the electrophoresing the sequencing reaction product. The info was provided as fluorimetric scans and subjected to sequence analysis software. The obtained nucleotide sequence of *P. aeruginosa* POA1, genes were analyzed using the BLAST program tool against the GenBank [37] accessible on the website of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Table (1). Specific designed oligonucleotide primers for PCR amplification of rhl genes

Gene	Forward primer	Sequence(5′→3′)	Reverse primer	Sequence(5′→3′)
rhlB	rhlB2-F	CATTTCCTCGACCTGGAGTC	rhlB2-R	ATCGAGAAAGCGTTGCAGTT
rhlI	rhlI2-F	TGCCGTTCATCCTCCTTTAG	rhlI2-R	GCAGGCTGGACCAGAATATC
rhlC	rhlC4-F	AACTGGCGGCGGCGTTTCC	rhlC4-R	AGTCCTGGTCGAGCAGCAGCA

2.4. Isolation and Purification of Rhamnolipid Biosurfactant

To remove the microbial cells, the filtrate was centrifuged for 20 minutes at 4 °C/8500 rpm. The obtained supernatant was acidified at pH 2.0 with a 6.0 N. HCl solution, and the supernatant was left to sit overnight at 4 °C for complete biosurfactant precipitation [38]. The precipitate was dissolved in a 0.1 M $NaHCO_3$ solution after centrifugation, followed by a biosurfactant extraction step at room temperature using a solvent with a 2:1 ratio of CH_3Cl–CH_3OH [13, 24, 39]. At 40 °C, the organic component was transferred to a rotary evaporator to remove the solvent, producing a viscous honey-colored biosurfactant that was collected per liter of effluent.

2.5. Applications of the Produced Rhamnolipid BS.

2.5 .1. Antimicrobial activity of the identified biosurfactant

The sample was tested in-vitro for antibacterial activity using the agar well diffusion method [40]. A variety of bacteria and fungi were used as *Streptococcus pneumonia* (RCMB 010010), *Staphylococcus aureus* (RCMB 010028), *Enterococcus faecalis* (RCMB 010068), *Bacillus subtilis* (RCMB 010067), Methicillin-Resistant *Staphylococcus aureus* (MRSA 2658 RCMB), *P. aeruginosa* (RCMB 010043), *Escherichia coli* (RCMB 010052), *Salmonella typhi* (RCMB 010072) and *Klebsiella pneumonia* (RCMB 010093) using nutrient agar medium. Antifungal activity was administered against *Geotricum candidum* (RCMB 05097), *Syncephalastrum racemosum* (RCMB 02763), *Candida albicans* (RCMB 05031), and *Aspergillus niger* (RCMB 02317) using (Sabouraud Dextrose Agar medium). Gentamycin and ampicillin, Amphotricine B, Vancomycin, were used as standards for antifungal, Gram-positive, and Gram-negative activity respectively. DMSO was used as a control. The concentration of 1.0 mg/ml of the compounds was tested against both fungi and bacteria.

2.5.2. Antitumor activity of biosurfactant

The American type culture selection provided the cell lines. The cells were grown in RPMI-1640 medium supplemented with 50 g/ml gentamycin and 10% in activator fetal calf serum. The cells were sub-cultured every two days and incubated in 5% CO_2 at 37 °C. The antitumor activity of Rhamnolipid against different carcinoma cell lines was tested at the Regional Centre for Microbiology and Biotechnology, according to Wilson, A.P. (2000) and Mosmann, T. (1983) [41-43].

2.5.3. Heavy and toxic metal recovery [44]

The four main heavy metals, as well as one toxic metal, were extracted from the solution prepared. Five basic heavy metals; Ag_2SO_4, $CoCl_2$, $FeCl_3$, $CuSO_4$ and $HgCl_2$ were tested. In a tube, 2.5 ml of standard metal with a concentration of 100 mg/ml was added, along with 0.25 ml crude rhamnolipid, and the mixture was allowed to rest for 1 hour until the dry weight was calculated after filtration. The ability of metal removal i.e. η was calculated as,

$$\eta = \frac{(\text{Initial heavy metal} - \text{Final heavy metal})}{\text{Initial metal concentration}} \times 100$$

2.6. Statistical Analysis

The statistical analysis was done in triplicates and the results were recorded as mean standard deviation. Mini tab (Version 11) software was used to perform one-way ANOVA statistical analysis.

3. Results and Discussion

3.1. Parameters of Optimization for Biosurfactant Production by P. aeruginous PAO1-B13

A previous study [13] revealed that agitation, pH, temperature, and oxygen availability, affect biosurfactant development through their effects on

cellular growth or activity. Several studies have focused on improving biosurfactant development by altering the factors that affect the amount and form of biosurfactant released by microorganisms. The production of rhamnolipid by *P. aeruginosa* increases in the stationary phase, as any other secondary metabolite [48- 50]. 7 days was chosen as the best incubation period for biosurfactant productivity which yielded 0.977 (mg/ml) production (Fig 1).

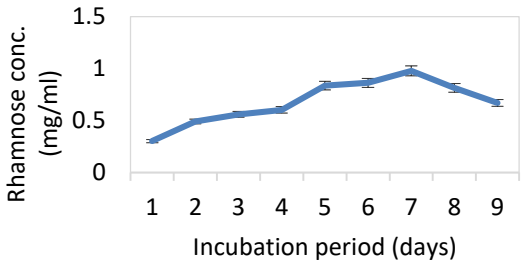

Fig. 1. Effect of incubation periods on biosurfactant production by *P. aeruginosa* PAO1-B13. There is a significant difference of (P = 0.001).

Also, [51] indicated that the maximum biosurfactant (rhamnolipid) production by *P. aeruginosa* OCD1 was obtained at a stationary growth phase after 7.0 days of incubation with an inoculum of 24 hrs culture. Similarly, [52] showed that maximum production of rhamnolipid on mannitol was obtained in stationary growth, revealing its secondary metabolite characteristics. The best incubation temperature for rhamnolipid productivity was found to be 37 °C which resulted in 0.872 (mg/ml) production (Fig 2). In the same accordance, *P. aeruginosa* S2 isolated from diesel-contaminated soil [53], and *P. aeruginosa* MR01 isolated from oil excavation areas in Iran [54] produced maximum biosurfactant at 37 °C. The majority of the strains like *P. putida* [55], *P. aeruginosa* RS29 [56], *Bacillus subtilis* MTCC441 [57], and *P. aeruginosa* WJ-1 [58] produce the maximum biosurfactant at 37 °C. In the case of *Pseudomonas*, a fluctuation in temperature caused the change in the contents of biosurfactant [59]. Additionally, [60] showed that *P. aeruginosa* sp. produces the maximum rhamnolipid at 35 °C while at 40 °C, bacterial growth and biosurfactant production were mostly inhibited. PH 6.8 was found to induce the highest biosurfactant productivity which gave 0.938 mg/ml production. Above or below this pH, a gradual decrease in productivity was noticed as shown in fig. (3). Lotfabad et al. [51] revealed that the highest biosurfactant production by *P. aeruginosa* OCD1 was obtained at pH 6.00. Concerning the effect of different inoculum sizes on biosurfactant productivity, it was declared that by increasing the inoculum size of *P. aeruginosa* PAO1-B13 up to 1 ml (containing 4.84×106 CFU/ml) biosurfactant productivity was increased by 0.93 mg/ml (Fig 4). Similarly, [61] also indicated that inoculum size (4.6×10^6 CFU/ml) at the concentration of 10% (v/v) has given the maximum production of biosurfactant by *B. subtilis* [51]. It was also discovered that the size of the inoculum had a significant impact on the development of biosurfactants by *P. aeruginosa* OCD1. With 1 vol percent inoculum, the maximum reduction in surface tension and the highest emulsification index was achieved. Over and below this inoculum volume, weak results are obtained. In addition, [62] showed that the highest production of the biosurfactant from *B. subtilis*, B7 was with an inoculum size up to 4000 µl (contains 19.36×10^6 CFU/ml). Concerning the effect of shaking at different RPMs on biosurfactant productivity in *P. aeruginosa* PAO1-B13, it was discovered that agitation greatly increased biosurfactant productivity, particularly at 150 rpm, when compared to the static state (Fig. 5).

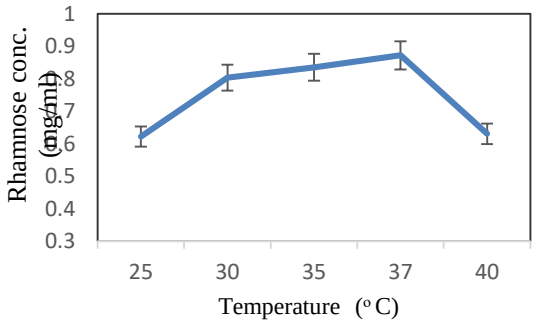

Fig. 2. Effect of different incubation temperatures on biosurfactant production by *P. aeruginosa* PAO1-B13. There is a statistically significant difference (P = <0.001).

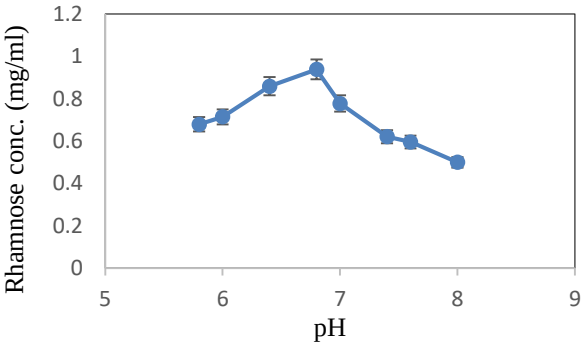

Fig. 3. Effect of different pHs on biosurfactant production by *P. aeruginosa* PAO1-B13. There is a statistically significant difference (P = <0.001).

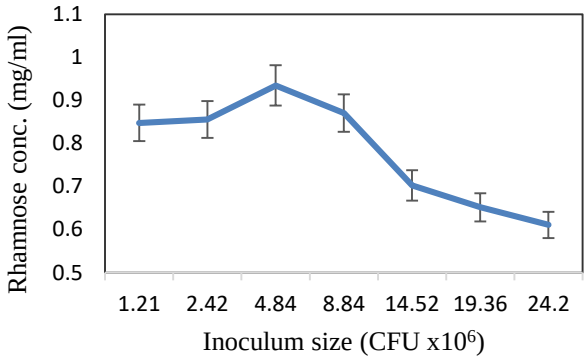

Fig. 4. Effect of different inoculums sizes on biosurfactant production by *P. aeruginosa* PAO1-B13. There is a significant difference of P = 0.001.

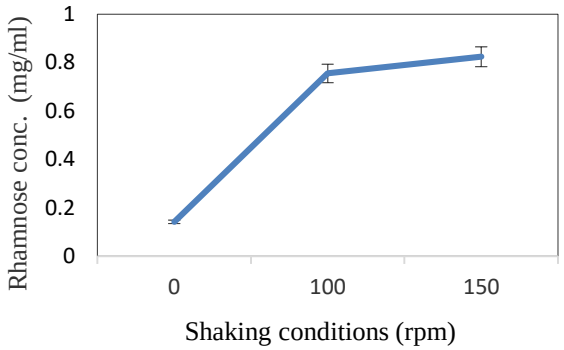

Fig. 5. Effect of static and shaking conditions on biosurfactant production by *P. aeruginosa* PAO1-B13. There is a statistically significant difference (P = <0.001).

Makkar and Cameotra [63] reported that, agitation range has been shown to affect the mass transfer efficiency of both oxygen and medium components, and is considered crucial to the cell growth and biosurfactant development of the strictly aerobic bacterium *P. aeruginosa*, particularly when grown under shaking condition. Results from batch fermentation under different agitation range (100–250 rpm) showed that, as the agitation rate increased from 100 to 200 rpm, rhamnolipid production increased and dry cell weight was also higher from 0.14 ± 0.01 and 0.94 ± 0.05 to 0.3 ± 0.01 and 1.8 ± 0.03 g/l, respectively. In the present study, olive oil waste and sunflower oil are used as the sole carbon sources for rhamnolipid production. The biosurfactant productivity increased up to 0.927 (mg/ml) with sunflower oil (Fig 6). The result is in full agreement with, Karkera et al. [64] who indicated that sunflower-seed oil was the best carbon source for production of rhamnolipid by *P. aeruginosa* san-ai. Reiling et al. [65] elucidated that using low-cost substrates, such as crude or waste materials, has a negative impact on biosurfactant assembly costs. The choice of low-cost raw materials is critical to the method's overall economy since they are responsible for a large portion of the final product cost (about 50%) and therefore minimize waste treatment costs [66]. The effect of different sunflower-seed oil concentrations on BS production by *P. aeruginosa* PAO1-B13, was calculated (Fig 7). By increasing concentration of sunflower-seed oil up to 0.75 ml oil/25 ml medium (3%, v/v), the biosurfactant productivity increased up to 2.052 mg/ml rhamnolipid. Any longer increase in oil concentration resulted in biosurfactant productivity reduction.

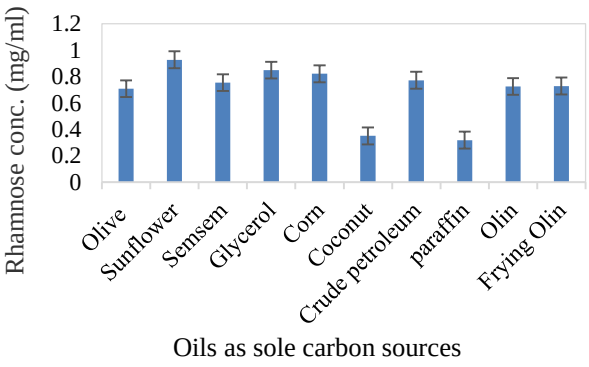

Fig. 6. Effect of various types of oil on biosurfactant production by *P. aeruginosa* PAO1-B13 there's a significant difference (P =0.010).

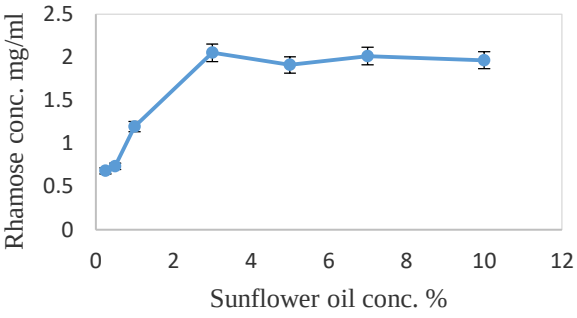

Fig. 7. Effect of different sunflower oil concentrations on biosurfactant production by *P. aeruginosa* PAO1-B13, there is a statistically significant difference (P = 0.010).

On the other hand, the best nitrogen source which stimulated the highest biosurfactant productivity by *P. aeruginosa* PAO1-B13, was NH_4NO_3 which was originally used in the production medium followed by $(NH_4)_2HPO_4$ and gave 2.682, 2.553 mg/ml rhamnolipid respectively (Fig 8). Das and Mukherjee [67] obtained similar results for *P. aeruginosa* R2 and discovered that ammonium nitrate (0.4%) was the best nitrogen source. In another study, Desai and Banat [13] showed that nitrate favored maximum biosurfactant production in *P. aeruginosa*, whereas, *A. paraffineus* preferred urea and Ammonium salts for biosurfactant production. Given the present investigation, Dl-valin showed a high production rate

of rhamnolipid; 5.99 mg/ml compared with control, followed by Glycine with a production rate 5.573 mg/ml. But the rest of the amino acids used during this study had a moderate effect on biosurfactant production by *P. aeruginosa* PAO1-B13. Moreover, aspartic acid results in complete inhibition of biosurfactant production (Fig 9). In the present investigation, 500 ppm of KCL has the highest biosurfactant productivity by *P. aeruginosa PAO1*-B13, among all the tested metallic ions. Moreover, the rest of the metallic ions at all concentrations used had a moderate effect on biosurfactant productivity (Fig 10). Mineral salts are essential factors in increasing rhamnolipid productivity [45]. Small concentrations of multivalent ions such as Mg, Ca, K, Na, and trace element salts were found to increase rhamnolipid production [68]. None of the tested vitamins or growth promoters at all concentrations used exhibited any further increase in the biosurfactant productivity by *P. aeruginosa* PAO1 (Fig. 11).

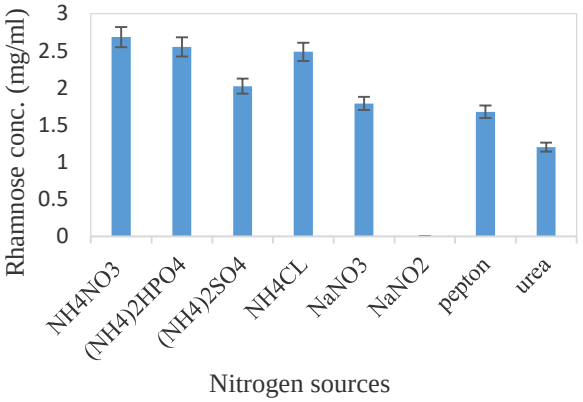

Fig. 8. Effect of different nitrogen sources on biosurfactant production by *P. aeruginosa* PAO1-B13, there is a statistically significant difference (P = 0.003).

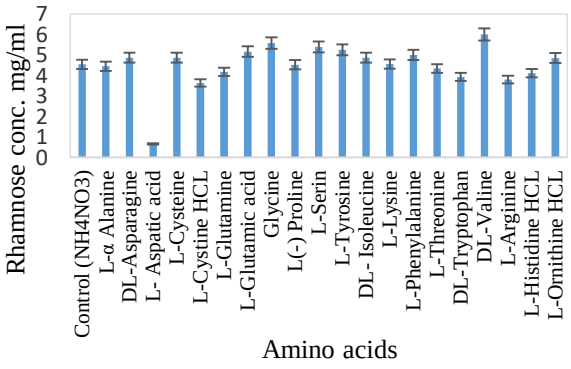

Fig. 9. Effect of different amino acids on biosurfactant production by *P. aeruginosa* PAO1. There is a statistically significant difference (P = <0.001).

3.2. Extraction of the BS

The recovery of biosurfactant produced by *Bacillus cereus* from complex fermentation broth was carried out. Biosurfactant was precipitated and extracted with a solvent system $CHCl_3$:CH_3OH in a ratio of 2:1. The organic phase was evaporated in a rotary evaporator to give a viscous honey extract. A 9.25 g/l of biosurfactant was obtained. [69] indicated that biosurfactant production by *P. aeruginosa* RS29 strain (6 g/L) in the presence of glycerol as the sole source of carbon was higher than biosurfactant production by *P. aeruginosa* NM strain (5.0 g/L) grown on glycerol. by [70, 71] Anthrone assay was used to determine the yield of biosurfactant developed by *P. aeruginosa* strain F23, which was found to be 2.8 g/L. As well as biosurfactant yields of 2g/L and 1.7g/L for *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* strain R2 were recorded by [72,67].

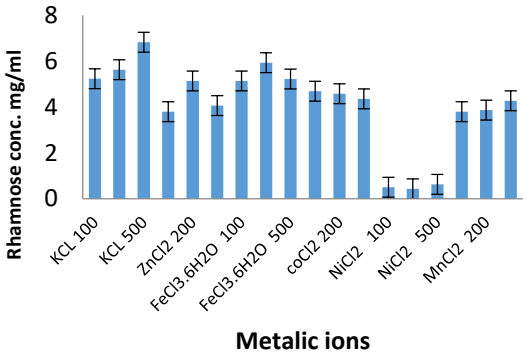

Fig. 10. Relation of different metallic ions on biosurfactant production by *P. aeruginosa* PAO1. There is a statistically significant difference (P = 0.008).

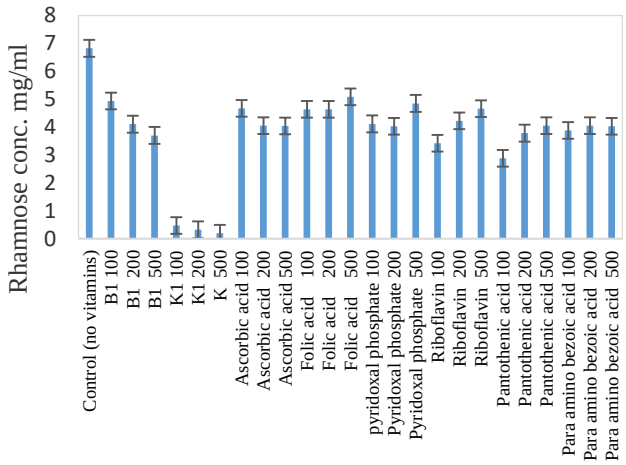

Fig. 11. Effect of different vitamins and growth promoters on the production of BS by *P. aeruginosa* PAO1. There is a statistically significant difference (P = 0.005).

3.3. Applications of the Biosurfactant Produced by P. aeruginosa PAO1-B13

3.3.1. *Antimicrobial activity of rhamnolipid.*

In this respect, several concentrations of the purified rhamnolipid were tested against some resistant pathogenic microorganisms like fungi (*Geotricum candidum*, *Syncephalastrum racemosum*, *Candida albicans*, and *Aspergillus niger*) and Gram-positive bacteria (*Staphylococcus aureus, Enterococcus faecalis, Streptococcus pneumonia, Bacillus subtilis, and* Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Gram-negative bacteria (*P. aeruginosa*, *Escherichia coli, Salmonella typhi, and Klebsiella pneumonia*). Data presented in (Table 2) and (Fig. 12). Asthana et al**.** [73] reported that the antimicrobial activity of rhamnolipid was higher against Gram-positive than Gram-negative bacteria. Many authors reported the antimicrobial effect of pyocyanin and rhamnolipid on some bacteria [74-76]. **Zhao** et al. [77] suggests that a rhamnolipid molecule with both its hydrophobic and hydrophilic groups could insert its fatty acid components into a cell membrane, causing a shift in the cell's conformation.

Table (2) Biological activity of the BS produced by *P. aeruginosa* PAO1-B13, against some pathogenic microorganisms

Sample / Tested microorganisms	Biosurfactant antimicrobial activity (mm)	Standard antibiotics (mm)
FUNGI		*Amphotericin B*
Aspergillus niger (RCMB 02317)	19.2 ± 0.58	22.6± 1.2
Candida albicans (RCMB 05031)	NIZ	20.7± 0.58
Geotricum candidum (RCMB 05097)	21.3 ± 1.2	24.3± 2.1
Syncephalastrum racemosum (RCMB 02763)	16.2 ± 2.1	20.1± 0.63
G + ve bacteria		*Ampicillin*
Staphylococcus aureus (RCMB 010028)	20.2 ± 1.5	25.2± 1.2
Bacillis subtilis (RCMB 010067)	23.4 ± 0.58	32.4± 1.5
Enterococcus faecalis (RCMB 010068)	16.3± 2.1	21.3± 2.1
Streptococcus pneumoniae (RCMB 010010)	NIZ	23.4± 0.63
Methicillin-Resistant *Staphylococcus aureus*		Vancomycine
(MRSA 2658 RCMB)	18.1± 1.2	23.4± 1.5
G - ve bacteria		*Gentamicin*
Pseudomonas aeruginosa (RCMB 010043)	NIZ	21.9± 2.1
Escherichia coli (RCMB 010052)	18.2± 0.58	22.4± 0.63
Salmonella typhi (RCMB 010072)	22.3± 1.5	25.2± 0.58
Klebsiella pneumonia (RCMB 010093)	20.3± 2.1	25.2± 1.2

The test was done using the diffusion agar technique, Well diameter: 6.0 mm .... (100 µl was tested), RCMB: Regional Center for Mycology and Biotechnology Antimicrobial unit test organisms * NIZ: No Inhibition Zone, data are expressed in the form of mean ± SD.

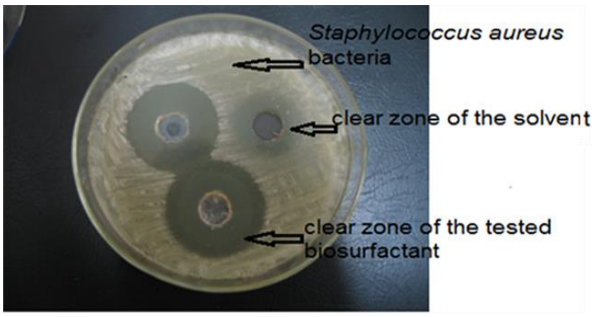

Fig. 12. Antimicrobial activity of rhamnolipid extract against Methicillin-Resistant *Staphylococcus aureus* (MRSA) pathogenic bacteria

3.3.2. Cytotoxicity activity of rhamnolipid biosurfactant

The present study reveals IC50 value of 23.2 and 40.4 µg/well against the Colon Carcinoma cells and Breast Carcinoma cells respectively (Fig 13 & 14). A maximum suppression was observed up to 34.17% and 38.56% at a concentration of 50 µg/well, respectively. The results also reveal that the biosurfactants extracted from *P. aeruginosa* PAO1-B13, show significant anti-proliferation activity against both Colon Carcinoma cell lines and breast carcinoma cells. Which suggests the utilization of the potential of those components in the pharmaceutical industries [78]. Also, the cytotoxic activity against Mammalian cells of African green monkey kidney (VERO) cells was revealed with 50% cell cytotoxic concentration (IC50) = 44.1 µg/well (Fig 15). Thanomsub et al. [79] showed significant anti-proliferation activity against cervical neoplastic cell line (HeLa) when used variable concentrations of purified biosurfactants from *P. aeruginosa* strain PB3A, that gave IC50 value of 125 µg against the HeLa cells and a maximum suppression was obtained up to 30.49% at a concentration of 1000 µg/ml. Pathaka and Pranav [80] reported that di-rhamnolipids have remarkable anti-proliferative activity against human breast cancer cell line (MCF-7) at a minimum inhibitory concentration (MIC) of 1 µg/mL as compared to a minimum inhibitory concentration of 5 µg/mL against human non-small lung cancer cell line (H460). A mono-rhamnolipid isolated from *P. aeruginosa* BN10 was found to have cytotoxic activity against human cancer cell lines [81]. In all of the studied leukemic cell lines, and the epithelial bladder carcinoma cell line, the mono-rhamnolipid RL-1 was the most active, causing 50 reciprocal inhibition of cell viability at lower concentrations than the di-rhamnolipid RL-2[81]. Di-rhamnolipid a (Rha2-C10-C10) and di-rhamnolipid b (Rha2-C10-C12) from *P. aeruginosa* strain B189 also inhibited the expansion of the human breast cancer cell line MCF-7 and thus the insect cell line C6/36, according to Dahrazma and Mulligan [82].

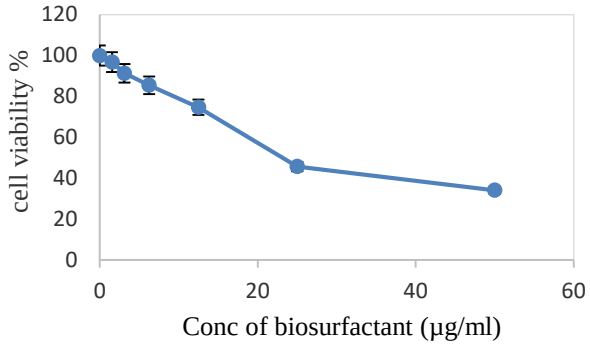

Fig. 13. IC_{50} analysis of biosurfactant against Colon Carcinoma cells.

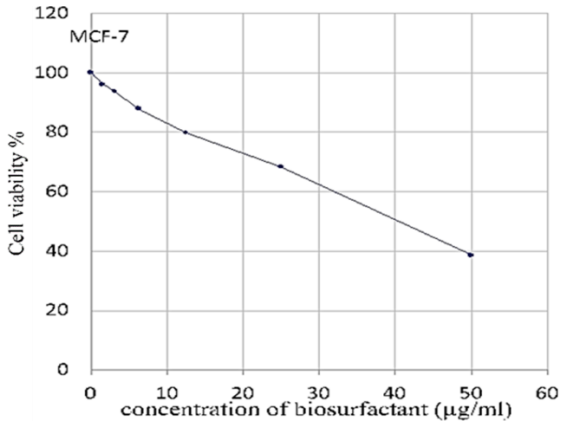

Fig. 14. IC50 analysis of biosurfactant against Breast Carcinoma cells.

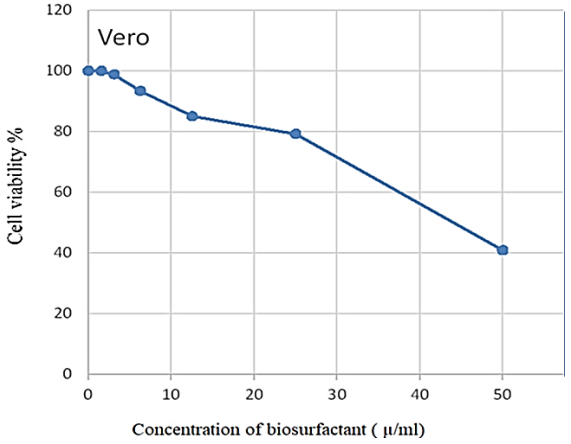

Fig. 15. IC50 analysis of biosurfactant against VERO cell line

3.3.3 Heavy metal recovery by rhamnolipid

Recovery of heavy metals by extracted rhamnolipid was performed. Where 1.0 ml crude rhamnolipid reduced heavy metal concentration of (Ag_2SO_4, $FeCl_3$, $CoCl_2$, $CuSO_4$ and $HgCl_2$) to 44, 48, 52, 48 and 56%, respectively. As shown in table (3) and fig (16a and b). Also, Stevenson et al.[83] reported that the 1.0 ml crude rhamnolipid was successfully applied for the toxic metals, and therefore the heavy metals' recovery, where rhamnolipid significantly eliminated heavy metal concentration to 73%, 65%, 71% for $FeCl_3$, $ZnSO_4$, Pb $(NO_3)_2$ respectively, and 43% in the case of toxic metal i.e. NaF. Hidayati et al. [81] showed that the very best recovery of Pb was up to 14.04% by Acinetobacter sp. Biosurfactant of *Pseudomonas putida* T1. Pornsunthorntawee et al [69] had the very best recovery of Zn and Cu up to six .5% and 2.01% respectively.

Table (3) Calculation table for heavy and toxic metal recovery

Metals	Wt. of filter paper (g)	The efficiency of metal recovery
Ag_2SO_4	0.14	44%
$FeCl_3$	0.13	48%
$CoCl_2$	0.12	52%
$CuSO_4$	0.13	48%
$HgCl_2$	0.11	56%

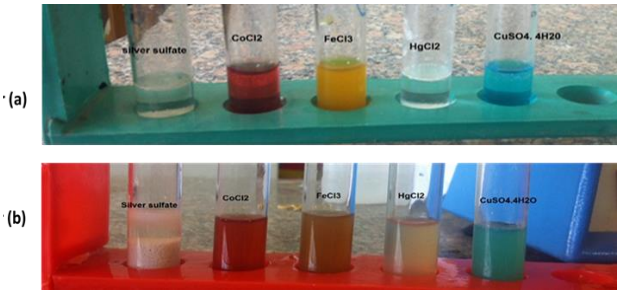

Fig. 16. Upper a) shows the heavy metal concentrations 100 mg/ml while lower b) shows the heavy metal recovery by biosurfactant.

3.4. DNA sequencing and phylogenetic analysis of rhl gene cluster.

The specified forward and reverse primers rh1B2, rh1C4and rh1I2 were used for amplification of genes (*rhlB*, *rhlC* and *rhlI*) respectively. The amplicons showed size of 924, 180, and 477 bp with *rhlB*, *rhlC* and *rhlI* respectively. Here, we report the identification and functional characterization of *rhl* gene cluster. The results show expression of three genes in *P. aeruginosa* PAO1-B13, *rhlB* gene size with (924 bp) encoding rhamnosyltransferase 1, the first glycosyltransferase involved in the production of the mono-rhamnolipid in *P. aeruginosa*, *rhlC* gene with size (180 bp), encoding rhamnosyltransferas 2, the second glycosyltransferase involved in rhamnolipid production, this enzyme involved in the assembly of di-rhamnolipid and also *rhlI* gene with size (477 bp). Locus of *P. aeruginosa* contains *rhlAB*, which encodes rhamnosyltransferase 1 [22], and *rhlR*, which comprises a quorum-sensing system in this pathogen [26] All rhamnolipid gene sequences have been deposited in the GenBank database with

accession numbers JN859048.1 and HM190303.1 for the rhlB gene, JN859048.1, CP002496.1 and DQ3454451 for the rhlI gene, and FN601394.1, FN601390.1 and FN601387.1 for the rhlC gene, and JN859048.1, CP00249In the same accordance, [84] indicated that, three genes in *P. aeruginosa* PAO1-B13, are responsible for the production of rhamnolipid biosurfactant *rhlA*, *rhlB*, and *rhlC* while *rhlA* is involved in the synthesis of the HAAs, the fatty acid dimers, from two 3-hydroxy fatty acid precursors; the membrane-bound *rhlB* rhamnosyltransferase 1 uses dTDP-L-rhamnose and an HAA molecule as precursors, giving monorhamnolipids. These monorhamnolipids are the substrates, together with dTDP-L-rhamnose, of the *rhlC* rhamnosyltransferase 2 to produce dirhamnolipids. In addition, Rahim et al. [21] identified rhlC, a gene responsible for the development of the rhamnosyltransferase involved in the production of dirhamnolipids in *P. aeruginosa.* RhlC is a 325-amino-acid protein with a molecular weight of 35.9 kilodaltons.

4. Conclusions

In this study, *Pseudomonas aeruginosa* PAO1-B13 was isolated from Cooktops of kitchen stove can produce rhamnolipid biosurfactant, The maximum rhamnolipid biosurfactant productivity was obtained at temperature, 37˚C; pH, 6.8; incubation, 7.0 days at 150 rpm; sunflower oil concentration, 3% (0.75 ml/25ml media) is the best carbon source; inoculum size, 4.84 x10^6 CFU; nitrogen source, ammonium nitrate; carbon sources, non; amino acid, Dl-valine; metallic ions, KCl (500 ppm); vitamins, non. Rhamnolipid produced by *Pseudomonas aeruginosa* PAO1-B13, was extracted with CH3Cl:C2H5OH in a ratio 2:1. The organic phase was evaporated in a rotary evaporator to give honey viscous BS. The cluster genes encoding for rhamnolipid biosurfactant production (rhlB, rhlC and rhlI) were isolated and sequenced , some applications for the produced rhamnolipid by *Pseudomonas aeruginosa* PAO1-B13, were carried out to evaluate the importance of the produced rhamnolipid biosurfactant as antimicrobial activity against some tested fungi and bacteria in addition to antitumor activity against both Breast and Colon Carcinoma cell lines. Also, Rhamnolipid exhibited a high recovery of heavy metals from suspensions in the field of bioremediation.

5. Conflicts of interest

There are no conflicts to declare.

6. Acknowledgment

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7. Abbreviations

rRNA: Ribosomal Ribonucleic Acid; HCT116: Human Colon Cancer Cell Line; MCF7: Breast Cancer Cell Line; BHM: Bussnell Hass Medium ; BS: Biosurfactant; RLs: rhamnolipid;.SD: Standard Deviation; DNA: Deoxyribonucleic Acid; BLAST: Basic Local Alignment Search Tool; NCBI: National Center for Biotechnology Information; bp: Base pair; ATCC: American Type Culture Collection; DMSO: Dimethyl Sulfoxide;; MDR: Multidrug Resistant; DCM: Dichloromethane;O.D.: Optical Density; IC50: Half Maximal Inhibitory Concentration; ROS: Reactive Oxygen Species; MPTP: Mitochondria Permeability Transition Pore;Pmole: Pico mole; mN/m: Milli Newten/meter; MELs: Mannosylerythritol lipids.

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