Physiological studies on a locally isolated rhamnolipid producer, *Pseudomonas* strain

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Aim
Bacterial strains were isolated from different soil samples and evaluated for their ability to produce biosurfactants. Preliminary identification was made of the selected strain Kh4, as well as of the type of produced biosurfactant. Finally, the most optimum conditions for improving growth and the percentage of reduction in the culture surface tension (ST) were investigated.

Materials and methods
The genus of the selected strain was preliminarily identified on the basis of some biological tests and characterized pigmentation on a specific medium. Rhamnolipid production was confirmed using both Siegmund Wagner and Dubois methods as well as thin-layer chromatography. Quantitative measurements of the ST of cell-free supernatants were determined using the Du Nouy ring method in order to estimate the amount of biosurfactant produced. Cultivation conditions were studied on the basis of both flask and 7 l stirred tank bioreactor levels.

Results
A total of 66 bacterial strains were isolated from Egyptian soil and assessed for biosurfactant productivity. The selected isolate, preliminarily identified as a *Pseudomonas* spp., has the ability to produce rhamnolipid and hence to decrease the ST of the fermentation medium from 60 to about 35 mN/m. The optimum fermentation medium, used for the growth and maximum decrease in ST, was determined. Some physiological parameters such as the initial pH, incubation temperature, and time were also studied. Furthermore, the effect of pH control during the fermentation production of rhamnolipid in a 7 l stirred bioreactor was also studied. Accordingly, a maximum decrease in the ST of the cell-free culture medium of 32 mN/m was reached, confirming the production of a satisfactory level of rhamnolipid biosurfactant.

Conclusion
These results qualified the locally isolated *Pseudomonas* spp. Kh4 for use in the bioremediation of hydrocarbon-contaminated sites as well as in petroleum industries.

Keywords:
bench top bioreactor, biosurfactants, *Pseudomonas* spp., rhamnolipid, surface tension

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Introduction

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at the interface [1]. They constitute an important class of industrial chemicals widely used in almost every sector of modern industry [2]. Almost all surfactants currently in use are chemically synthesized. However, increasing environmental awareness has led to seriously considering biological surfactants as the most promising alternative to the existing products [3].

Microbial-derived surfactants, or biosurfactants, are surface active compounds containing hydrophobic and hydrophilic moieties, produced by a wide variety of microorganisms such as bacteria, yeast, fungi, and actinomycetes [4]. Biosurfactants have gained considerable interest in recent years and have become an important product of biotechnology for industrial and medical applications. The reason for their popularity as high-value microbial products is primarily their specific action, low toxicity, relative ease of preparation, and widespread applicability [5]. They can be used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, food ingredients [1], and as detergents in various industrial sectors such as petroleum and petrochemicals [6]. They are also used in the manufacture of organic chemicals, foods and beverages [7], cosmetics, and pharmaceuticals, as well as in mining and metallurgy [8], for environmental control [9], and many others. In addition, biosurfactants have many advantages over synthetic ones, including bioavailability, structural diversity, specific activity at extreme temperatures, pH and salinity, productivity on cheap and renewable substrates, capacity for modification, and mass production through biotechnology and genetic engineering [10,11]. Moreover, biosurfactants are more efficient than synthetic surfactants as smaller concentrations are needed to disperse solutions with insoluble phases [12].

Unlike chemically synthesized surfactants, which are classified according to the nature of their polar grouping, biosurfactants are categorized mainly by their chemical composition and their microbial origin [13]. In general, biosurfactants can be broadly classified into low molecular weight (glycolipids, lipopeptides, and flavolipids) [14] and high molecular weight molecules (polysaccharides, proteins, lipopolysaccharides, and lipoproteins) [15].

However, despite the advantages and the diverse potential applications of biosurfactants, attempts at commercial production have been unsuccessful because of the low yields obtained [16,17]. Therefore, to reduce the production cost of biosurfactants, it is important to increase their yield. This could be achieved through different routes, including the search for new biosurfactant-producing strains and the optimization of their production conditions [18].

The aim of this work was to isolate a novel bacterial strain with potent biosurfactant production ability from local Egyptian soil. The optimum cultural conditions were also investigated in order to improve its productivity and thereby reduce its production cost.

Materials and methods

Collection of soil samples

Bacterial strains were isolated from soil samples taken from a depth of 10 cm below the surface of the earth. The soil samples were collected from three different regions as follows:

Sample 1: Soil, contaminated with petroleum oil, obtained from the close vicinity of Khalda petroleum oil well (West desert, Suez-Egypt).
Sample 2: Soil, partially contaminated with petroleum oil, obtained from the land surrounding the above-mentioned petroleum oil well.
Sample 3: Soil, uncontaminated with petroleum oil, obtained from the gardens of the National Research Centre (NRC, Giza, Egypt).

Isolation of bacterial strains

Bacterial strains were isolated from the previously mentioned soil samples according to the method of Bodour *et al*. [19] as follows: 90 ml of sterile normal saline solution was added to 10 g of each soil sample individually. The suspensions were incubated at 30°C and 200 rpm for 7 days as a preliminary treatment of the soil samples. At different time intervals, aliquots of 10 ml were taken from each suspension and serially diluted in sterile normal saline solution under aseptic conditions. An equal volume of 0.1 ml was drawn from each dilution tube and evenly distributed on the surface of solid nutrient agar medium in sterile Petri dishes. These Petri dishes were incubated at 30°C for 48 h and the developed colonies were subcultured, maintained on slants of the same medium, and stored at 4°C for further studies.

Test microorganisms

Biosurfactant production ability of the locally isolated bacterial strains was tested and compared with that of two different test microorganisms known for their high biosurfactant productivity, which were considered as positive controls:

Control A: *Pseudomonas aeruginosa* NRRL B-800.
Control B: *Bacillus subtilis* ATCC 21332.

The first microorganism was obtained from the culture collection of Northern Regional Research Laboratory (NRRL) (Peoria, Illinois, USA), whereas the second strain was purchased from the American Type Culture Collection (Rockville, Maryland, USA).

Preliminary screening of bacterial isolates

Bacterial isolates were subjected to a preliminary screening test in order to assess for their biosurfactant production ability by individually inoculating 0.1 ml of each strain in 6 ml of glycerol medium [20] composed of (g/l) $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (3.0), $MgSO_4.0.7H_2O$ (0.2), and 10 ml glycerol (pH 7.0) for 3 days.

Preliminary identification of the selected isolate

The selected isolate was preliminarily identified using some biological tests carried out using ERIC Electronic RapID Compendium kits (version: 1.0.75; Remel Inc., Lenexa, Kansas, USA). The procedures described by the manufacturer were followed and the colors of the different biochemical reactions were read and analyzed automatically using ERIC windows-based software for RapID systems. Moreover, the isolate enhanced pigment production by its cultivation on Sabouraud maltose agar medium [21] for 3 days at 37°C. The latter is composed of (g/l) maltose (40), peptone (10), and agar 15 (pH 5.8).

Preliminary identification of rhamnolipid production

Test 1: Rhamnolipid production was preliminarily identified using the Siegmund and Wagner method [22], which relies on testing rhamnolipid production using the light blue Siegmund–Wagner (SW) medium. The SW medium was first prepared by adding 10 ml of 2% (w/v) citrimide solution

(cetyltrimethylammonium bromide) and 5 ml of 1% (w/v) methylene blue solution to the components of mineral salts medium (MSM) to reach a final volume of 1 l. The MSM medium [19] is composed of (g/l) glucose (20), $NaNO_3$ (2.5), $MgSO_4.0.7H_2O$ (0.4), NaCl (1.0), KCl (1.0), $CaCl_2.0.2H_2O$ and H_3PO_4 (85%) (10 ml), and trace elements solution (1 ml); the medium was adjusted at pH 7.0–7.2. The trace elements solution is composed of (g/l) $FeSO_4.0.7H_2O$ (0.5), $ZnSO_4.0.7H_2O$ (1.5), $MnSO_4.H_2O$ (1.5), H_3BO_3 (0.3), $CuSO_4.0.5H_2O$ (0.15), and $Na_2MoO_4.0.2H_2O$ (0.1). The glucose content of the medium was autoclaved separately and added under aseptic conditions to the rest of the medium's constituents. The SW medium was solidified by the addition of 15 g/l bacteriological agar and then poured into sterile Petri dishes. The tested strain was cultivated on the surface of the medium and incubated at 37°C for 5 days. Rhamnolipid production was identified by the formation of a dark blue zone around the producing culture.

Test 2: Rhamnolipid production was also confirmed by investigating the presence of rhamnose by thin layer chromatography and using the method of Dubois [23] as follows: 1 ml of 5% redistilled phenol solution (w/v) was added to each 1 ml of the clear supernatant of the fermentation broth and then 5 ml of concentrated H_2SO_4 was added. The mixture was shaken and left for 10 min at room temperature.

Production media and cultivation conditions for shake flask fermentation

Different constitutive media were used for the fermentation production of rhamnolipid by the selected isolated strain. Each medium was adjusted at pH 7.0–7.2 using NaOH or HCl solutions, divided into 50 ml triplicates in 250 ml conical flasks, plugged with cotton wool, and sterilized by autoclaving at about 121°C and 1.5 atm for 15 min. Some components of the media, such as glucose or soy bean, were preferably autoclaved separately and then added to the medium under aseptic conditions. These flasks were inoculated with 10% v/v suspension of the tested isolate, obtained after 24 h incubation, at 200 rpm and 35°C, in MSM medium described above, and then incubated in a reciprocating incubator shaker (New Brunswick Scientifics Co., New Brunswick, New Jersey, USA) at 200 rpm and 30°C for 72 h.

The fermentation media, the components (g/l) of which are mentioned in Table 1, are as follows:

Medium no 1: Basal mineral salts medium [24].
Medium no 2: Glycerol yeast extract medium [25].

Table 1 Chemical composition of different constitutive fermentation media used for cell growth and rhamnolipid production by *Pseudomonas* spp.

Medium component (g/l)	Medium no				
	1	2	3	4	5
$K_2HPO_4.7H_2O$	4.8	1.0			
KH_2PO_4	1.5		3.0	1.4	0.2
$(NH_4)_2SO_4$	1.0		1.0		
$Na_3(C_6H_5O_7).2H_2O$	0.5				
$MgSO_4.7H_2O$		0.5	0.2	0.6	0.2
Na_2HPO_4				2.2	
$FeSO_4.7H_2O$				0.01	
NaCl				0.05	
$CaCl_2$				0.02	
NH_4NO_3					3.0
Glucose	20			20	
Glycerol		30	10		
Soya bean extract					40
Yeast extract	0.1	5.0		0.02	0.5

Medium no 3: Glycerol medium [20].
Medium no 4: Mineral salts yeast extract medium [5].
Medium no 5: Soy bean yeast extracts medium (SBY medium) [26].

Production media and cultivation conditions for stirred tank bioreactor fermentation

The medium used in this experiment was the SBY medium (medium no 5) adjusted at 7.5 and inoculated with 10% v/v suspension of the selected isolate Kh4 obtained after 24 h incubation at 200 rpm and 35°C in MSM medium described above.

Soy bean extract was autoclaved separately and added to the medium along with the previously mentioned inoculum size. The cultivation was carried out at 35°C in two bench top 7 l bioreactors (bioflo-3000; New Brunswick Scientifics Co.), each with four, equally spaced, baffles and a working volume of 5 l. Agitation was performed at 200 rpm using three, four-blade rushton turbine impellers on a single shaft driven by a motor of 1/4 hp. Aeration, using filtered sterile air provided from a compressor, was adjusted at 1 v/v/min. Foam was suppressed, when necessary, by the addition of antifoam reagent SP1 (Th. Goldschmildt AG, Essen, Germany). During the experiment, the dissolved oxygen percentage concentration was analyzed by a polarographic electrode (Ingold, Germany). Samples of 20 ml each were drawn from each vessel with a syringe through an in-line air filter. These samples were assayed for their surface tension (ST), cell growth, and final pH.

Determination of bacterial growth

The growth of the producing bacterial strain was determined spectrophotometrically at 600 nm using a

double beam spectrophotometer (UV/V double beam spectrophotometer; Labomed Instruments, Korea). When necessary, the samples were diluted with unseeded medium so that the optical density readings were in an appropriate range. The dry weight of the cells was also calculated and the following relationship was deduced: CDW calculated in g/l is equivalent to 0.2-fold the OD measurements at 600 nm. Therefore, the growth of the cells was monitored according to their optical density at 600 nm and expressed in terms of their total dry weight in g/l.

Determination of surface tension

ST measurements were determined on cell-free broth of each isolate obtained by centrifuging the corresponding culture at 10 000 rpm in a cooling centrifuge (Biofuge Primo R, Heraeus, Germany) for 20 min These measurements were determined using a Du Nouy ring-type tensiometer (Kruss GmbH, Hamburg, Germany) at room temperature using distilled water for calibrating the instrument [5,27]. Before measuring the ST of a new culture sample, the cup and the ring were serially washed with water and acetone and then left to dry. The ring was preferably flamed until redness to avoid any contamination [28].

Results and discussion

Preliminary screening test of bacterial isolates

Biosurfactant production, in satisfactory amounts, was tested for a total of 66 isolates, using the above-mentioned glycerol medium and compared with that of the test microorganisms. The ability of the isolates to grow on this medium, which eventually proves their ability for biosurfactant production, was investigated on the basis of the optical density measurements at a wavelength of 620 nm. According to Santa Anna *et al*. [20] and Maneerat and Phetrong [29], the bacterial strains can only grow on this specific medium if they are able to produce a certain amount of biosurfactant to decrease the ST between the glycerol content, which represents the sole carbon source in the medium, and the aqueous phase, containing the nitrogen source along with the trace elements, hence making all of the medium's constituents available for the microorganism's use.

The results of this test, summarized in Table 2, showed that 32 strains, most of them isolated from soil sample no 1, were able to grow on glycerol medium. Of them, nine were found more promising, as compared with the test strains growth on the same medium, and hence were further subjected to the quantitative screening test.

Quantitative screening test

In Table 3, isolates from each soil sample were sorted according to their descending ability for biosurfactant production, based on the quantitative measurement of their cell-free, culture broth (glycerol medium) ST, and compared with that of the two identified microorganisms. ST was ascertained using the Du Nouy ring method explained above. The lower the ST of the broth, the greater the production of biosurfactant, and vice versa.

The results showed that a higher % reduction in ST was mainly detected when the fermentation medium was inoculated with bacterial strains, isolated from soil sample no 1, highly contaminated with hydrocarbons (because of its collection from the close vicinity of a petroleum well). Among them, strain Kh4 recorded the highest decrease in ST, from 60 to 35 mN/m, representing a reduction % of more than 41%, which was equivalent to that produced by the identified rhamnolipid producer *P. aeruginosa* NRRL B-800 strain. Lower percentages of ST decrease, ranging between 40 and 39.1%, were detected in the culture filtrate of strains Kh5, Kh8, and Kh16 and were therefore equivalent to that obtained by the identified surfactin producer *B. subtilis* ATCC 21332. However, much lower % reduction of ST, ranging from 35 to 35.83%, was recorded in the fermentation medium

Table 2 Screening results of bacterial strains isolated from different soil samples

Soil sample no	Number of isolates	Number of isolates producing biosurfactant	(%) of isolates producing biosurfactant
1	27	27	100
2	10	2	20
3	29	3	10

Table 3 Surface tension reduction ability of some selected bacterial isolates

Bacterial source	Soil sample									Control	
Sample no	1						2	3			
Isolate code	Kh4	Kh5	Kh8	Kh16	Kh7	Kh11	Ds1	NRC1	NRC3	A[a]	B[b]
ST (mN/m)	35	36	36	36.5	39	39.5	38.5	39	40	35	36
Reduction in ST (%)	41.66	40	40	39.16	35	34.16	35.83	35	33.33	41.66	40

ST, surface tension; [a]Control A: *Pseudomonas aeruginosa* NRRL B-800; [b]Control B: Bacillus subtilis ATCC 21332.

inoculated with strains Kh7, Kh11, and Ds1. The latter was previously isolated from soil sample no 2, which was partially contaminated with hydrocarbons as it was collected from the land surrounding the above-mentioned petroleum well. Strains NRC1 and NRC3, which were previously isolated from sample no 3, resulted in only 35 and 33.33% decrease in their cultures' STs. Sample no 3, collected from the gardens of the NRC, was also most probably partially contaminated with a certain percentage of hydrocarbons originating from plant manure, car exhaust, and others. According to these results, strain Kh4 was selected as the higher biosurfactant-producing isolated strain and therefore subjected to further studies.

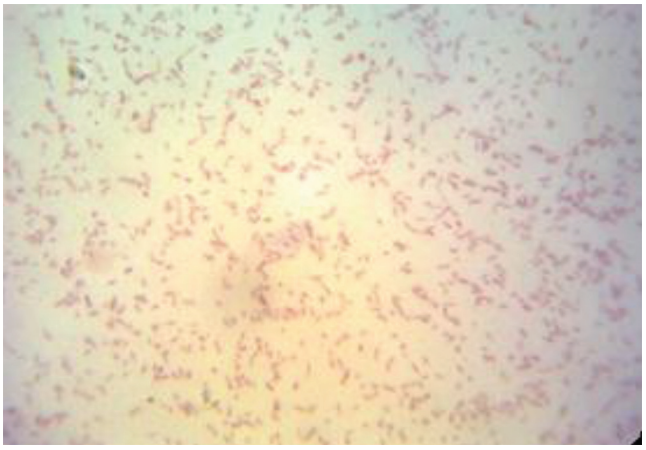
Preliminary identification of the selected strain

The characteristic blue-green pigmentation observed, which was enhanced when the strain was grown on Sabouraud maltose agar medium, suggested that Kh4 most probably belongs to the genus *Pseudomonas*, which is characterized by the production of two specific pigments in different proportions: the yellow fluorescein and the blue pyocyanin [30]. This suggestion was further confirmed using some biochemical tests, including Gram staining, which identified the Kh4 isolate as gram-negative, short-rod bacterium (Fig. 1).

Preliminary identification of the biosurfactant produced by the selected strain

Preliminary identification of the biosurfactant produced by the *Pseudomonas* spp. isolate Kh4 was carried out using the SW method. SW agar is a medium especially developed for the detection of anionic extracellular rhamnolipid known to be, most specifically, produced by *Pseudomonas* spp. Therefore, SW plates were prepared, and spot inoculated with a loopful of the selected Kh4 strain, and then incubated at 37°C for 5 days. A dark blue zone was noticed surrounding the grown culture. This zone reveals the production of extracellular glycolipids (rhamnolipid), which, due to its anionic nature, form a dark blue insoluble compound with the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue present in the medium. Moreover, the samples were tested by thin layer chromatography against standard rhamnose sugar, followed by the Dubois method for the colorimetric determination of sugars. The development of the characteristic orange color reveals the presence of rhamnose, consequently confirming rhamnolipid production.

Figure 1



Microscopic examination of Gram-stained isolated *Pseudomonas* spp.

Effect of different cultivation parameters on cell growth and rhamnolipid production under shake flask condition

Effect of different fermentation media

This experiment was carried out for investigating the most suitable fermentation medium for the biosurfactant production. Five different constitutive media were prepared and inoculated, as described above, and then incubated at 30°C for 72 h on an incubator shaker. Analyses of the changes in the ST of the culture filtrates were carried out on triplicate flasks at the end of the incubation period. The results, represented in Table 4, proved that when the Kh4 strain was grown on medium no 5 the greatest % reduction in the ST of the culture medium of 43.33% was recorded as the latter decreased from 60 to 34 mN/m. This could be because this medium contains soy bean and yeast extracts, which are rich organic sources of C and N, as well as amino acids, sugars, mineral salts, vitamins, and others. The medium also contains NH_4NO_3, which has been reported by many investigators, such as Manresa *et al*. [31], Arino *et al*. [32], Santa Anna *et al*. [20], and Deziel *et al*. [33], to be very effective in the production of rhamnolipids by *Pseudomonas* spp. Moreover, Syldatk *et al*. [34] stated that nitrogen limitation, caused by the depletion of nitrogen (not by limiting its original content in the medium) results in an overproduction of rhamnolipids. In contrast, Barber and Stuckey [35] proved that the assimilation of nitrate as the nitrogen

Table 4 Effect of different constitutive fermentation media on cell growth and surface tension reduction ability of the *Pseudomonas* spp. isolate, cultivated in 250 ml Erlenmeyer flasks

Studied parameters	Medium no				
	1	2	3	4	5
CDW (g/l)	2.45	2.53	2.65	2.61	2.70
Initial ST (mN/m)	60	55	56	60	60
Final ST (mN/m)	37	34	35	38	34
ST reduction (%)	38.33	38.18	37.50	36.66	43.33

ST, surface tension.

source is very slow as it first undergoes a dissimilatory nitrate reduction to ammonium and is then assimilated through the glutamine–glutamate metabolism. They added that this slow assimilation process of nitrates would simulate a condition of limiting nitrogen. The high percentage of ST reduction obtained when medium no 5 was used could be attributed to these two explanations. Lower % reduction in ST was detected in the culture filtrate of medium no 1 to medium no 4.

Furthermore, the results also showed that there was a good correlation between the amount of cell growth and the % reduction in the ST of the cell-free culture broth as the highest CDW of 2.7 g/l was also recorded in the SBY medium. Growth association with rhamnolipids, produced by a *Pseudomonas* spp., has been previously reported by many researchers such as Kitamoto *et al*. [36].

Therefore, medium no 5 (SBY) was selected for rhamnolipid production by the isolate Kh4.

Effect of different initial pH of the fermentation medium

To perform this experiment, the initial pH of medium no 5 was adjusted at different values ranging from 4 to 9 and then inoculated and incubated as previously mentioned. The results, illustrated in Table 5, showed that the maximum % reduction in ST of about 42 to 43% and a maximum CDW of ~2.62 and 3.15 were recorded at neutral pHs of 7 and 7.5, respectively. However, the results also showed that the % reduction in the ST of the cell-free fermentation broth as well as the recorded CDW (g/l) decreased as the initial pH of the medium was shifted to more alkalinity and was much lower when the latter was shifted to more acidity, reaching an insignificant decrease in ST of less than 2% at pH 4.

Effect of different incubation temperatures

To select the most suitable temperature that supports the highest rhamnolipid production, the selected strain was incubated under different temperatures ranging between 25 and 45°C, which is the normal range for the mesophilic group of bacteria. Flasks of 250 ml capacity, containing 50 ml of medium no 5, adjusted at pH 7.5, were inoculated with the selected strain, as previously described, and shaken at 200 rpm for 72 h.

The results, represented in Table 6, indicate that increasing the fermentation temperature resulted in an increase in the CDW (g/l) and % reduction of ST up to 35°C, above which further increase was found to be unfavorable for cell growth and rhamnolipid production as the % ST reduction results were found to be greatly affected.

Effect of different incubation times

The cell growth of the selected isolate and its ability to decrease the ST of medium no 5 (pH 7.5) due to rhamnolipid production were monitored over an incubation period of 144 h at 35°C. The recorded results, illustrated graphically in Fig. 2, showed that rhamnolipid production onset was only detected after 24 h of incubation. The latter was revealed by a small % decrease in ST of 6.6%. This % reduction in ST

Table 5 Effect of differential pH of the fermentation medium on cell growth and surface tension reduction ability of the *Pseudomonas* spp. isolate cultivated in 250 ml Erlenmeyer flasks

pH	CDW (g/l)	Initial ST (mN/m)	Final ST (mN/m)	ST reduction (%)
4	0.84	57	56	1.75
5	1.70	58	50	13.79
5.5	1.78	59	44	25.42
6	1.87	59.5	40	32.77
6.5	2.07	60	41	31.66
7	2.62	60	35	41.66
7.5	3.15	60	34	43.33
8	2.14	61	40	34.42
8.5	2.11	63	43	30.64
9	1.82	65	48	26.15

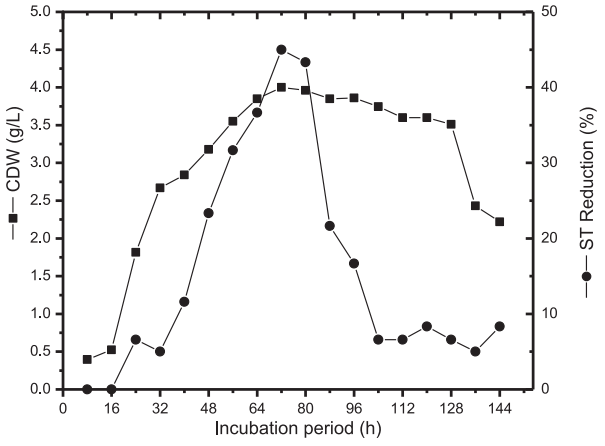
ST, surface tension.

Table 6 Effect of different cultivation temperatures on cell growth and surface tension reduction ability of the *Pseudomonas* spp. isolate cultivated in 250 ml Erlenmeyer flasks

Temperature (°C)	CDW (g/l)	Initial ST (mN/m)	Final ST (mN/m)	ST reduction (%)
25	1.97	60	44	26.66
30	3.12	60	34	43.33
35	3.97	60	33	44.54
40	2.04	58.9	38.5	34.63
45	1.38	57.5	47	18.26

ST, surface tension.

Figure 2



Effect of different incubation periods on cell growth and surface tension reduction ability of the *Pseudomonas* spp. isolate, cultivated in 250 ml Erlenmeyer flasks.

increased linearly with time, reaching a maximum of 45% after 72 h of incubation. After this incubation period, a gradual decrease in the % ST reduction, and therefore in rhamnolipid production, was observed. The recorded decrease in the medium's ST between 104 and 144 h was found to be in the insignificant ranges of 6.6 and 8.3%.

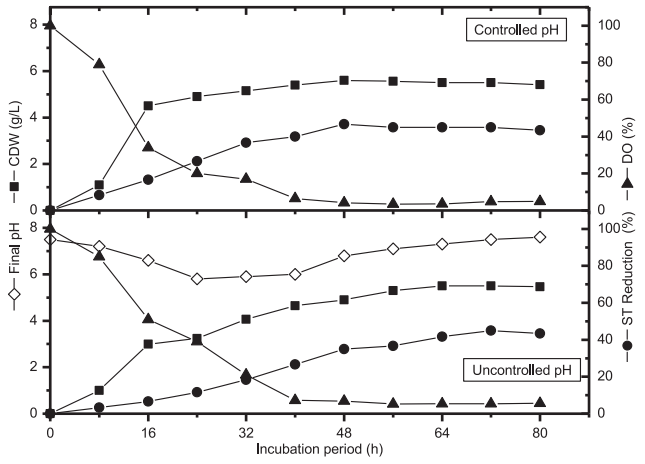
On the other hand, the cell growth was also found to increase gradually in the medium, reaching a maximum of about 4 g/l after 72 h of incubation. After that, cell lysis was suggested, as an obvious decrease in the CDW was observed until the end of the experiment.

From these results, we concluded that the rhamnolipid biosurfactant was produced by the selected isolate Kh4 as a secondary metabolite at the end of its exponential phase of growth, which coincided with the maximum reduction in the ST of the cell-free broth from 60 to 33 mN/m, representing 45% reduction.

Effect of pH-controlled stirred tank bioreactor on cell growth and rhamnolipid production

The aim of this experiment was to study the effect of controlling the pH of the SBY medium in a 7 l stirred tank bioreactor throughout the previously optimized fermentation period on cell growth, on the medium's final ST, and hence on rhamnolipid production. The pH of the culture was either monitored or controlled, at pH 7.5, by the intermittent addition of 1 N acid and base solutions through automatically operated dosing pumps. The results, illustrated in Fig. 3, revealed that, during the first 24 h of incubation, the pH of the uncontrolled fermentation medium initially dropped to the acidic range and then increased gradually, reaching 7–7.6 after 72 h, a phase that more or less coincided with the maximum growth and % ST reduction of 5.5 g/l and 45%, respectively, revealing therefore maximum rhamnolipid production. However, when the culture pH was controlled at 7.5, the exponential, and consequently the stationary, phases of growth were reached earlier. As a result of this, the incubation period, required for maximum growth of 5.6 g/l as well as rhamnolipid production, expressed by the higher % decrease in ST of 46.6%, was satisfactorily reduced from 72 to 48 h.

Figure 3



Effect of different incubation periods on cell growth and surface tension reduction ability of the *Pseudomonas* spp. isolate, cultivated in 7 l stirred tank bioreactor.

Conclusion

Strain Kh4, isolated from a soil contaminated with petroleum oil, was preliminarily identified as a *Pseudomonas* spp. This strain has the ability to grow and lower the ST of different media by the assimilation of different complex, cheap carbon and nitrogen sources, such as soy bean extract, yeast extract, glycerol and glucose, as well as NH_4NO_3. The produced biosurfactant, which caused this decrease in the cell-free broth, was identified as rhamnolipid using the Siegmund–Wagner and Dubois methods. The results of the experiments showed that the highest % reduction in ST of 45% was recorded when this isolate was cultivated on SBY medium, of initial pH adjusted at 7.5, for 72 h at 35°C. However, when the isolated strain was cultivated in a 7 l stirred tank bioreactor, the above-mentioned incubation period of 72 h was satisfactorily reduced by 30% when the pH of the fermentation medium was controlled at 7.5 throughout the course of the experiment.

These results qualified the locally isolated *Pseudomonas* spp. Kh4 to be used for the bioremediation of hydrocarbon-contaminated sites as well as in petroleum industries.

The next step of this study will focus on the rhamnolipid biosurfactant extraction and quantification.

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Conflicts of interest

There are no conflicts of interest.

References

1 Mulligan CN. Environmental applications for biosurfactants. Environ Pollut 2005; 133:183–198.

2 Deleu M, Paquot M. From renewable vegetables resources to microorganisms: new trends in surfactants. C R Chimie 2004; 7:641–646.

3 Suresh Kumar A, Mody K, Jha B. Evaluation of biosurfactant/bioemulsifier production by a marine bacterium. Bull Environ Contam Toxicol 2007; 79:617–621.

4 Datta S, Sonali S, Dipa B. Optimization of culture conditions for biosurfactant production from *Pseudomonas aeruginosa* OCD. J Adv Sci Res 2011; 2:32–36.

5 Abouseoud M, Yataghene A, Amrane A, Maachi R. Biosurfactant production by free and alginate entrapped cells of *Pseudomonas fluorescens*. J Ind Microbiol Biotechnol 2008; 35:1303–1308.

6 Abu-Ruwaida AS, Banat I, Haditirto S, Khamis A. Nutritional requirements and growth characteristics of a biosurfactant-producing *Rhodoccoccus* bacterium. World J Microbiol Biotechnol 1991; 7:53–60.

7 Raaijmakers JM, de Bruijn I, de Kock MJ. Cyclic lipopeptide production by plant-associated Pseudomonas spp.: diversity, activity, biosynthesis, and regulation. Mol Plant Microbe Interact 2006; 19:699–710.

8 Bordoloi NK, Konwar BK. Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons. J Hazard Mater 2009; 170:495–505.

9 Issazadeh K, Sara KR, Saeed Z, Mohammad RR. Antagonism of *Bacillus* species against *Xanthomonas campestrispv. campestris* and *Pectobacterium carotovorum subsp. carotovorum.* Afr J Microbiol Res 2012; 6:1615–1620.

10 Illori MO, Amobi CJ, Odocha AC. Factors affecting biosurfactant production by soil degrading Aeromonas spp. isolated from a tropical environment. Chemosphere 2005; 61:985–992.

11 Raza ZA, Khan MS, Khalid ZM. Evaluation of distant carbon sources in biosurfactant production by a gamma ray induced *Pseudomonas putida* mutant. Process Biochem 2007; 42:686–692.

12 Dehghan-Noude G, Housaindokht M, Bazzaz BS. Isolation, characterization, and investigation of surface and hemolytic activities of a lipopeptide biosurfactant produced by Bacillus subtilis ATCC 6633. J Microbiol 2005; 43:272–276.

13 Guerra-Santos LH, Kapelli O, Flechter A. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl. Microbiol Biotechnol 1986; 24:443–448.

14 Smyth TJP, Perfumo A, Marchant R, Banat LM. Isolation and analysis of low molecular weight microbial glycolipids. Timmis KN, editor *Handbook of hydrocarbon and lipid microbiology*. Berlin, Heidelberg: Springer-Verlag; 2010; 3705–3723.

15 Smyth TJP, Perfumo A, Clean MC, Marchant R, Banat LM. Isolation and analysis of lipopeptides and high molecular weight biosurfactants. Timmis KN, editor *Handbook of hydrocarbon and lipid microbiology*. Berlin, Heidelberg: Springer-Verlag; 2010; 3689–3704.

16 Nitschke M, Pastore GM. Production and properties of a surfactant obtained from Bacillus subtilis grown on cassava waste water. Biores Technol 2006; 97:336–341.

17 Mukherjee S, Das P, Sivapathasekaran C, Sen R. Enhanced production of biosurfactant by a marine bacterium and statistical screening of nutritional parameters. Biomed Eng J 2008; 42:254–260.

18 Gong G, Zheng Z, Chen H, Yuan C, Wang P, Yao L, Yu Z. Enhanced production of surfactin by *Bacillus subtilis* E8 mutant obtained by ion beam implantation. Food Technol Biotechnol 2009; 47:27–31.

19 Bodour AA, Drees KP, Maier RM. Distribution of biosurfactant producing bacteria in undistributed and contaminated arid south western soil. Appl Environ Microbiol 2003; 69:3280–3287.

20 Santa Anna LM, Sebastian GV, Menezes EP, Alves TLM, Santos AS, Pereira NJr, Freire DGM. Production of biosurfactants from *Pseudomonas aeruginosa* PAI isolated in oil environments. Brazil J Chem Eng 2002; 19:159–166.

21 Martineau B, Forget A. Routine use of Sabouraud maltose agar for the rapid detection of the bluish-green pigment of Pseudomonas aeruginosa. J Bacteriol 1958; 76:118–119.

22 Siegmund I, Wagner F. New method for detecting rhamnolipids exerted by Pseudomonas species grown on mineral agar. Biotechnol Tech 1991; 5:265–268.

23 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956; 28:350–356.

24 Tuleva, BK, Ivanov GR, Christova NE. Biosurfactant production by a new *Pseudomonas putida* strain. Z Naturforsch C 2002; 57:356–360.

25 Rashedi H, Assadi MM, Jamshidi E, Bonakdarpour B. Production of rhamnolipids by *Pseudomonas aeruginosa* growing on carbon sources. Int J Environ Sci Technol 2006; 3:297–303.

26 Thanomsub B, Pumeechockchai W, Limtrakul A, Arunrattiyakorn P, Petchleelaha W, Nitoda T, *et al*. Chemical structures and biological activities of rhamnolipids produced by Pseudomonas aeruginosa B189 isolated from milk factory waste. Bioresour Technol 2007; 98:1149–1153.

27 Bodour AA, Maier RM. Application of a modified drop-collapse technique for biosurfactant quantitation and screening of biosurfactant producing microorganisms. J Microbiol Methods 1998; 32:273–280.

28 Youssef NH, Duncan KE, Nagle DP, Savage KN, Knapp RM, McInerney MJ. Comparison of methods to detect biosurfactant production by diverse microorganisms. J Microbiol Methods 2004; 56:339–347.

29 Maneerat RS, Phetrong K. Isolation of biosurfactant producing marine bacteria and characteristics of selected biosurfactant. Songklanakarin J Sci Techno l 2007; 29:781–791.

30 King EO, Ward MK, Raney DE. Two simple media for the demonstration of pyocyanin and fluorescein. J Lab Clin Med 1954; 44:301–307.

31 Manresa MA, Batisda J, Mercade ME, Robert M, De Andres C, Espuny MJ, Guinea J. Kinetic studies on surfactant production by *Pseudomonas aeruginosa* 44T1. J Ind Microbiol Biotechnol 1991; 8:133–136.

32 Arino S, Marchal R, Vandecasteele JP. Identification and production of a rhamnolipidic biosurfactant by a *Pseudomonas* species. Appl Microbiol Biotechnol 1996; 45:162–168.

33 Deziel E, Lepine F, Milot S, Villemur R. rhlA is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas* aeruginosa: 3-(3-hydroxyakano yloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiol 2003; 149:2005–2013.

34 Syldatk C, Lang S, Wagner F, Wray V, Witte L Chemical and physical characterization of four interfacial-active rhamnolipids from Pseudomonas spec. DSM 2874 grown on n-alkanes. Z Naturforsch C 1985; 40:51–60.

35 Barber WP, Stuckey D. Nitrogen removal in a modified anaerobic baffled reactor (ABR): 1, Denitrification. Water Res 2000; 34:2413–2422.

36 Kitamoto D, Isoda H, Nakahara T. Functions and potential applications of glycolipid biosurfactants — from energy-saving materials to gene delivery carriers. J Biosci Bioeng 2002; 94:187–201.