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Production of a new lipoprotein biosurfactant by *Streptomyces* sp. DPUA1566 isolated from lichens collected in the Brazilian Amazon using agroindustry wastes

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ABSTRACT

The present study describes a new biosurfactant produced by *Streptomyces* spp. isolated from lichens from the Brazilian Amazon region. Forty-one *Streptomyces* spp. strains were screened for their ability to produce biosurfactants. A full 2^4-factorial design was used to investigate the effects of pH, percent aeration, agitation and temperature on surface tension and emulsification index after fermentation by *Streptomyces* sp. DPUA1566 isolate, which ensured the highest biosurfactant concentration (1.9 g/l) in a medium containing 10 g/L soybean waste frying oil and 20 g/L corn steep liquor at pH 8.5, 150 rpm, 28 °C and air saturation of 80%. The biosurfactant reduced the surface tension of water from 72 to 28 mN/m, with a critical micelle concentration of 0.08%, and was effective over wide ranges of temperature, pH and salt concentration. The biomolecule, which was characterized as a lipoprotein and denominated bioelan, did not exhibit toxicity against vegetable seeds or brine shrimp. *Streptomyces* sp. DPUA1566 proved to be a promising source of biosurfactant from low-cost waste with potential application either in bioremediation processes or in pharmaceutical and cosmetic industries.

1. Introduction

Biosurfactants are natural surface-active compounds produced by a variety of microorganisms, whose amphiphilic molecules are able to reduce surface tension and interfacial tension of aqueous solutions and hydrocarbon mixtures (Santos et al., 2016). The molecular structure of biosurfactants consists of glycolipids, lipopeptides, phospholipids, neutral lipids and other substances (Bezerra et al., 2018).

Research on biosurfactants has been widened due to their potential use in different sectors such as food, agriculture, pharmaceutical, oil petrochemical, paper and pulp industries (Rocha e Silva et al., 2018). Biosurfactants have recently been attracting attention as promising natural surfactants owing to their advantages over chemical surfactants such as lower toxicity, biodegradability, higher stability in extreme environments and ecological acceptability (Almeida et al., 2016). In recent years, a number of studies have been published on their production by different bacteria, among which *Alcaligenes faecalis, Cronobacter sakazakii* and *Streptomyces* sp. stand out (Bharali et al., 2011; Jain et al., 2012; Khopade et al., 2012a, 2012b; Lamilla et al., 2016, 2018; Santos et al., 2018).

However, biosurfactant production is hindered by low yields and high cost of raw materials, which can account for up to 30% of total production costs; therefore, inexpensive byproducts are increasingly used as carbon and nitrogen sources (Sarubbo et al., 2015), among which are glycerol from biodiesel production, molasses, waste frying oils and ground-nut oil refinery residue, with the addition of corn steep liquor and cassava wastewater (Santos et al., 2016).

Different substrates, including agroindustry residues, have already been tested to produce biosurfactants by the *Streptomyces* genus such as glucose (Colin et al., 2013), glycerol (Elkhawaga, 2018), sunflower oil (Maniyar et al., 2011; Deepa et al., 2015), rapeseed oil (Elsayed et al., 2015), palm oil (Zambry et al., 2017), toluene (Kokare et al., 2007), *n*-

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hexadecane (Lamilla et al., 2018), sugarcane vinasse (Colin et al., 2017) and sugarcane molasses (Korayem et al., 2015).

Recently, a glycoprotein biosurfactant was produced by *Streptomyces* sp. DPUA1559 cultivated in mineral medium containing residual frying soybean oil in our laboratories (Santos et al., 2018).

Considering the need for potential biosurfactant producers using low-cost substrates, the aim of the present study was to investigate the production of biosurfactants by *Streptomyces* spp. isolated from lichens from the Brazilian Amazon using industrial and agro-industrial wastes as substrates.

2. Materials and methods

2.1. Microorganisms and inoculum preparation

Strains of *Streptomyces* spp. isolated from lichens from the Brazilian Amazon region, which are still under characterisation, were kindly provided by the Microorganism Collection of the Department of Parasitology (DPUA) of the Federal University of Amazonas, Manaus, Brazil. They were maintained at 30 °C for 15 days in ISP-2 medium (Pridham et al., 1957) without glucose (0.4% yeast extract; 1% malt extract; 2% agar, pH 7.0). Spores were inoculated in 250-ml Erlenmeyer flasks containing 50 ml of this medium and let to germinate for 48 h in an orbital shaker at 28 °C and 150 rpm. The resulting suspension was used as inoculum for subsequent experiments. The microorganisms were stored in cryotubes (10% v/v glycerol) at − 18 °C and used throughout the present study.

2.2. Preliminary screening for biosurfactant production in defined media

Forty-one *Streptomyces* spp. strains were screened for their ability to produce biosurfactants in two media. Medium A was composed of 10 g/l glycerol as the carbon source and 10 g/l peptone as the nitrogen one. Medium B was composed of 10 g/l *Caryocar brasiliense* vegetable oil as the carbon source and 8 g/l peptone and 20 g/l yeast extract as the nitrogen ones. Both media were prepared in a mineral salt medium with the following composition (g/l): K_2HPO_4 (4.75), NH_4Cl (1.0), $MgSO_4{\cdot}7H_2O$ (0.6), $FeSO_4$ $7H_2O$ (0.01), $ZnSO_4{\cdot}7H_2O$ (0.01), $CaCl_2{\cdot}H_2O$ (0.01) and $MnCl_2$ (0.01), and their pH was adjusted to 7.0. 250-ml Erlenmeyer flasks containing 70 ml of these production media were inoculated with a 1.0% (v/v) cell suspension with 0.8 optical density at 600 nm, corresponding to an inoculum of 10^8 colony-forming units (CFU) per ml, and cultures were carried out at 28 °C and 150 rpm. All experiments were carried out in triplicate.

2.3. Selection of low-cost culture medium

Streptomyces spp. were then tested for their ability to produce biosurfactants in four different low-cost media, in order to select the most performant one. Medium I was made up of 10 g/l soybean oil as the carbon source, 20 g/l corn steep liquor as the nitrogen one in basal mineral salt (MBS) medium; Medium II of 10 g/l soybean waste frying oil as the carbon source and 20 g/l corn steep liquor as the nitrogen one; Medium III of 10 g/l waste motor oil as the carbon source, 20 g/l peptone as the nitrogen one in MBS medium; and Medium IV as Medium II in MBS medium. After pH adjustment to 7.0, cultures were carried out under the same conditions as those of preliminary screening in defined media.

2.4. Growth curve

The growth curve was followed for the best biosurfactant producer (*Streptomyces* sp. DPUA1566). The bacterial strain was cultured at 28 °C and 150 rpm in 250-ml flasks using a 1.0% (v/v) inoculum. Cell concentration, pH, surface tension, emulsification index and product concentration were measured at different time intervals up to 144 h.

2.5. Experimental design

The biosurfactant produced by *Streptomyces* sp. DPUA1566 cultivated in medium supplemented with 10 g/l soybean waste frying oil and 20 g/l corn steep liquor was selected for the experiments. A full 2^4-factorial design was used to determine the effects and interactions of pH (5.5, 7.0 and 8.5), aeration (air saturation) (60%, 70% and 80%), agitation (100, 150 and 200 rpm) and temperature (28, 32 and 36 °C), selected as the independent variables, on surface tension and emulsification indexes of motor oil and soybean oil, selected as responses. All statistical and graphical analyses were carried out using the Statistica 6 program (StatSoft Inc., Tulsa, OK, USA).

2.6. Biomass determination

After centrifuging known-volume samples of fermented broth at 5000 × *g* for 20 min to separate the supernatant, biomass was washed twice with distilled water, and cell dry weight determined after drying at 105 °C for 24 h. Cell mass concentration was calculated by dividing this weight by the sample volume.

2.7. Surface tension and critical micelle concentration

Surface tension was determined in the above cell-free supernatant samples using a tensiometer, model Sigma 700 (KSV Instruments, Helsinki, Finland), and employing the Du Nouy ring method at room temperature. The critical micelle concentration (CMC) was assumed to correspond to the concentration at which micelles began to form leading to sudden changes in surface tension and detergency. CMC was automatically determined by measuring the surface tension of the purified biosurfactant in distilled water up to a constant value (Cooper et al., 1981).

2.8. Emulsification index

The emulsification index (EI) of the culture broth was determined by the addition of 2 ml of a hydrocarbon substance (*n*-hexadecane, motor oil, waste motor oil and corn, soybean, sunflower, canola vegetable oils) to 2 ml of cell-free broth in a 15-ml graduated tube. The mixture was mixed vigorously for 2 min and allowed to stand for 24 h at room temperature. EI after this time (EI_{24}) was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100 (Cooper and Goldenberg, 1987), and expressed as a percentage.

2.9. Effect of environmental factors on biosurfactant activity

The influence of different environmental factors on biosurfactant activity was investigated in the cell-free broth in terms of variations in surface tension and emulsification index. In particular, the effect of salinity was investigated at 28 °C and pH 7.0 adding NaCl at different concentrations (2–12%, w/v), that of temperature (4, 28, 70, 100 and 120 °C) at pH 8.5 during 60 min, that of heating time (10, 20, 40 and 60 min) at 90 °C and pH 7.0, and that of pH (2, 4, 6, 8, 10, 12 and 14) at 28 °C after adjustment with 6.0 M NaOH or HCl (Silva et al., 2010).

2.10. Separation and recovery of biosurfactant

Biosurfactant was separated from the cell-free culture broth, which was previously acidified to pH 2.0 with 6.0 M HCl. Liquid-liquid extraction was performed with two equal volumes of a chloroform: methanol (2:1, v/v) mixture (Javaheri et al., 1985). The precipitated biosurfactant was collected by centrifugation at 3000 × *g* for 2 min. The organic phase was removed, re-suspended in distilled water, adjusted to pH 7.0 and dried at 37 °C to determine the concentration of isolated biosurfactant (g/l).

2.11. Biosurfactant characterisation by thin-layer chromatography

A sample (0.1 g) of the recovered biosurfactant was dissolved in methanol and analysed using thin-layer chromatography on silica gel plates (G 60; Merck, Darmstadt, Germany). Chromatograms were developed with chloroform:methanol:acetic acid (65:15:2, v/v) and detection was performed using the following methods: (1) exposure to iodine vapour for lipid staining, (2) exposure to Molish reagent for sugar detection and (3) exposure to 1% ninhydrin solution for free amino groups. Reagents were sprayed, and plates heated for 30–40 min at 110 °C until colour appearance (Parra et al., 1989; Horowitz et al., 1990).

2.12. Biochemical composition of biosurfactant

Total protein concentration was determined by the Bradford (1976) method, using Coomassie Brilliant Blue G and absorbance measurements at 595 nm with bovine serum albumin as the standard. Lipid extraction was performed using the method described by Bligh and Dyer (1959).

2.13. Fatty acid composition of biosurfactant

Fatty acids composition of the isolated biosurfactant was analysed using a gas chromatograph, model MASTER GC (Dani Instruments, Cologno Monzese, Italy), equipped with FID detector, after esterification to their corresponding methyl ethers as described by Hartman and Lago (1973). Analyses were carried out with a DB-WAX capillary column (30 m × 0.53 mm × 0.25 mm), with helium as the carrier gas (5.0 ml/min) and temperature programming from 800 °C (40 °C/min) up to 1100 °C for a total time of 250 min. The sample volume was 1 µl. Chromatographic peaks were identified by comparing the chromatograms with those of the standard mixture of fatty acid methyl esters (Sigma Chemical Co., St. Louis, MO, USA).

2.14. FTIR spectra of dried biosurfactant

Fourier transform infrared spectroscopy (FTIR) was used to elucidate the biosurfactant chemical structure through functional group identification. The sample for FTIR analysis was prepared by grinding 1.0 mg of freeze-dried biosurfactant with 100 mg of dry KBr and pressing the mixture with a 7500-kg weight for 30 s to obtain a pellet. FTIR spectrum was recorded in the 4000–400 cm^{-1} region in a Varian 640-IR spectrometer equipped with a cooled DLaTGS detector.

2.15. Biosurfactant toxicity towards Artemia salina

Toxicity of the isolated biosurfactant was assayed using brine shrimp (*Artemia salina*) as the toxicity indicator. Brine shrimp eggs were obtained from a local store, and larvae were used within one day of hatching. After dilution of a biosurfactant solution at the CMC (80 mg/l) with 33 mg/l saline water up to 1, 10 and 50 mg/l, assays were conducted in 10-ml glass tubes containing 10 brine shrimp larvae in 5 ml of saline water per tube. The cell-free broth containing the biosurfactant was also tested. The brine shrimp larvae in each tube were tested using 5 ml of each biosurfactant solution at different concentration. The tubes were examined for 24 h to determine the mortality rate (Meyer et al., 1982). The toxicity threshold concentration was defined as the lowest biosurfactant concentration per 100 ml of saline water able to kill brine shrimp within 24 h. Each test was run in triplicate, using saline water as the control.

2.16. Biosurfactant phytotoxicity assay

Biosurfactant phytotoxicity was evaluated by a static test involving the seed germination and root elongation of lettuce (*Lactuca sativa* L.) and cabbage (*Brassica oleracea* L.) (Tiquia et al., 1996). Solutions of the isolated biosurfactant were prepared with distilled water at concentrations of 1, 10, 100 and 80 mg/l (CMC). Toxicity was determined in sterilised Petri dishes (1 cm × 10 cm) containing filter paper. After pre-treatment with sodium hypochlorite, ten seeds were inoculated in each Petri dish containing 5 ml of test solution. After five days of incubation at 28 °C in the dark, seed germination, root elongation (≥ 5 mm) and germination index (GI) were determined as follows:

$$\text{Relative seed germination}(\%) = \frac{n_s}{n_c}\text{x}100 \quad (1)$$

where n_s it the number of seeds germinated in the sample and n_c that in the control,

$$\text{Relative root length}(\%) = \frac{L_s}{L_c}\text{x}100 \quad (2)$$

where L_s is the mean root length in the sample and L_c that in the control,

$$\text{GI}(\%) = [(\%\text{seed germination}) \times (\%\text{root length})]\text{x}100 \quad (3)$$

2.17. Oil displacement analysis

The oil displacement test is often used to measure the diameter of the clear zone appearing after dropping a surfactant-containing solution on an oil-water interphase, and the binomial diameter allows evaluating the effectiveness of a given biosurfactant to reduce the surface tension. In the present study, the oil displacement test was adapted from the method described by Youssef et al. (2004). Briefly, after addition of 40 ml of Milli-Q water to a Petri dish with 15-cm diameter, 2.0 ml of motor oil were added at the centre of the dish, and 10 µl of cell culture supernatant were added onto the surface of the oil layer. Mean diameters of clear halos under visible light induced by cell culture were measured with a digital caliper (Digimess, São Paulo, SP, Brazil) and expressed as fraction of Petri dish diameter.

2.18. Statistical analysis

The Analysis of Variance (ANOVA) was used to check statistically significant differences between the average values of responses induced by variations in the independent variables. For this purpose, after preliminary comparison among data that composed each midpoint with the help of box plot, the one-way ANOVA test was used. Differences were considered statistically significant when the significance level (p) was < 0.05.

3. Results and discussion

3.1. Screening for biosurfactant production

The search for promising biosurfactant-producing microorganisms has been growing in the last years, with special concern to strains of actinobacteria, although theses bacteria are not known as traditional biosurfactant producers (Baoune et al., 2018; Deepa et al., 2015; Duddu et al., 2015).

Screening of *Streptomyces* spp. isolates for biosurfactant production was carried out using two culture media. Surface tension of the production medium decreased from its initial value of 59.0 mN/m to less than 30 mN/m at the end of the incubation period, hence proving the ability of the strains to grow and use glycerol and *Cariocar brasiliense* vegetable oil as carbon sources for biosurfactant production (Table 1). Based on the screening results, 24.4% of the *Streptomyces* spp. DPUA strains (1542, 1543, 1547, 1550, 1559, 1566, 1572, 1573, 1595 and 1611) were selected for their high biosurfactant production potential, and the DPUA1566 one proved to be the best producer (25.3 mN/m reduction of surface tension). A similar result was reported for the

Table 1
Surface tension of cell-free media after 144-h cultivations of different biosurfactant-producing isolates of *Streptomyces* spp. Results are expressed as means ± standard deviation.

Streptomyces DPUA	Surface tension (mN/m)		*Streptomyces* DPUA	Surface tension (mN/m)	
	Medium A[a]	Medium B[b]		Medium A	Medium B
1542	50.1 ± 0.5	29.5 ± 0.2	**1578**	50.3 ± 0.5	40.3 ± 0.5
1543	51.7 ± 0.7	26.3 ± 0.4	**1579**	50.6 ± 0.6	49.9 ± 0.3
1545	45.6 ± 0.6	33.9 ± 0.3	**1580**	48.7 ± 0.7	47.5 ± 0.5
1546	39.0 ± 0.5	32.3 ± 0.5	**1581**	50.9 ± 0.5	45.0 ± 0.5
1547	46.6 ± 0.3	27.4 ± 0.7	**1582**	42.3 ± 0.4	49.4 ± 0.7
1550	45.0 ± 0.9	27.4 ± 0.6	**1583**	42.9 ± 0.5	44.2 ± 0.5
1552	38.1 ± 0.5	45.4 ± 0.7	**1585**	44.3 ± 0.6	39.1 ± 0.8
1553	40.9 ± 0.7	47.3 ± 0.5	**1586**	50.2 ± 0.8	40.9 ± 0.5
1554	47.3 ± 0.8	40.0 ± 0.2	**1587**	49.02 ± 0.7	49.0 ± 0.6
1556	42.9 ± 0.9	39.3 ± 0.8	**1591**	45.9 ± 0.9	31.8 ± 0.3
1557	47.2 ± 0.6	42.4 ± 0.5	**1595**	47.0 ± 0.8	27.1 ± 0.2
1559	49.3 ± 0.3	29.2 ± 0.5	**1598**	50.0 ± 0.5	41.0 ± 0.5
1560	43.6 ± 0.5	36.6 ± 0.4	**1600**	46.5 ± 0.5	42.8 ± 0.8
1561	45.7 ± 0.8	42.5 ± 0.3	**1602**	48.8 ± 0.7	43.1 ± 0.5
1563	43.6 ± 0.3	44.6 ± 0.8	**1605**	48.7 ± 0.8	35.6 ± 0.7
1564	43.3 ± 0.2	42.8 ± 0.7	**1606**	48.4 ± 0.5	40.6 ± 0.6
1566	39.4 ± 0.4	25.2 ± 0.6	**1609**	45.1 ± 0.9	31.0 ± 0.5
1568	46.6 ± 0.5	31.1 ± 0.4	**1610**	44.8 ± 0.5	49.8 ± 0.3
1570	45.0 ± 0.3	38.5 ± 0.6	**1611**	48.8 ± 0.5	27.0 ± 0.5
1572	29.6 ± 0.5	43.3 ± 0.5	**1612**	45.3 ± 0.5	43.8 ± 0.3
1573	41.1 ± 0.5	26.4 ± 0.8	**Average**	58.5 ± 0.5	58.9 ± 0.5

[a] Medium A: 10 g/l glycerol and 10 g/l peptone.
[b] Medium B: 10 g/l *Caryocar brasiliense* vegetable oil, 8 g/l peptone and 20 g/l yeast extract.

biosurfactant produced by *Streptomyces* sp. B3 isolated from marine environment, with a reduction of medium surface tension as high as 29 mN/m (Khopade et al., 2012a).

3.2. Selection of low-cost culture medium

Table 2 shows the production of biosurfactant by the selected ten *Streptomyces* spp. DPUA strains using corn steep liquor, waste motor oil, soybean waste frying oil and glycerol as substrates. The best substrates for biosurfactant production were selected based on the crude biosurfactant concentration and reduction of cell-free broth surface tension.

Even though all strains and substrates tested allowed for satisfactory bacterial growth and biosurfactant production, except for motor oil, the strain *Streptomyces* sp. DPUA1566 exhibited the highest growth and ensured the lowest surface tension (24.7 mN/m); therefore, it was selected as a biosurfactant producer. The highest biosurfactant concentration (1.90 g/l) was achieved with this strain in Medium II made up of 10 g/L soybean waste frying oil supplemented with 20 g/L corn steep liquor, which was then selected as culture medium in subsequent experiments.

As is well known, the selection of an appropriate substrate is a crucial step in biosurfactant production. As raw materials account for 10–30% of the overall cost (Bezerra et al., 2018) its success strongly depends on the development of cheap processes based on the use of low-cost substrates. In this respect, whereas waste motor oil was unsuccessful, soybean waste frying oil and corn steep liquor may be interesting alternative ingredients for biosurfactant production, because they are much cheaper than others. A wide variety of industrial and agro-industrial wastes have been used for this purpose. To give only a

Table 2
Surface tension and biosurfactant concentration detected in different culture media after 144-h cultivations of ten *Streptomyces* spp. DPUA isolates. Results are expressed as means ± standard deviation.

Isolate DPUA	Medium I[a]		Medium II[b]		Medium III[c]		Medium IV[d]	
	Surface tension (mN/m)	Biosurfactant concentration (g/l)	Surface tension (mN/m)	Biosurfactant concentration (g/l)	Surface tension (mN/m)	Biosurfactant concentration (g/l)	Surface tension (mN/m)	Biosurfactant concentration (g/l)
1542	42.7 ± 0.8	0.03 ± 0.01	39.7 ± 0.8	0.10 ± 0.06	52.5 ± 0.4	0.01 ± 0.01	36.4 ± 0.7	0.6 ± 0.21
1543	44.4 ± 0.7	0.02 ± 0.01	41.2 ± 0.6	0.10 ± 0.03	44.3 ± 0.3	0.01 ± 0.01	32.4 ± 0.6	0.90 ± 0.15
1547	46.7 ± 0.9	0.01 ± 0.01	37.3 ± 0.5	0.10 ± 0.05	45.5 ± 0.8	0.01 ± 0.01	34.2 ± 0.8	0.80 ± 0.12
1550	38.3 ± 0.6	0.12 ± 0.07	32.6 ± 0.3	0.30 ± 0.08	42.8 ± 0.9	0.02 ± 0.01	26.7 ± 0.7	1.50 ± 0.14
1559	42.8 ± 0.8	0.03 ± 0.02	36.7 ± 0.8	0.20 ± 0.07	42.2 ± 0.5	0.02 ± 0.01	28.4 ± 0.8	1.30 ± 0.11
1566	41.0 ± 0.6	0.02 ± 0.01	26.1 ± 0.3	1.90 ± 0.20	50.1 ± 0.4	0.01 ± 0.01	24.7 ± 0.4	1.80 ± 0.20
1572	45.9 ± 0.7	0.01 ± 0.01	33.2 ± 0.4	0.40 ± 0.01	48.0 ± 0.6	0.01 ± 0.01	38.7 ± 0.6	0.30 ± 0.05
1573	37.3 ± 0.6	0.15 ± 0.09	37.5 ± 0.5	0.10 ± 0.01	56.3 ± 0.9	0.01 ± 0.01	39.3 ± 0.5	0.20 ± 0.02
1595	41.2 ± 0.5	0.02 ± 0.01	30.0 ± 0.8	0.90 ± 0.02	53.0 ± 0.5	0.01 ± 0.01	29.0 ± 0.5	1.20 ± 0.05
1611	40.0 ± 0.4	0.04 ± 0.02	34.1 ± 0.5	0.10 ± 0.01	48.7 ± 0.3	0.01 ± 0.01	33.5 ± 0.7	0.50 ± 0.02
Mean	52.8 ± 0.6	ND	52.3 ± 0.4	ND	59.3 ± 0.7	ND	58.8 ± 0.8	ND

ND: not determined.
[a] Medium I: soybean oil (10 g/l) + corn steep liquor (20 g/l) in mineral salt medium.
[b] Medium II: soybean waste frying oil (10 g/l) + corn steep liquor (20 g/l).
[c] Medium III: waste motor oil (10 g/l) + peptone (20 g/l) in mineral salt medium.
[d] Medium IV: soybean waste frying oil (10 g/l) + peptone (20 g/l) in mineral salt medium.

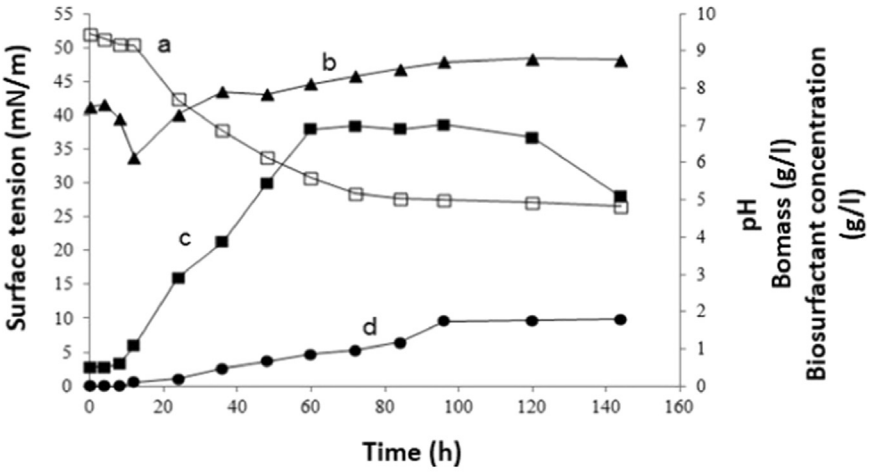

Fig. 1. (a) Surface tension□, (b) pH, ▲, (c) biomass concentration, ■, (d) biosurfactant concentration, •, during *Streptomyces* sp. DPUA1566 cultivation in low-cost medium made up of 10 g/l soybean waste frying oil and 20 g/l corn steep liquor.

few examples, *Pseudomonas aeruginosa* and *Candida sphaerica* were able to produce 8.0 and 4.5 g/l biosurfactants on a mineral medium formulated with glycerol (Khopade et al., 2012a) and a ground-nut oil refinery residue in combination with corn steep liquor (Sobrinho et al., 2008), respectively. Korayem et al. (2015), who tested various raw materials as substrates, found that biosurfactant secretion by *Streptomyces* sp. 5S was dependent on the carbon source. Other studies demonstrated that organic nitrogen sources are better than the inorganic ones (Elkhawaga, 2018; Kalyani et al., 2014).

3.3. Growth curve and biosurfactant production

The growth curve and biosurfactant production by *Streptomyces* sp. DPUA1566 in a low-cost medium containing 10 g/l soybean waste frying oil as the carbon source and 20 g/l corn steep liquor as the nitrogen one were followed for 144 h to establish the relationship between cell growth and biosurfactant surface activity (Fig. 1).

Maximum biosurfactant production (1.9 g/l) was achieved in the stationary growth phase after 96 h, suggesting that the biosurfactant was likely produced mainly as a growth-associate primary metabolite during the exponential phase and only in minor extent during the stationary one. Growth-associated production has already been described for biosurfactants produced by *P. aeruginosa*, *Bacillus subtilis* and *Rhodococcus erytropolia* (Xia et al., 2011). Khopade et al. (2012a) observed that biosurfactant production by *Streptomyces* species B3 and surface tension reduction were dependent on growth.

As far as the effect of pH is concerned, *Streptomyces* sp. DPUA1566 was able to produce biosurfactant in a pH range of 7.5–8.9, with maximum biosurfactant concentration at pH 8.5 (Fig. 1).

3.4. Experimental design

A 2^4-factorial design was used to identify those factors that played a significant role in biosurfactant production by *Streptomyces* sp. DPUA1566 cultivated in the medium supplemented with 10 g/l soybean waste frying oil and 20 g/l corn steep liquor. For this purpose, the pH, temperature, agitation and air saturation were selected as the independent variables and the reduction of surface tension and emulsification indices of waste motor oil and soybean oil as the responses. As can be seen in Table 3, surface tension and emulsification indexes varied markedly, from 50.8 to 26.1 mN/m and from 10.8% to 95.2%, respectively.

Fig. 2 illustrates the effects, in the form of Pareto charts (with a 95% of confidence level), of the above variables as well as the interactions among them on each of the selected responses. From the statistical standpoint, pH was by far the variable that mostly influenced all the three responses, followed by aeration and pH-aeration interaction. In particular, Fig. 2(a) shows that an increase in pH from 5.5 to 8.5 and a simultaneous increase in percent aeration from 60% to 80% (v/v) led to a reduction in surface tension of cell-free culture medium, while the pH-aeration interaction led to an increase. Fig. 2(b) shows that the same pH increase and the pH-aeration interaction had positive influences on the soybean oil emulsification index, while an increase in percent aeration from 60% to 80% (v/v) a negative one. Finally, Fig. 2(c) shows that an increase in pH, a decrease in percent aeration and the aeration-agitation or pH-aeration interaction led to statistically significant increases in waste motor oil emulsification index.

A 70.1% increase in the emulsification index was also observed with the biosurfactant produced by *Streptomyces griseoplanus* NRRL-ISP 5009, SM1, when the initial pH of culture medium was increased from 5 to 7, whereas no significant variation occurred under alkaline conditions (Elkhawaga, 2018).

Since the agitation speed (rpm) was reported to have an impact on biosurfactant production influencing mass transfer of both oxygen and medium components (Santos et al., 2016), it is likely that its increase favoured the release of biosurfactant attached to cell wall, thereby increasing its concentration in the culture medium. This result agrees with the observations of Zambry et al. (2017), who obtained maximum biosurfactant production by the *Streptomyces* isolate R1 cultivated in a 3-L bioreactor, corresponding to the lowest surface tension (40.5 mN/m), just at the highest agitation speed.

As far as the effect of temperature is concerned, the results of the present study revealed that the production of biosurfactant by *Streptomyces* sp. DPUA1566 reached its maximum at 28 and 32 °C. However, since the higher the temperature, the more expensive the process, 28 °C was selected as an optimum for further experiments. According to Elkhawaga (2018), temperature was one of the critical parameters in biosurfactant production by *S. griseoplanus* NRRL-ISP 5009, SM1, that achieved its maximum value when the isolate was grown at 30 °C.

Korayem et al. (2015), using experimental designs, found that nutrients such as treated molasses, as well as environmental factors such as incubation time and inoculum size significantly influenced biosurfactant production by an isolate of *Streptomyces*, whose cultivation in the optimized medium increased the emulsification index from 31.7% to 42.7%.

3.5. Surface tension and critical micelle concentration

A low critical micelle concentration (CMC) and the ability to lower surface tension of aqueous solutions are considered important properties of a potent surface-active agent (Silva et al., 2014). A CMC of 0.08% (800 mg/l) was determined for the isolated biosurfactant by measuring surface tension of solutions at different concentrations (Fig. 3). At such

Table 3
2^4-Full factorial design and experimental results of *Streptomyces* sp. DPUA1566 cultivations in medium supplemented with 10 g/l soybean waste frying oil and 20 g/l corn steep liquor after 144 h. Results are expressed as means ± standard deviation.

Run	pH	Percent aeration (%)	Agitation speed (rpm)	Temperature (°C)	Surface Tension (mN/m)	Emulsification index (%)	
						Waste motor oil	Soybean oil
1	5.5	60	100	28	28.1 ± 0.5	90.3 ± 2.5	58.0 ± 0.9
2	8.5	60	100	28	26.1 ± 0.6	93.0 ± 1.9	60.5 ± 2.7
3	5.5	80	100	28	50.8 ± 0.5	38.2 ± 3.0	10.8 ± 2.5
4	8.5	80	100	28	27.8 ± 0.7	91.8 ± 1.8	59.3 ± 2.0
5	5.5	60	200	28	44.6 ± 0.4	50.4 ± 1.6	14.4 ± 0.5
6	8.5	60	200	28	28.4 ± 0.3	90.8 ± 0.9	57.8 ± 1.2
7	5.5	80	200	28	42.6 ± 0.4	65.2 ± 1.5	16.1 ± 1.0
8	8.5	80	200	28	26.9 ± 0.5	92.4 ± 2.1	60.0 ± 2.0
9	5.5	60	100	36	31.3 ± 0.2	89.4 ± 2.3	53.1 ± 0.8
10	8.5	60	100	36	32.0 ± 0.5	87.6 ± 0.5	51.8 ± 1.7
11	5.5	80	100	36	46.6 ± 0.6	48.7 ± 0.9	16.9 ± 1.0
12	8.5	80	100	36	26.4 ± 0.6	92.9 ± 1.5	59.9 ± 2.5
13	5.5	60	200	36	28.7 ± 0.5	90.7 ± 0.5	59.2 ± 1.3
14	8.5	60	200	36	32.9 ± 0.7	83.1 ± 1.8	52.3 ± 2.0
15	5.5	80	200	36	36.3 ± 0.4	80.3 ± 2.7	46.5 ± 0.9
16	8.5	80	200	36	26.7 ± 0.5	92.8 ± 1.5	59.2 ± 1.5
17	7.0	70	150	32	26.1 ± 0.4	95.1 ± 1.0	61.8 ± 0.5
18	7.0	70	150	32	26.2 ± 0.3	94.4 ± 0.9	60.9 ± 1.8
19	7.0	70	150	32	26.5 ± 0.3	93.8 ± 2.0	60.1 ± 1.6
20	7.0	70	150	32	26.3 ± 0.5	94.6 ± 1.4	60.7 ± 2.0

a CMC, surface tension was reduced from 72 mN/m in pure water to 28 mN/m, a value that falls within the range reported in the literature for other purified biosurfactants at their CMCs (27–35 mN/m) (Santos et al., 2016). On the other hand, Elkhawaga (2018) observed a surface tension reduction from 68 to only 40 mN/m when *S. griseoplanus* NRRL-ISP5009, SM1, biosurfactant was added to water up to a concentration of 110 mg/l.

3.6. Emulsification index (EI_{24})

The emulsifying activity of a biosurfactant is its capability of retaining emulsion of hydrocarbons or oils in water, which, in addition to surface activity, is crucial for several environmental and industrial applications (Campos et al., 2013). The isolated biosurfactant efficiently emulsified hydrocarbons and oils. As suggested by high emulsification indices up to 24 h, the formation of a stable emulsion was observed with soybean oil (60%), *Cariocar brasiliense* oil (65%), motor oil and waste motor oil (100%), whereas canola oil (35%) and *n*-hexadecane (30%) were not effectively emulsified. These results indicate that *Streptomyces* sp. DPUA1566 biosurfactant has a high emulsification specificity toward long-chain hydrocarbons, like that reported by Shavandi et al. (2011) for a biosurfactant produced by the *Rhodococcus* strain TA6. Emulsification activities as high as 84.1–95.8% and 60–90% were reported for biosurfactants produced by actinomycetes (Zambry et al., 2017) and *Streptomyces luridus* So3.2 (Lamilla et al., 2018), while Elkhawaga (2018) observed emulsification activities of *S. griseoplanus* NRRL-ISP 5009, SM1, biosurfactant toward xylene and olive oil of 60–50% and 80–70%, respectively.

3.7. Effects of environmental factors on biosurfactant activity

Table 4 summarizes the effects of pH, temperature, sodium chloride concentration and time of heating at 90 °C on surface tension reduction activity and emulsification indices of cell-free broth containing *Streptomyces* sp. DPUA1566 biosurfactant toward waste motor oil, motor oil, *n*-hexadecane and soybean oil. The biosurfactant abilities to reduce surface tension and emulsify were stable during 1-h incubation at temperatures ranging from 4 to 120 °C, despite a slight tendency toward a surface tension increase. These results taken together give an idea of how the use of this biosurfactant would be useful in industries in which heating is of paramount importance to achieve sterility.

Biosurfactant activity was retained over a very wide pH range (6 − 12), with minimal variation in surface tension and emulsification indices, which is another important issue regarding its application spectrum. On the other hand, the higher values of surface tension observed at pH 2.0–4.0 (33.0–32.6 mN/m) suggest the occurrence of some alteration in surfactant structure under acidic conditions, in agreement with the results of Silva et al. (2010).

The biosurfactant capacity to reduce surface tension and emulsify the selected hydrophobic compounds did not statistically significantly vary ($p > 0.05$) with increasing NaCl concentration up to 12% (Table 4), which suggests that it was not precipitated or “salted out” under these conditions. Such a tolerance to high ionic strength is suitable for oil-related applications, most of which are carried out under high salt conditions. High stability over wide ranges of temperature, pH and salinity was described for biosurfactants from *Streptomyces* sp. isolate R1 (Zambry et al., 2017) and *S. griseoplanus* NRRL-ISP5009, SM1 (Elkhawaga, 2018).

3.8. Biosurfactant characterisation

The biosurfactant extracted from cell-free broth was submitted to thin-layer chromatography and staining with specific reagents able to react with amino groups (ninhydrin) and lipids (iodine vapours), which revealed that the isolated biosurfactant was made up of 84% proteins and 15% lipids, while the gas chromatographic characterisation of lipid fraction showed a fatty acid composition of C18:1 (80%), C18:2 (10%), C18:0 (4.1%), C16:1 (4.1%) and C16:0 (1.4%) as the major components and C20:1 (< 0.1%), C20:0 (< 0.1%), C14:0 (< 0.1%) and C12:0 (< 0.1%) in quite minor amounts.

The FT-IR spectrum of the isolated biosurfactant (Fig. 4) evidenced a broad stretching peak at 3300 cm^{-1} (I), which is characteristic of the hydroxyl and amine groups. Absorption at around 2960 cm^{-1} (II) and 2850 cm^{-1} (III) can be assigned to the symmetric stretch (C–H) of CH_2 and CH_3 groups of aliphatic chains, an absorption band at 1670 cm^{-1} (IV) to the intense stretching of the C=O bond, a stretching peak at around 1080 cm^{-1} to the carboxyl groups, and that at around 1558 cm^{-1} (V) to the amide one, which is typical of the protein portion of biosurfactant. These results suggest that *Streptomyces* sp. DPUA1566 biosurfactant was a lipoprotein that we denominated Bioelan. The partially purified biosurfactant from *Streptomyces* sp. isolate R1 was also characterized as a lipopeptide (Zambry et al., 2017), whereas the

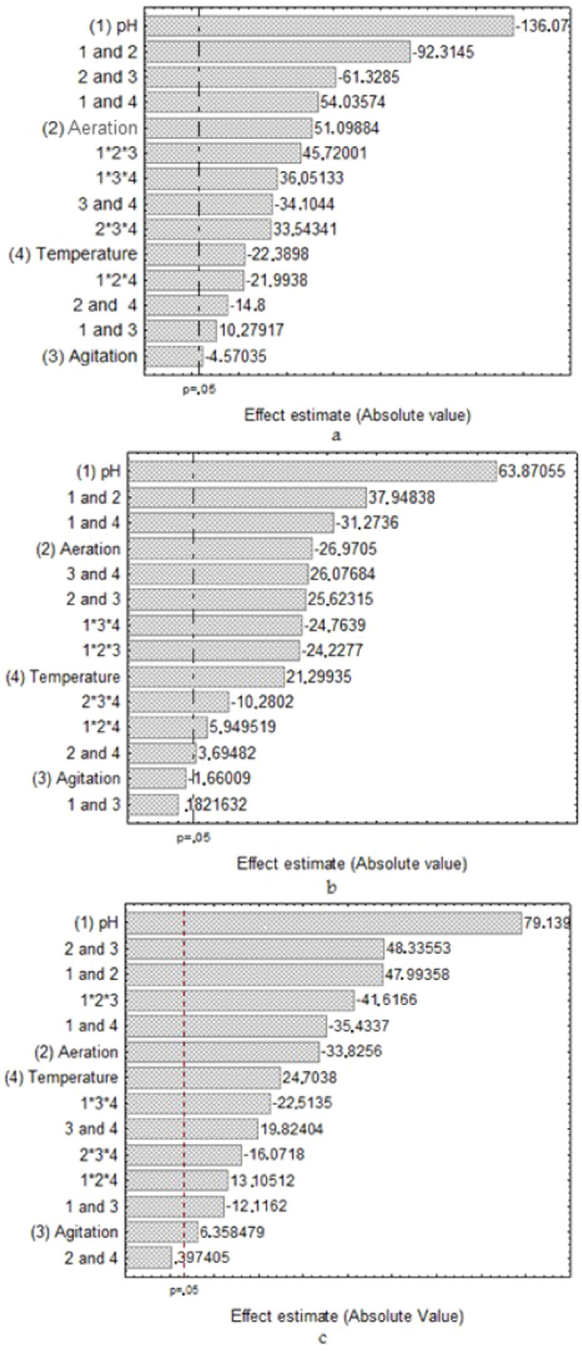

Fig. 2. Pareto chart of standardized effects of pH, temperature, agitation speed and aeration on a) medium surface tension, b) waste motor oil emulsification index, and c) soybean oil emulsification index, according to the 2^4-full factorial design of Table 3.

bioemulsifier produced by *Streptomyces luridus* So3.2 was described as a fatty acid (Lamilla et al., 2018).

3.9. Oil displacement activity of biosurfactant

The oil displacement test, which is indicative of surface and wetting properties of aqueous solutions (Youssef et al., 2004), is an indirect measurement of surfactant surface activity on oil, in that, the larger the clear zone diameter, the stronger the surface activity (Rodrigues et al., 2006). This test carried out in the presence of *Streptomyces* sp. DPUA1566 biosurfactant led to a clear zone area as large as 94.9 cm^2, corresponding to a dispersion rate of 95.0% of the initial diameter of the oil. This value pointed out high surface activity and suggests that *Streptomyces* sp. DPUA1566 biosurfactant has suitable properties for application in bioremediation of hydrocarbon-contaminated sites. Similar results were obtained for *Streptomyces luridus* So3.2 the biosurfactant (Lamilla et al., 2018).

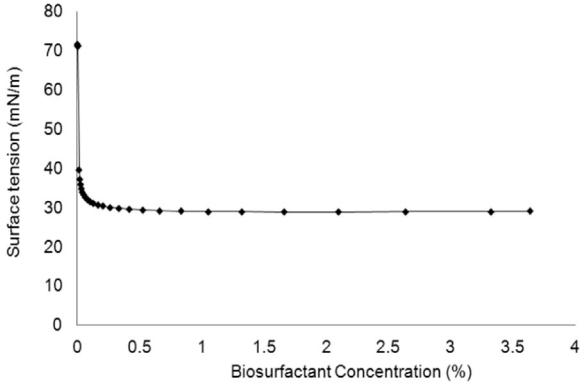

Fig. 3. Surface tension versus concentration of the isolated biosurfactant produced by *Streptomyces* sp. DPUA1566 in medium supplemented with 10 g/l soybean waste frying oil and 20 g/l corn steep liquor.

Table 4
Influence of pH, temperature, salt concentration and heating time at 90 °C on surface tension reduction activity and emulsification indices of cell-free broth containing *Streptomyces* sp. DPUA1566 biosurfactant obtained from cultivation in medium supplemented with 10 g/l soybean waste frying oil and 20 g/l corn steep liquor toward different oily substances. Results are expressed as means ± standard deviation.

	Surface tension (mN/m)	Emulsification index (%)			
		Waste motor oil	Motor oil	*n*-Hexadecane	Soybean oil
pH					
2	33.0 ± 0.5	62.7 ± 2.0	61.0 ± 1.8	10.2 ± 1.5	48.8 ± 2.2
4	32.6 ± 0.7	65.3 ± 1.7	63.5 ± 1.0	12.4 ± 2.1	54.0 ± 1.0
6	27.6 ± 0.6	90.0 ± 1.4	88.7 ± 1.7	16.1 ± 1.7	62.1 ± 1.7
8	26.5 ± 0.4	95.6 ± 1.5	91.1 ± 2.3	20.9 ± 2.2	66.2 ± 2.1
10	26.6 ± 0.5	94.5 ± 2.3	92.0 ± 1.5	21.1 ± 0.9	66.6 ± 1.7
12	27.0 ± 0.6	93.2 ± 1.8	91.6 ± 1.9	18.8 ± 1.4	60.5 ± 1.8
Temperature (°C)					
4	26.2 ± 0.8	95.5 ± 1.0	92.8 ± 1.7	20.4 ± 1.5	62.1 ± 2.1
28	26.5 ± 0.4	95.1 ± 2.2	93.0 ± 2.0	20.9 ± 2.2	65.3 ± 2.0
40	26.5 ± 0.7	94.2 ± 2.0	92.3 ± 1.9	20.4 ± 1.4	66.0 ± 2.4
60	26.7 ± 0.5	94.2 ± 2.1	91.7 ± 2.4	19.5 ± 2.0	65.9 ± 1.4
80	27.6 ± 0.6	94.0 ± 1.7	92.0 ± 1.8	18.8 ± 1.6	63.8 ± 1.5
100	27.8 ± 0.7	90.8 ± 1.4	90.0 ± 1.7	17.5 ± 1.3	64.7 ± 1.3
120	27.8 ± 0.5	88.6 ± 1.8	87.3 ± 1.5	16.0 ± 0.9	50.8 ± 1.7
NaCl (%)					
0	26.2 ± 0.8	94.9 ± 1.0	93.0 ± 1.7	20.7 ± 1.4	65.9 ± 2.3
2	26.2 ± 0.7	94.2 ± 1.9	92.5 ± 2.2	21.1 ± 1.7	66.0 ± 1.5
4	26.4 ± 0.6	94.2 ± 1.4	92.8 ± 2.4	20.3 ± 0.9	65.4 ± 2.1
6	26.3 ± 0.4	93.8 ± 2.4	91.6 ± 2.1	20.8 ± 1.7	65.9 ± 1.0
8	26.5 ± 0.7	92.9 ± 1.8	90.3 ± 1.9	21.8 ± 2.1	62.8 ± 2.0
10	27.4 ± 0.6	90.8 ± 0.9	89.7 ± 2.3	19.9 ± 2.2	61.4 ± 2.4
12	27.5 ± 0.5	90.0 ± 1.7	88.9 ± 1.7	21.9 ± 1.5	60.8 ± 1.9
Heating time at 90 °C (min)					
10	26.8 ± 0.4	94.9 ± 2.2	92.5 ± 2.0	21.1 ± 1.4	62.1 ± 2.2
20	27.7 ± 0.5	94.2 ± 1.8	91.9 ± 1.4	20.2 ± 2.0	58.8 ± 1.3
40	27.2 ± 0.8	93.8 ± 2.0	92.2 ± 1.7	19.4 ± 2.1	57.0 ± 1.8
80	29.0 ± 0.6	92.4 ± 1.4	90.3 ± 2.1	19.0 ± 1.7	49.0 ± 1.7
100	30.8 ± 0.4	90.0 ± 1.7	90.0 ± 2.0	18.8 ± 1.8	42.3 ± 2.0
120	31.4 ± 0.5	89.3 ± 1.9	89.4 ± 2.4	18.7 ± 0.9	40.5 ± 2.3

3.10. Biosurfactant toxicity

The toxicity of the isolated biosurfactant was first tested in a short-term bioassay, in which it did not display any lethality to brine shrimp

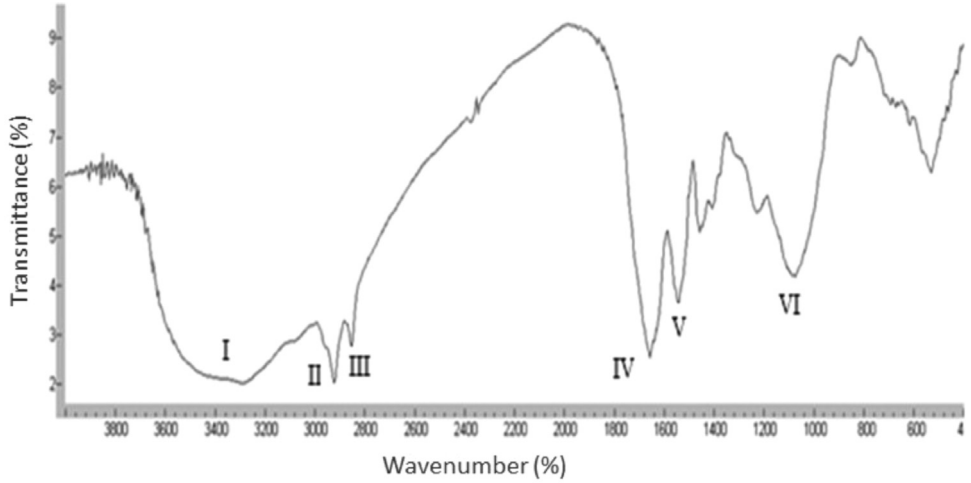

Fig. 4. FT-IR spectrum of the biosurfactant produced by *Streptomyces* sp. DPUA1566 cultivated in medium supplemented with 10 g/l soybean waste frying oil and 20 g/l corn steep liquor.

after 24 h. Contrariwise, it was reported low toxicity of *P. aeruginosa* biosurfactant to *Artemia salina* larvae (Silva et al., 2010) and acute toxicity of *Gordonia* sp. JE1058BS biosurfactant to two species of marine larvae, *Mysidopsis bahia* (shrimp) and *Menidia beryllina* (fish) (Saeki et al., 2009).

The germination index (GI), which combines measures of relative seed germination and relative root elongation, was finally used to assess the biosurfactant phytotoxicity to *Lactuca sativa* L. and *Brassica oleracea* L. GI values of 189, 110, 105, 96% were found for the former and of 201%, 128%, 113% and 113% for the latter at biosurfactant concentrations of 10, 100, 800 (CMC) and 1000 mg/l, respectively. Moreover, leave growth and elongation of secondary roots were observed under all the conditions tested. As GI ≤ 80% is considered an indicator of phytotoxicity (Tiquia et al., 1996), these results demonstrated no phytotoxicity of *Streptomyces* sp. DPUA1566 biosurfactant.

4. Conclusions

The bacterium *Streptomyces* sp. DPUA1566 isolated from lichens from the Brazilian Amazon was found to produce a lipoprotein biosurfactant. The best production was obtained using a mixture of soybean waste frying oil and corn steep liquor as substrate of production medium. The biosurfactant proved to have effective surface tension reduction capacity and emulsification activity toward hydrocarbons and vegetable oils. Its thermal stability, tolerance to wide ranges of pH and salt concentration and absence of toxicity makes this biosurfactant a promising candidate for applications in biotechnological, environmental, cosmetic, food and pharmaceutical industries.

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