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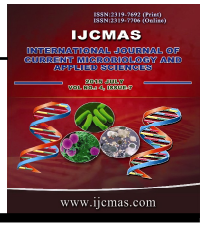

Original Research Article

Enhanced Production of an Glycolipid Biosurfactant Produced by *Candida glabrata* UCP/WFCC1556 for Application in Dispersion and Removal of Petroderivatives

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Keywords

Candida glabrata, Glycolipid, Removal of petroderivatives, Renewable-resources

A B S T R A C T

Glycolipid biosurfactants are produced by yeasts provide significant opportunities to replace chemical surfactants with those that are sustainable biologically. The present study investigated the potential of *Candida glabrata* UCP/WFCC1556, the new yeast isolated from semi-arid soil, that was identified by morphological, biochemical and molecular methods, and was found to be able to produce an potent biosurfactant on water soluble renewable substrates whey (WH) and corn steep liquor (CSL) using Central Composite Designs (CCD). The results showed that the biosurfactant produced reducing the surface tension to 28.53 mN/m, and interfacial tension to 1.02 mN/m with Critical Micelle Concentration (CMC) of 1% (w/v). The biosurfactant showed stables emulsions at temperature (0-100°C), pH (2-12), and NaCl concentrations (2-12%w/v) and was characterized by Fourier transform infrared (FT-IR) spectroscopy exhibited an absorption peak represented by C-O-C stretch which is characteristic of carbohydrates. Also was detected other absorption peak constituent fatty acids and lipids (2850-2960 cm^{-1}). Biochemical determinations indicated that the biosurfactant is a glycolipid (lipids 65% w/v and carbohydrates 25% w/v), and showed a hydrophobic tail constituted by palmitic acid (22.8%), stearic acid (25.3%), oleic acid (26.8%), and linoleic acid (25.1%) , respectively. The biosurfactant shown ability to remove and disperse hydrophobic pollutants, and it is a suitable candidate for bioremediation processes in the future.

Introduction

Removing hydrophobic pollutants from the environment has been a major technological challenge because conventional treatment technologies are very often unable to do so efficiently (Morais and Abud, 2012).

The micro-organisms and its metabolites are able of minimize pollution caused by petroleum hydrocarbons and derivatives, making it a strategic alternative in replacement the conventional technologies. Consideration of the use of biosurfactants as an alternative in bioremediation processes arises because of their emulsifier action and capacity to disperse hydrocarbons and derivatives, thereby accelerating the degradation of various oils by micro-organisms present in water and soil microbiota (Thavasi *et al.,* 2011).

Biosurfactants are compounds produced within microbial cells or are extracellular compounds produced by biotransforming renewable raw material (Singh, 2012). Their molecules have hydrophobic and hydrophilic portions with properties for reducing surface and interface tension which makes them suitable for applications in many industries such as in the food industry, the cosmetics industry, agriculture and for the bioremediation of soils and water (Saharan *et al.,* 2011).

The groups of surface active molecules are categorized as glycolipids, lipopeptides, lipopolysaccharides, phospholipids and particulate biosurfactants (Bhosale *et al.,* 2014). Glycolipid biosurfactants are produced by yeasts and bacteria and provide significant opportunities to replace chemical surfactants with those that are sustainable biologically. A glycolipid comprises a hydrophilic carbohydrate section and a hydrophobic fatty acid chain (Marchant and Banat, 2012).

Biosurfactants are of natural origin, diverse in structure, can make large scale production possible and can be active in extreme conditions (Satpute *et al.,* 2010). Another advantage lies in the fact that they are not derived from petroleum (Lima *et al.,* 2010).

The problem of producing biosurfactants economically can be significantly reduced by using alternative sources of nutrients, which are easily available and inexpensive (Cameotra *et al.,* 2010). Biotechnology combined with fermentation processes enables byproducts or waste from industries and agro-industries to be used as nutritional sources for micro-organisms (Accorsini *et al.,* 2012). However, the main problem with this is related to selecting substrates, such that they contain a correct balance of nutrients to enable cell growth of the microorganism and to producing compounds that are of commercial interest (Jamal *et al.,* 2012).

Materials and Methods

Yeast isolation and identification

The yeast was isolated from soil sample (Serra Talhada-PE, Brazil) in suspensions containing 1g in 10 mL of sterile distilled water. 0.1 mL of dilutions (10^{-3} and 10^{-6}) were transferred in triplicate to Petri dishes containing Sabouraud dextrose agar medium and chloramphenicol (Kastner *et al.,* 1994). The microscopic morphological identification of yeast was carried out using the microculture technique in corn meal agar medium (17.0g) in distilled water (1000 mL) with 1% of Tween 80. Other tests were also involved in the preliminary identification evidenced by carbon source fermentation (zymogram) and assimilation of nitrogen sources (auxonogram). Identity was confirmed by molecular analysis of the ITS2 region of the DNA by polymerase chain reaction (PCR). Primers derived from rRNA

genes of fungi can be used for universal fungi PCR (White *et al.*, 1990). In this study, the universal fungal primers used for amplifiying the ITS2 region were forward primer ITS3 (5`-GCATCGATGAAGAA CGCAGC-3`) corresponding to the 5.8S rRNA gene and reverse primer ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) corresponding to the 28S rRNA gene. The amplicon was purified using a Pure Link Kit (Invitrogen) and DNA sequencing was carried out at the Federal University of Pernambuco. The nucleotide sequences obtained were compared with those of Candida sequences available in GenBank, by the tool BLAST-http/blast.ncbi.nlm.nih.gov. The yeast after identification was preserved by freeze-drying and freezing in glycerol solution, was registered in the Culture Collection UCP (Universidade Católica de Pernambuco)/ World Federation for Culture Collections (WFCC).

Agro industrials substrates

The hydrophilic sources tested in this study for producing biosurfactant were corn steep liquor (CSL) [a byproduct which is rich in amino acids, from the corn industry], whey (WH) [rich in proteins, from the industrial plant of São Bento do Una, Pernambuco, Brazil] and cassava wastewater (CWW), which is rich in carbohydrates.

Biosurfactant production

The biosurfactant production was carried out using the medium proposed by Andrade *et al.* (2009) (cassava wastewater - CWW 10% $v.v^{-1}$, ammonium sulfate 0.2% $v.v^{-1}$ and urea 0 1% $v.v^{-1}$), and later the medium was modified replacing the CWW to hydrophilic substrates whey(WH) and corn steep liquor (CSL). We tested different sources associated a single medium, and all assays the nitrogen sources was used (0.2% ammonium sulfate and 0.1% urea). Fermentations were carried out using Erlenmeyer flasks containing 100 mL of the medium for biosurfactant production (Table 1) added 5% $v.v^{-1}$ of the pre-inoculum (yeast grown in Sabouraud broth (D.O600nm= 0.8). The flasks were incubated in orbital shaker at 150 rpm, temperature of 28°C, for 72h.

Surface and interfacial tension of the biosurfactant

The cell-free culture containing the biosurfactant was obtained by centrifugation at 10.000g, 15 min, and 28 °C and was determined the surface tension in an automatic tensiometer (Kuyukina *et al.*, 2001), and the interfacial tension was measured using n-hexadecane (Darvishi *et al.*, 2011). The extracellular production of biosurfactant was evaluated in the culture medium as: a) the supernatant containing yeast cells; b) the cell-free supernatant, and c) the supernatant after extracting of the biosurfactant. The time in course of the optimal for biosurfactant production was established at 120h when was observed the lower surface tension of the cell-free broth.

Extraction and purification of biosurfactant

The biosurfactant was removed from the supernatant by precipitation with 70% ammonium sulfate saturation. The material was kept overnight at 5°C. After this period the supernatant was centrifuged (10,000 g for 15 min), discarded and the extract was subjected to dialysis with deionized water for 24 h, which was changed every 4 h to remove contaminants adhering to the sample. The precipitate was collected and lyophilized (Navon-Venezia *et al.*, 1995).

Emulsifying property, CMC, charge and stability

The biosurfactant was evaluated for its potential as an emulsifier using engine oil, burnt engine oil, diesel oil, soybean oil, corn oil, canola oil and n-hexadecane (Cooper and Goldenberg, 1987). The negative control was emulsified using distilled water. The Critical Micelle Concentration (CMC) of biosurfactant required to maintain surface activity was also investigated. The CMC was automatically determined by measuring the surface tensions of the purified biosurfactant in distilled water up to a constant value of surface tension have been reached. The charge present in the biosurfactant hydrophilic portion was evaluated by the double diffusion technique at low viscosity (1% agar solution) (Meylheuc *et al.,* 2001) and confirmed by zeta potential analysis. The stability of the biosurfactant was measured by surface tension after adding different NaCl concentrations (2, 4, 6, 8, 10 and 12%), at different temperatures (0, 5, 70, 100 and 120°C), and pH (2, 4, 6, 8, 10 and 12).

Chemical composition of the biosurfactant

The total proteins of purified biosurfactant were determined using the kit from Labtest Diagnóstica S.A., Brazil. The total carbohydrates were determined according Dubois *et al.* (1956). The lipids were extracted with chloroform: methanol in different proportions (1:1 and 1:2 v/v). The organic extracts were then evaporated under vacuum and the lipid content determined by gravimetric estimation following the methodology of Manocha *et al.* (1980). The chemical composition of biosurfactant was confirmed by analysis using an infrared spectrometer with Fourier transform (FT-IR) recorded on a Bruker IFS 66 instrument and the results are expressed in cm^{-1} in the region 4000–400 cm^{-1}.

Identifying fatty acids in the hydrophobic portion of biosurfactant

The fatty acids present in the hydrophobic portion of the biosurfactant were identified after they were converted into methyl esters following a modification of the method of Durham and Kloos (1987) and they were identified by gas chromatography. The biosurfactant (10mg) was transferred to tubes containing 3 mL of boron trifluoride – methane at 14% and 3 ml of benzene and incubated at 60°C over-night. Distilled water (4mL) was added to the sample and shaken in vortex (5 min), centrifuged (5.000g for 10 min. at 5ºC). The benzene was removed after centrifugation by nitrogen. The methyl esters of fatty acids were resuspended in n-hexane and analyzed by gas chromatography using a capillary column HP – 20M (25m x 032 mm x 0.3 mM). Helium was the carrier gas with split less injection. An initial temperature of 40°C column was maintained for 1 min, increased to 150°C at 5°C per min, then increased to 220°C at 1.7°C per min. The temperature detector was maintained at 220°C and the injector at 200°C. The carrier gas used was nitrogen. The injected sample volume was 1 µL in split mode 1 to 20. The pattern is used to identify the FAME Mix C4- C24 SUPELCO (code 18919). The fatty acids were identified by comparing their retention times with those of standard fatty acid methyl esters.

Experimental designs

The effect of concentrations of independent factors of whey (WH) and corn steep liquor (CSL) were analyzed on the dependence of surface tension using central composite designs (CCD) with 4 factorial points, 4

axial points and 4 central points. Three factorial designs (CCD) were performed to determine the optimal concentrations for biosurfactant production. The levels of experimental design 2^2 were coded as -1, +1 and 0 (zero is considered the midpoint) and the axial points (1.41 and -1.41) were calculated using the following equation:

$$\frac{X_{i=} x_i - x_0}{\Delta x_i}$$

Where, Xi is the coded value of the independent variable, xi is the actual value of the independent variable, x0 is the actual value of the independent variable on the center point and Δxi is the step change value (Luna *et al.*, 2011). The optimized conditions were adjusted to a second order polynomial equation based on variance analysis (ANOVA).

Application of biosurfactant to removal and dispersion of petroderivatives

The ability of the biosurfactant to disperse burnt engine burning was tested following the method of Morikawa *et al.* (2000). Deionized water (30mL) was poured onto Petri dishes and subsequently 0.5 mL of engine oil was added. At the center of the Petri dish containing water and oil, 1 mL of cell-free metabolic liquid containing the biosurfactant was added. The controls were made with distilled water. The halo formation characterized by a clear zone indicates its ability to disperse hydrophobic compounds. The experiments were performed in triplicate. The percentage of the dispersion was calculated from the by diameter of the Petri dish on the basis of the mean diameter of the clear zones. The analysis of the potential for removing biosurfactant was performed with burnt engine oil which was impregnated in sandy soil (from beach of Boa Viagem, Recife-PE). The treatment was performed with biosurfactant present in cell-free metabolic liquid for 48 h, 150rpm at 28°C in line with the method of Nitschke and Pastore (2002).

Results and Discussion

Identification of the yeast isolated from semi-arid soil

The yeast isolated from soils of the semi-arid was identified using morphological and biochemical profiles. The absence of pseudo hyphae in the cellular structure of the isolate was noted on cultivation in corn meal agar, only blastoconidia being seen. The yeast assimilates as a source of growth and energy only glucose in low oxygen tensions proven by changes in the pH and gas formation of the medium. Sucrose, maltose, lactose and rhamnose were not assimilated. *Candida* species can be identified by microbiological, biochemical and molecular methods. According to Piens *et al.* (2003), the biochemical identification of *Candida glabrata* is determined by the ability of this yeast to assimilate only glucose and trehalose as sugar, which differentiates it from other species. Among the *Candida* species, this is the one that has no pseudo-filaments in its structure (Fidel *et al.*, 1999).

Molecular methods have often been used to confirm the identity of micro-organisms (Bouchara *et al.*, 1996). In this context, the confirmation of a genus and species of yeast was given by molecular analysis indicating that the isolate had 95% identity for *Candida glabrata* after amplification using universal fungal primers ITS3 and ITS4 yielding PCR products of 400-bp to *C. glabrata* amplicon (Figure 1). The results of molecular identification obtained in this study were similar to those reported by Chen *et al.* (2000), using a PCR product with primers ITS3/ITS4 corresponding to 99% of the identity of *Candida glabrata* after amplification of 413 bp segments

Selection of the production medium

Hydrophilic substrates and their combination were previously tested for biosurfactant production (Table 1). The medium consisting of the combination of whey-WH (10%) and corn steep liquor-CSL (5%) supplemented with nitrogen sources (0.2% ammonium sulfate and 0.1% urea) resulted in the minimum surface tension obtained, a measurement in the cell-free metabolic liquid which showed a reduction from 72 (distilled water) to 35.66 mN/m.

Optimization of the production medium

The medium described that induced the production of biosurfactant (whey 10% $v.v^{-1}$ as a carbon source and corn steep liquor 5% $v.v^{-1}$ as a carbon source\nitrogen) was used to reach the optimal concentrations studied in the three experimental designs (Table 2).

In the first experimental design, there was no significant reduction of the surface tension in any of the concentrations tested. The minimum surface tension obtained was 38.44 mN/m in the medium containing the minimum level of the CSL 4% $v.v^{-1}$ and the maximum level of the WH 12% v.v-1. In the second experimental design, the surface tension decreased for values around 28 mN/m at the central point of the experimental design containing intermediate levels of WH 20% $v.v^{-1}$ and CSL 3% $v.v^{-1.}$ According to Pacwa-Płociniczak *et al.* (2011), the effectiveness of the surfactant is measured by the minimum value of surface tension to values below 30 mN/m and interfacial tension values below 1 mN/m. The biosurfactant produced in this second experimental design, in medium containing intermediate levels of WH 20% $v.v^{-1}$ and CSL 3% $v.v^{-1}$ was shown to be more efficient than the biosurfactant produced by *Candida albicans* (surface tension of 32mN/m) (Cameotra *et al.*, 2010) and has a similar capacity when compared with the sophorolipid biosurfactant of *Torulopsis bombicola* (Interface tension of 1.8mN/m) (Christofi and Ivshina, 2002).

In the third experimental design, the minimum surface tension obtained was 36.97mN/m in the medium containing intermediate levels of WH 30% $v.v^{-1}$ and the maximum level of CSL 3.41% $v.v^{-1}$. In this design, the WH concentration was investigated at higher concentrations (22.95 - 37.05% $v.v^{-1}$), while the CSL had the minimum concentrations (0.41 - 3.41% $v.v^{-1}$) (Table 3).

According to the Pareto Chart the effects standardized for a 95% confidence level represented by p value, demonstrates that for the tested concentrations of whey [7–12% ($v.v^{-1}$)] and corn steep liquor [3–6.4% ($v.v^{-1}$)] in the first experimental design, none of the independent variables had an impact, as their association did not significantly influence the reduction in the surface tension (Figure 2A). In the second experimental design, where the whey concentration (12–27% of $v.v^{-1}$) was increased and the concentration of the corn steep liquor (1–4% $v.v^{-1}$) was reduced, the Pareto Chart shows that all the factors evaluated in the quadratic (Q) and linear (L) functions were above the p values, as their associations were statistically representative for all the factors analyzed. However, the association between the factors in intermediate concentrations (whey 20% $v.v^{-1}$ and corn steep liquor 3% $v.v^{-1}$) caused a statistically negative effect which influenced the reduction of surface tension, this being the most relevant factor for maximum biosurfactant production (Figure 2B). The analysis of the Pareto Chart in the third experimental design showed that whey and corn steep liquor in the quadratic function (Q) were statistically significant. However, whey and corn steep

liquor both in linear function (L), as well as in their interaction, were not significant for the reduction of surface tension (Figure 2C).

In view of the results described above, the levels tested in the central point of the second experimental design indicate the probable reach of the optimization of the concentration of the medium for the production of biosurfactants, given that other experimental designs tested in this study did not result in lower values when they reduced the surface tension.

Properties of the biosurfactant in optimized medium

The characteristics and properties of biosurfactant produced in the optimized medium were investigated. The confirmation of the extracellular production was given by the increase of surface tension after the biosurfactant extraction of 50.06 mN/m (before extraction) for 28.53mN/m (after extraction). The value of the surface tension remained approximately the same in the liquid containing cells of *C. glabrata* and in the cell-free metabolic liquid thus confirming that the cell removal is not related to the removal of biosurfactant and that the production was extracellular.

Studies were conducted to evaluate the stability of the biosurfactants against conditions due to the possible inactivation of its properties The biosurfactant produced by *C. glabrata* in the optimal medium has the potential to form stable emulsions with canola oil (81.25%), burnt engine oil (80.27%). soybean oil (78.02%), corn oil (75.63%), engine oil (75.00%) and diesel (70.15%). Canola oil and burnt engine oil were the hydrophobic substrates that most propitiated the formation and stabilization of the emulsions. However, the least satisfactory result was obtained with the use of n-hexadecane. The biosurfactant maintained stable activity after the addition of 2-12% NaCl. However, its efficiency was maintained in reducing surface tension only in pH 2-4 and demonstrated variation in the biosurfactant activity after being subjected to different temperatures. According to Nitschke and Pastore (2002), some biosurfactants support concentrations of 10% NaCl, while saline solutions of 2-3% are sufficient to inactivate conventional surfactants. The biosurfactant produced in this study by *Candida glabrata* UCP/WFCC 1556 in a medium consisting of whey and corn steep liquor was able to maintain its activity in saline concentrations higher than those cited in the literature.

The biosurfactant of *C. glabrata* showed a hydrophilic portion of the molecule with a negative charge which indicates that it is an anionic surfactant. The CMC of the biosurfactant was computed as 1 % which is within the range of CMC values reported for different types of biosurfactants produced by other *Candida* species.

Chemical characterization of the biosurfactant

The type of biosurfactant depends on the raw material used in the culture medium as a carbon source for microorganisms (Makkar *et al.,* 2011).

Chemically, the biosurfactant produced in this study consisted of 68% lipids, 25% carbohydrates. The Fourier Transform Infrared Spectroscopy (FTIR) of the spectrum of the biosurfactant exhibited an absorption peak at 1100cm^{-1} represented by a C-O-C stretch which is characteristic of carbohydrates. The spectrum of FTIR represents a peak typically associated with the portion of aliphatic hydrocarbons, constituent fatty acids and lipids (2850-2960 cm^{-1}). The spectrum of absorption showed peaks at 3300cm^{-1} corresponding to the N-H

stretch and OH associate group that is characteristic carbon-containing compounds within an amino group (Naumann, 2000). The results obtained identified the biosurfactant as a glycolipid. Glycolipid surfactants produced by *Candida* species reported in the literature have also yielded similar FTIR absorption spectra (White *et al.,* 1990). The purified glycolipid biosurfactant tail showed a mainly fatty acid composition: C16:0 (palmitic acid) 22.8%, C18:0 (stearic acid) 25.3%, C18:1 (oleic acid) 26.8%, and C18:2 (linoleic acid) 25.1%, respectively. In this study specific fatty acid composition of the tail of glycolipid biosurfactant portion from *C. glabrata* UCP/WFCC1556 a similar fatty acid composition, but not identical was observed.

Statistical validation

Variance analysis (ANOVA) was performed based on the second experimental design to find the effect contribution of the independent variables by Fisher variation (F-value) and by the p value, thus confirming the significance of the variables as shown in table 4. The observed high F-value indicated by the variable corn steep liquor in the quadratic term explained the variation that is given on the estimated mean of the squares with a confidence level of ($p < 05$). The small p-values were associated with higher F-values, implying that the effects were larger than that error.

The value of the correlation coefficient ($R^2 = 0.94956$) showed that only 5.04% of the total variation was not explained by the prediction equation (Equation 2) considering that the closer the value of R^2, the higher is the correlation between the observed and predicted values for the model. The quadratic model predicted having the surface tension as a response variable and this is represented by Equation 2.

(Eq. 2)

$$TS= 102.339 -16.241 X + 3.606 X^2 – 4.792Y + 0.129Y^2 – 0.235XY$$

Application of the biosurfactant in dispersing and removing oil

The biosurfactant present in the cell-free metabolic liquid produced by *C. glabrata* WFCC 1556 was applied in dispersion tests of oil in water and removing oil in soil. The biosurfactant promoted 80% of dispensability of burnt engine oil in water evidenced by the formation of large clear area inside the plate and is effective in removing 95.7% of burnt engine oil impregnated in sand (Figure 3). The biosurfactant obtained has a high potential for application in bioremediation processes as an aid to treating environments contaminated with hydrophobic pollutants (petroleum and its derivatives).

This study proposed the development of biotechnological an economically feasible process that is able to minimize environmental impacts by using renewable sources such as nutrients to stimulate biosurfactant production with a new strain of *Candida glabrata* UCP/WFCC 1556. The biosurfactant has had shown an anionic character, ability for reduce surface and interface tension and emulsifier property. Such characteristics are more efficient than some synthetic surfactants. The biosurfactant obtained was a new one, with high potential for application in bioremediation processes as an aid to treating environments contaminated with hydrophobic pollutants (petroleum and its derivatives). This study confirms the potential of biosurfactant and promising applications to dispersion and removal of hydrophobic compounds.

Table.1 Production of biosurfactant by *Candida glabrata* UCP 1556 using hydrophilic renewable-resources by surface tension evaluation

Hydrophilic substrates	**Surface tension (mN/m)**
Cassava flour wastewater $_{(10\%)}$ + ammonium sulfate $_{(0.2\%)}$ +urea $_{(0.1\%)}$	42.00
Corn steep liquor$_{(10\%)+}$ ammonium sulfate $_{(0.2\%)}$ +urea $_{(0.1\%)}$	36.03
Whey $_{(10\%)+}$ ammonium sulfate $_{(0.2\%)}$ +urea $_{(0.1\%)}$	39.95
Association of hydrophilic substrates	**Surface tension (mN/m)**
*Cassava flour wastewater$_{(10\%)}$ + Corn steep liquor$_{(5\%)}$	38.12
*Whey$_{(10\%)}$ + Corn steep liquor$_{(5\%)}$	35.66
*Corn steep liquor $_{(10\%)}$ + Sucrose$_{(5\%)}$	41.51
*Cassava flour wastewater$_{(10\%)}$ + Whey $_{(5\%)}$	39.27

* Adedd 0.2% ammonium sulfate and 0.1% urea (Andrade *et al.*, 2009)

Table.2 Levels of the three 2^2 experimental design to determination of optimal concentrations on biosurfactant production

	First Design CCD				
	Level				
Factors	**-1.41**	**-1**	**0**	**1**	**1.41**
Whey % ($v.v^{-1}$)	7.18	8	10	12	12.82
Corn steep liquor- % ($v.v^{-1}$)	3.59	4	5	6	6.41
	Second Design CCD				
	Level				
Factors	**-1.41**	**-1**	**0**	**1**	**1.41**
Whey % ($v.v^{-1}$)	12.95	15	20	25	27.05
Corn steep liquor- % ($v.v^{-1}$)	1.59	2	30	4	4.41
	Third Design CDC				
	Level				
Factors	**-1.41**	**-1**	**0**	**1**	**1.41**
Whey % ($v.v^{-1}$)	22.95	25	30	35	37.05
Corn steep liquor % ($v.v^{-1}$)	0.41	1	2	3	3.41

Table.3 Optimize of the biosurfactant production medium using three factorial designs 2^2 evaluated by response variable surface tension

	Surface tension (mN/m)		
	Level		
Assay	First Factorial	Second Factorial	Third Factorial
1	40.99	36.87	37.52
2	41.70	39.15	38.49
3	38.44	33.76	37.62
4	45.63	31.33	40.00
5	43.22	32.44	36.97
6	38.91	36.45	39.03
7	41.81	34.97	45.68
8	37.66	32.43	40.04
9	40.02	28.63	39.03
10	43.90	28.53	39.90
11	40.34	28.63	40.04
12	40.58	28.63	40.30

Table.4 Analysis of variance (ANOVA) for the surface tension

Response variable	Effects (independents variables)	Squares mean	F-Values	Confidence level (p)
Surface Tension (mN /m)	Corn steep liquor $_{(T.L)}$	3. 77124	187.003	0.000846
	Corn steep liquor $_{(T.Q)}$	83.23225	4127.219	0.000008,
	Whey $_{(T.L)}$	21.10795	1046.675	0.000065
	Whey $_{(T.Q)}$	66.92569	3318.629	0.000012
	Assoc. Corn steep liquor $_{(T.L)}$ and Whey.$_{(T.L)}$	5.54603	275.010	0.000477
		5.64086	279.712	0.000361
	Adjustment error	0.02017		
	Pure error			

* TL and TQ (linear and quadratic term respectively);

* Assoc. corn steep liquor $_{(TL)}$/Whey $_{(TL)}$ - Association corn steep liquor, and whey linear

Figure.1 Electrophoresis in agarose gel (0.8%) showing the amplification product with specific universal "primers" of the ITS2 region and macroscopic and morphological identification. (M) Molecular weight marker of 100bp; (1) *Candida glabrata* amplicon before purification; (2) *Candida glabrata* amplicon after purification; (3) macroscopic identification and (4) morphological identification

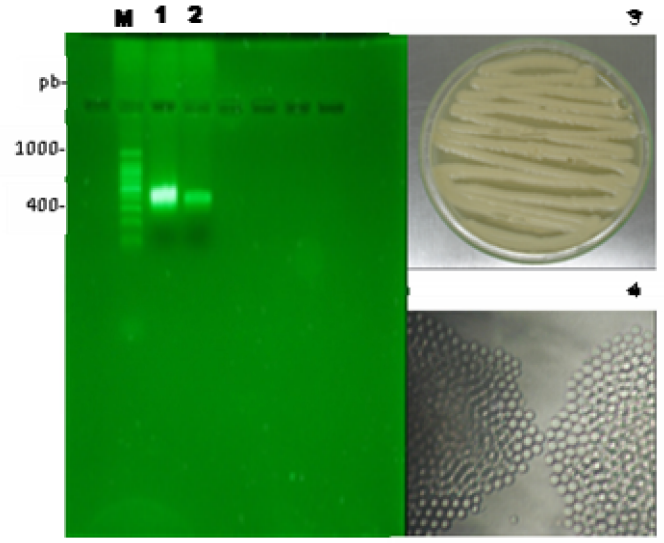
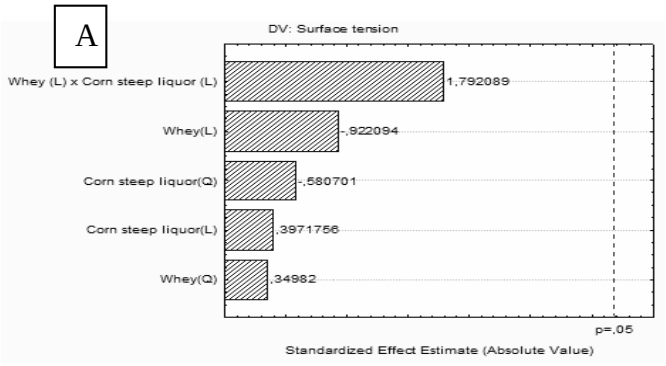
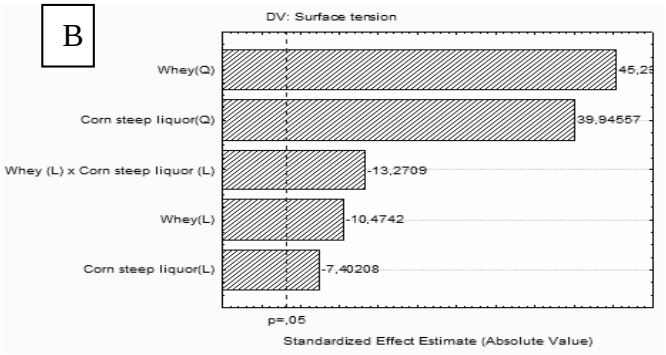


Figure.2 Pareto Chart of the three factor central composite design to evaluate of the effect of the concentrations of whey (WH) and corn steep liquor (CSL) on surface tension. (A) first experimental design, (B) second experimental design and (C) third experimental design





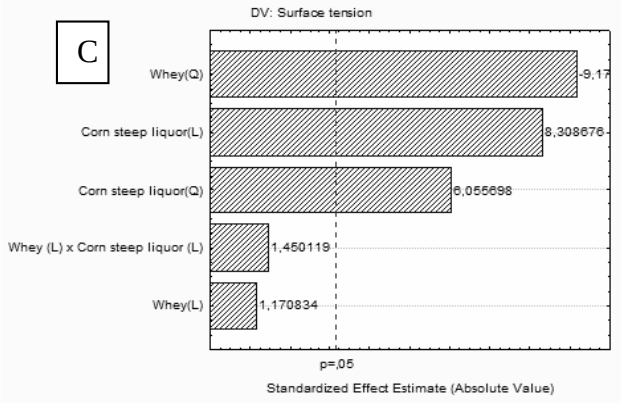
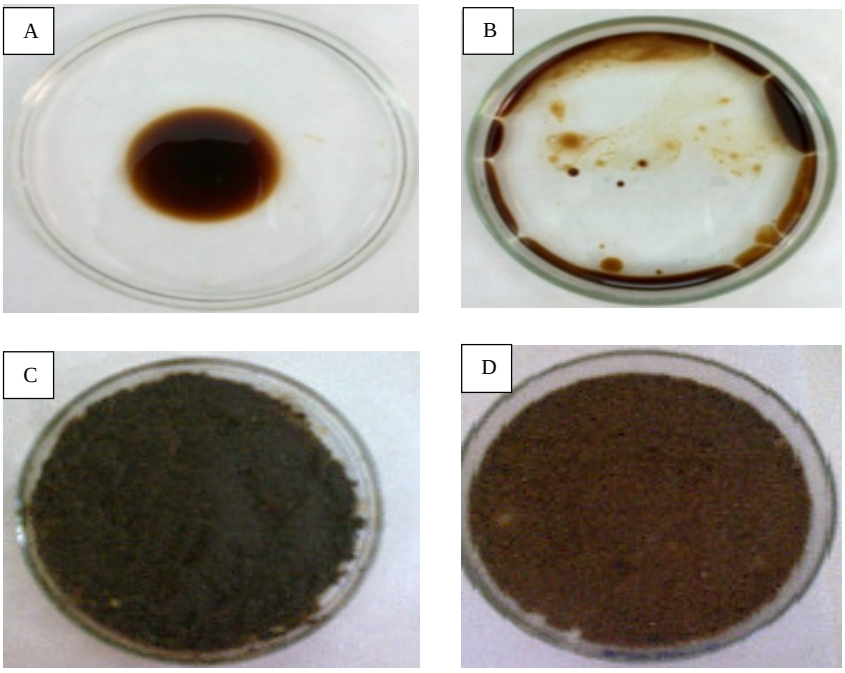


Figure.3 Application of the biosurfactant produced by *Candida glabrata* WFCC 1556 in the dispersion and removal of engine burning oil: (A) Dispersion control [water in oil]; (B) Dispersion of engine burning oil in water by biosurfactant; (C) Removal control [soil impregnated with engine burning oil and water] and (D) Removal of the engine burning oil in soil by biosurfactant



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