British Biotechnology Journal
4(6): 708-719, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Production and Evaluation of Biosurfactant by *Serratia marcescens* UCP 1549 Using Industrial Wastes

T. S. Alves[1], J. P. Salgado[2], R. F. S. Andrade[3,4], D. Montero-Rodríguez[3,4], W. B. Ferreira[2], M. M. Almeida[2], G. M. Campos Takaki[4] and H. W. C. Araújo[1*]

[1]Department of Chemistry, State University of Paraíba, UEPB, Campina Grande, PB, Brazil.
[2]Department of Sanitary and Environmental Engineering, State University of Paraíba, UEPB, Campina Grande, PB, Brazil.
[3]Center of Biological Sciences, Post-graduation Course, Federal University of Pernambuco UFPE, Recife PE, Brazil.
[4]Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, UNICAP, Recife, PE, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. Authors HWCA and GMCT designed the study. Authors MMA and WBF performed the statistical analysis. Authors HWCA wrote the protocol. Authors TSA, RFSA and JPS wrote the first draft of the manuscript. Authors TSA, RFSA, JPS and DMR managed the analyses of the study. All authors read and approved the final manuscript.

Short Research Article

Received 27th February 2014
Accepted 2nd May 2014
Published 18th June 2014

ABSTRACT

Aims: Biosurfactants are surface-active agents of microbial origin that have a property of lowering the surface tension between two liquids. This study aimed to the production of biosurfactant by *Serratia marcescens* UCP 1549 in medium containing agro-industrial wastes for making possible its industrial application in the near future and to propose its environmental applications.
Place and Duration of Study: Center of Science and Technology from State University of Paraíba - UEPB, Campina Grande – PB, Brazil and Nucleus of Research in Environmental Sciences and Biotechnology (NPCIAMB), Catholic University of

**Corresponding author: Email: hwcasullo@ig.com.br;*

Pernambuco - UNICAP, Recife-PE, Brazil between June 2011 and July 2012.
Methodology: According to the proposed factorial design, the culture medium was developed and used for the production of biosurfactant and incubated at 28ºC, 155 rpm during 48 h. The produced biosurfactant was evaluated by emulsifying index, emulsifying activity and surface tension using hydrophobic substrates as vegetable oils after frying, n-hexadecane and diesel.
Results: The best results for emulsification index were found between 79.92 and 100% of emulsification and for activity were values between 2.304 and 5.000 EA (emulsification activity) using vegetable oil after frying as substrate. In relation of the surface tension, the best value obtained was 33.10mN/m in the condition of the central point of the experimental design.
Conclusion: The results show that *Serratia marcescens* UCP 1549 was capable of producing a biosurfactant with emulsifying property from industrial wastes in the studied conditions in this work.

Keywords: Serratia marcescens; biosurfactant; surface tension; emulsification; industrial wastes.

1. INTRODUCTION

In the industrialized world, the contamination by organic compounds became one of major problems to be faced. In the 21th century, a wide of diversity of toxic and hazardous substances has been introduced into the environment, in particular, those resulting from discharges of industrial effluents and from accidents involving oil spills and its derivatives. [1] affirm that the increase of problems linked to environmental pollution caused, in recent years, increased awareness about the importance of restricting indiscriminate releases of pollutants and the need to remedy these impacted locations.

The Brazilian industrial systems cover a wide diversity agricultural or farming activity because the country is considered a major supplier of food to the world. However, liquid wastes generated by the food industry have great physical and chemical complexity, which complicates their treatment and may cause risks to the environment where they are discarded [2]. According to [3], industrial wastes have aroused great interest when used as alternative substrates for the supply of raw material of low cost in the production of biosurfactants, as it lessens the environmental impact as well as business costs with treatment of this wastes, thus eliminating two problems with a single product.

The use of renewable substrates for biotechnological processes is touted as an alternative to economic viability, because the raw material is considered a significant part of production costs. It's estimated that the amount of raw material in biotechnological processes represent 10 to 30% of the production costs [4,5,6].

The potential application of biosurfactants is based on the properties of emulsification, wetting, solubilizing, separation, corrosion inhibition, reduction liquids viscosity, among others. Its use has been increasingly accepted in the market due to their wide applicability in various areas (food pharmaceutical, ceramic, paper and metal industries, wastewater treatment, among others). The most promising application is the cleaning of ships and oil tanks in bioremediation of oil spills and oil [7].

Therefore, this study was aimed to produce a biocompound based in industrial wastes through the use of microorganisms for the treatment of oily and petroleum derivatives substances to reduce the environmental impacts caused by them.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Microorganism and culture conditions

Serratia marcescens UCP 1549 was isolated from soil contaminated with petroleum from banana cultivation area of Recife-Pernambuco, Brazil. The strain was deposited in the Culture Collection of the Nucleus of Research in Environmental Sciences and Biotechnology (NPCIAMB), Catholic University of Pernambuco, and registered in World Federation Culture for Collection (WFCC). The strain is maintained in Nutrient Agar at 5ºC. The growth was carried out in Luria–Bertani solid medium [10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, and 15g/L agar, supplemented with 5g/L of glucose] for 24 h at temperature of 28ºC. Cells were transferred to Luria–Bertani broth for 24h at 300 rpm at 28^{o}C to obtain the pre-inoculum.

2.1.2 Substrates

The substrates used for the biosurfactant production were liquid waste of cassava from of the processing plant of municipality of Carnaíba-PE, vegetable soya oil after frying and paraffin.

2.1.3 Production of biosurfactant

The production of the biosurfactant was carried out in Erlenmeyer flasks of 250mL containing 100mL of the medium composed by liquid waste of cassava, vegetable oil after frying and paraffin in according to the concentrations established by factorial design, with pH 7, 0. All flasks were inoculated with 5% of the pre-inoculum and incubated in orbital shaking during 48h, 150rpm, 28ºC. After the fermentation, the cells were separated of the metabolic liquid by centrifugation (10000g, 15 min at 10ºC), with subsequent filtration in Millipore 0.45 µm for complete separation of the biomass. The cell-free metabolic liquid was used for the following determinations: surface tension, activity and emulsification index.

2.1.4 Factorial design

Factorial design of 2^3 was conducted to evaluate the influence of the concentrations of independent variables liquid waste of cassava, vegetable oil after frying and paraffin on the response variable surface tension according with Table 1. The analysis was carried by the software STATISTIC version 6.0 of Stat Soft ®. The maximum and minimum values used in Table 1 of the matrix for the factorial design were based on Araujo 2009 [8].

Table 1. Design matrix of experiment

Parameters	Lower Level	Central Point	Higher Level
	-1	0	+1
Liquid waste of cassava (%)	5	6	7
Paraffin (%)	2	4	6
Vegetable soya oil after frying (%)	3	5	7

2.2 Methods

2.2.1 Determination of surface tension (TS) and Critical Micelle Concentration CMC

The determination of surface tension was carried out in the cell-free broth obtained by the centrifugation of the cultures at 5000 *g* for 20 min at room temperature, using a Sigma 700 digital surface tensiometer (KSV Instruments LTD – Finland) and working on the principle of the Du Nuoy ring method [9]. The CMC was determined by measuring the surface tension of dilutions of the isolated biosurfactant in distilled water up to a constant surface tension value. Stabilization was allowed to occur until the standard deviation of 10 successive measurements. The CMC value was obtained by plotting the surface tension against the surfactant concentration and was determined as g/L of biosurfactant.

2.2.2 Determination of Emulsification Activity (EA) and emulsification index (E_{24})

Determination of emulsification activity (EA) was also tested under all conditions of the factorial design for each condition using the same above-mentioned substrate and was performed according to the methodology proposed by [10], stirring solution of 2.0mL of liquid metabolic, 2.0 mL and 2.0 mL of buffer solution, mixed by vortexing for 2 min and making the reading of the optical density in a spectrophotometer at 540nm.

The determination of E_{24} was performed in all conditions of the factorial design using different substrates for each condition as diesel oil, vegetable oil after frying and n-hexadecane. It was determined by adding 2.0mL of substrates in 2.0mL of the supernatant, mixed by vortexing for 2 minutes and allowed to stand for 24h. The index was calculated as a percentage of the height of the emulsified layer (cm) divided by total height of the column of liquid (cm) [11].

2.2.3 Isolation of biosurfactant

The isolation of the biosurfactant was performed on cell-free supernatant through precipitation, lowering pH of the free-cell liquid metabolic to pH equal two using solution of HCl (5N), and left resting overnight, the following day, the mix was centrifuged and the supernatant was discarded and the precipited was taken to the stove at 37ºC for 24 hours [12].

2.2.4 Biosurfactant composition

The proteins were quantified by Labtest kit (Brazil) using albumin as standard, carbohydrates by phenol-sulfuric acid method using glucose as standard and lipid were quantified after extraction using chloroform and methanol following methodology [13].

3. RESULTS AND DISCUSSION

3.1 Production of Biosurfactant by *Serratia marcescens*

Researchs in the area of biosurfactants have been expanded as result of the biotechnological potential of microorganisms, enabling the development of more economical processes [10,14]. In this context, the Table 2 shows the factorial design to evaluate the concentration of liquid waste of cassava, vegetable oil after frying and paraffin for maximum production of the biosurfactant by *Serratia marcescens* in economic medium. The detection of production biosurfactant was evalued by variable response to surface tension (mN/m) and emulsification index (E_{24}). The best result was observed with a focus on reducing the surface tension of water from 70 to values around 33 mN/m in condition of the central point of design in medium containing liquid waste of cassava (6%), paraffin (4%) and vegetable oil after frying (5%). According to [15], the effectiveness of the surfactant is measured by minimum value of surface tension to values below 30mN/m. Similar studies for production of biosurfactant using corn steep liquor as culture medium decreased to surface tension 33.35mN/m [8].

The emulsification index (E_{24}) using vegetable oil after frying, n- hexadecane and diesel as substrates, showed highly significant values in all conditions of planning with the exception of condition 8 using diesel. The best emulsification index (100%) was found in the central points of planning using as the substrate soy oil after- frying (Table 2).

Table 2. Production of biosurfactant by *Serratia marcescens* UCP/WFFC 1549 using the factorial design 2^3

Conditions	Variables				Results		
	Liquid waste of cassava	Paraffin	Vegetable Oil After frying	Surface Tension (mN/m)	% IE_{24} (vegetable oil after frying)	% IE_{24} (n -hexadecane)	% IE_{24} (diesel)
1	-1	-1	-1	34.10	95.83	37.50	23.33
2	1	-1	-1	36.10	79.92	54.16	50.00
3	-1	1	-1	43.00	96.76	54.16	50.00
4	1	1	-1	37.20	87.50	33.33	46.67
5	-1	-1	1	36.50	79.92	45.83	20.00
6	1	-1	1	35.50	87.50	50.00	30.00
7	-1	1	1	35.80	95.83	45.83	46.67
8	1	1	1	36.00	97.68	54.16	13.33
9	0	0	0	33.50	100.00	20.83	43.33
10	0	0	0	33.40	100.00	41.66	46.67
11	0	0	0	33.20	100.00	40.24	45.65
12	0	0	0	33.10	100.00	38.33	45.65

In accordance with the Pareto diagram (Fig. 1), the interactions between paraffin and vegetable oil after frying and the liquid waste of cassava and paraffin were statistically significant and have contributed positively with the reducing of the surface tension, as well as the effect of vegetable soya oil after frying. However, the independent variables paraffin and liquid waste of cassava and interaction vegetable oil after frying were statistically representative and contributed negatively to the reduction of surface tension at the concentrations established by factorial design of 2^3 on surface response with a confidence level of 95%.

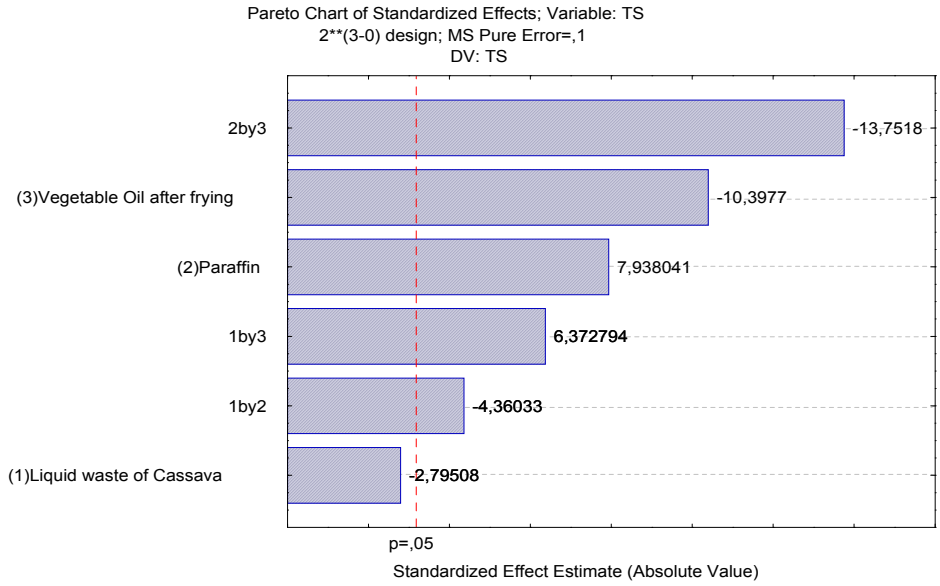

Fig. 1. Pareto diagram to evaluate the effect of different concentrations of liquid waste of cassava, vegetable oil after frying and paraffin about variable response surface tension

The response variable emulsification index (E_{24}) was also statistically analyzed by Pareto diagram. The effects of the independent variables (liquid waste of cassava, paraffin and vegetable oil after frying and their interactions using the substrates to emulsification: vegetable oil after frying (Fig. 2) and n-hexadecane (Fig. 3). The statistical analysis shows that all variables tested, as well as their interactions were not significant. However, when used diesel substrate (Fig. 4), the interactions between liquid waste of cassava/paraffin, liquid waste of cassava / vegetable oil after frying, and the concentration of vegetable oil after frying were significant but negative values for the property of the biosurfactant emulsion. Nevertheless, the concentration of paraffin was statistically representative and influenced positively in the production of emulsification index, the interactions liquid waste of cassava / vegetable oil after frying, and the liquid waste of cassava concentration were not statistically representative.

Bioemulsifier are characterized by formation of macro and micro stable emulsions of oil in water or water in oil. This work confirmed emulsification due to the formation of emulsions between the two fluid phases with different degrees of polarity, metabolic cell-free liquid (polar) and hydrocarbon (nonpolar) occurring dispersion of a liquid in another as showed in Fig. 5 presenting at the central point of the 2^3 factorial design (Table 2).

3.2 Emulsification Activity

According to the results shown in Fig. 6, it can be seen that the emulsifying activity ranged from 2.304 to 5.000 EA, and the larger values have been found when used the substrates vegetable oil after frying and diesel. [16] demonstrated that the emulsifying capacity of the biosurfactant produced by *Escherichia coli* JM 101 was not significant, once that the formed emulsions showed activities around 0.9 EA. As control of the emulsification activity of test activities, some commercial chemical surfactants were tested and presented activities values

below 2.58 EA, therefore, the values of emulsification activity obtained in this study were quite significant.

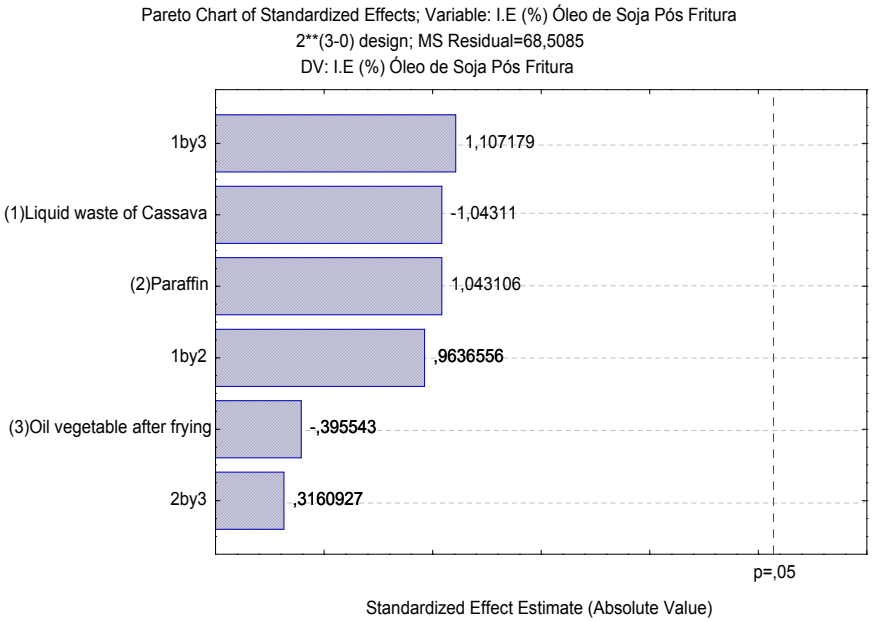

Fig. 2. Pareto diagram to evaluate the effect of the variable emulsification index (%) of the liquid metabolic free of cells using vegetable oil after frying as substrate

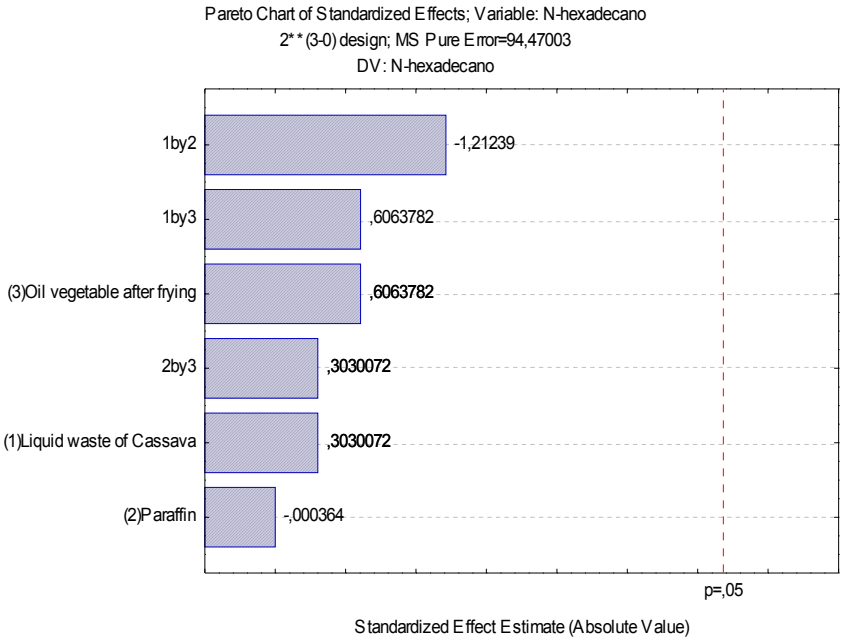

Fig. 3. Pareto diagram to evaluate the effect of the variable emulsification index (%) of the liquid metabolic free of cells using n-hexadecane as substrate

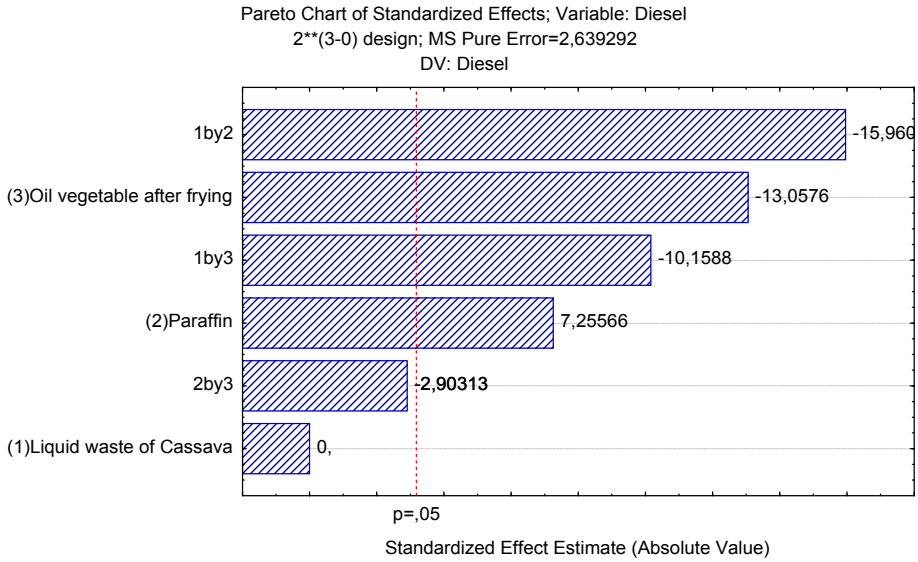

Fig. 4. Pareto diagram to evaluate the effect of the variable emulsification index (%) of the liquid metabolic free of cells using diesel as substrate

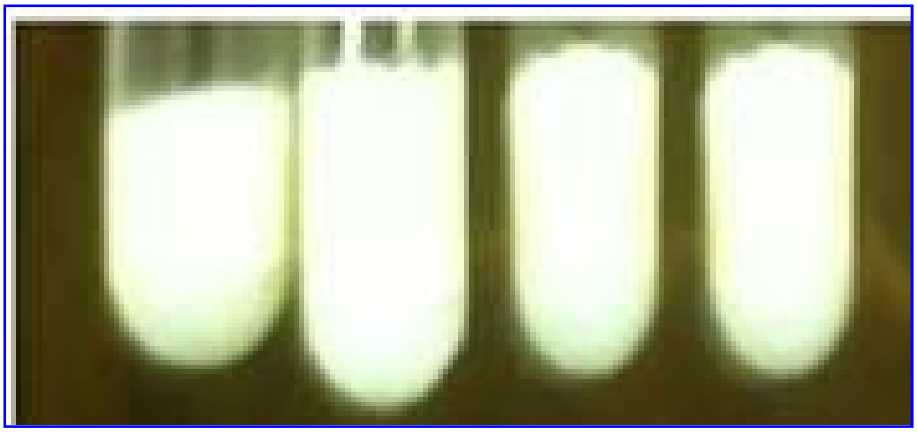

Fig. 5. Emulsion of biosurfactant produced on the central point condition with vegetable oil after frying

3.3 Critical Micelle Concentration (CMC)

The CMC was obtained from the biosurfactant concentration of 2.5 % (Fig. 7) after surface tension of 33.80 to 33.10mN/m. The effect of surface tension reduction is directly related to the concentration of biosurfactant to achieve the values of CMC [5]. The CMC of the biosurfactant (efficiency measure) varies between 1000 to 2000mg/L [17], obtained the crude biosurfactant CMC was estimated around 512mg/L and surface tension of biosurfactant minimum of 30.1mN/m for the lipopeptides produced by *Bacillus natto* TK - 1. [18] obtained a CMC of 120 and 140mg/L and surface tensions of 33.3 and 33.0mN/m using *Pseudomonas sp.*

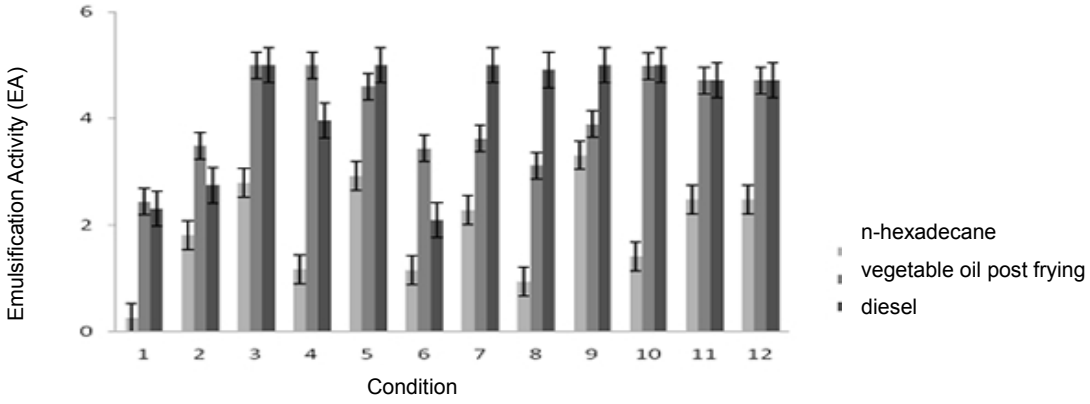

Fig. 6. Emulsification activity (EA) of biosurfactant produced by *Serratia marcescens* UCP 1549 under the conditions of 2^3 factorial design

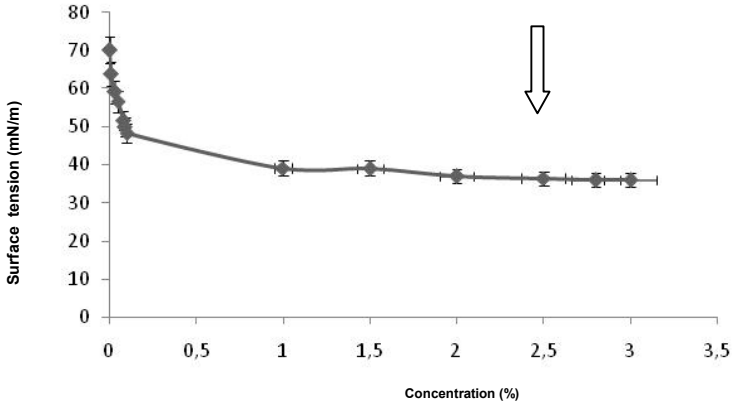

Fig. 7. Critical Micellar Concentration (CMC) of the biosurfactant produced by *Serratia marcescens* UCP 1549 after 48 hours of cultivation medium composed of liquid waste of cassava (6%), paraffin (4%) and vegetable oil after frying (5%)

3.4 Growth Curve

In this work, the bacterium *Serratia marcescens* UCP 1549 was cultured in medium containing 6% liquid waste of cavassa, 5% vegetable oil after frying and 4% of paraffin as substrates (best condition obtained at the central point). Fig. 8 shows the production of the biosurfactant during the growth of *S. marcescens* UCP 1549 in the above-mentioned media at intervals 4 hours during 72hours. The results indicate that the growth and formation of biosurfactant, increase with time simultaneously it can be observed the reduction in surface tension that occurs from the exponential growth phase, obtaining minimum values after 48 hours and the lowest surface tension observed in the stationary phase with a value of 33.10 mN/m corroborating with [8] using corn steep liquor as substrate.

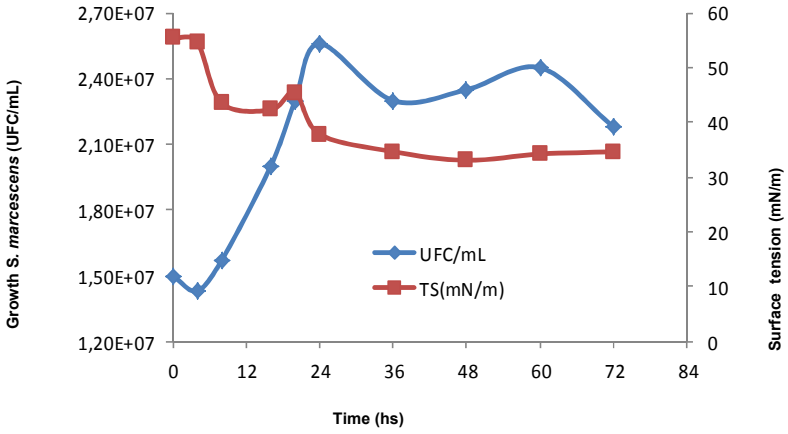

Fig. 8. Profile of growth and reduction of surface tension during growth of *Serratia marcescens* UCP 1549 in medium containing 6% liquid waste of cassava, vegetable oil after frying 5% and 4% paraffin

Araujo et al. [8] used *S. marcesces* UCP 1549 and corn steep liquor residue as culture medium and obtained a reduction of the surface tension of 25.82mN/m. Cunha et al. [19] obtained an initial reduction of the medium 67.8 to 34.4mN/m after 96 hours of cultivation using gasoline as a substrate in the production of the biosurfactant with *Serratia sp.* SVGG16. The biosurfactant production by *Pseudomonas fluorescens* using oil as a substrate, there was a reduction in the surface tension of 70 to 30.04mN/m [20].

The commercial chemical surfactant such as Triton X - (t-octylphenoxypolyethoxyethanol - sigma) and Span 20 (sorbitan monolaurate, Sigma) were tested by Matsuura [21] at concentrations of 100 and 500mL /L and had a surface tension 31.1mN/m and 29.3mN/m respectively. It has been found that it's possible to reuse waste as substrate for biosurfactant production by *S. marcescens* UCP 1549 being one of the first steps to reduce production costs.

3.5 Isolating and Composition of the Biosurfactant

The step of isolating of the biosurfactant is considered part of higher costs for its production, to reduce costs in the biosurfactant production process, the isolation of the biopolymer was carried out by precipitation by lowering the pH to 2 and putting in overnight, for 24 hours, separating the supernatant from the precipitate and putting it in the oven at 37ºC to dry it and then characterize it is possible to obtain different types of biosurfactants one species of microorganism [22,6].

The extraction allowed isolation of the biopolymer, which appeared as a light brown precipitated soluble in water, preliminary chemical characterization of this biosurfactant was 48% lipid, 11% carbohydrate and 22% protein presenting as problable lipopeptide [12].

4. CONCLUSION

In recent years, the increase in the number of publications on biosurfactant production by bacteria shows that this technology won more space and importance. However, biosurfactant production on an industrial scale is still limited due to the costs involved in its production process. The optimization of the fermentation process is the key factor to improve production efficiency and reduce costs. Several studies focused on the influence of carbon and nitrogen sources, showing that they are important in the production of biosurfactants parameters, since the type and concentration of the substrate used can increase or decrease the synthesis of the biosurfactant and even modify it structurally. One strategy that being widely used to decrease the cost of production is the use of renewable sources of substrates as carbon source. It has been estimated that the use of these decrease the total cost of production by 10 to 30%, besides being a possible solution for reuse of industrial residues. This work demonstrated the potential by *Serratia marcencens* UCP 1549 in metabolize industrial wastes (liquid waste of cassava, paraffin and vegetable oil after frying) as a source of carbon and nitrogen for growth thus generating inputs with high biotechnological value. The results showed that the produced biocompound was able to reduce the surface tension and to form emulsions of good quality, especially with the substrate vegetable oil after frying. For successful scaling-up of production of biosurfactants by bacteria, able to make them economically competitive with chemical surfactants, developments are needed about the topics covered in this work, opening new perspectives for increasing the efficiency of production, making it possible to industrial application of these compounds in the near future.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rizzo ACL, Leite SGF, Soriano AU, Santos RLC, Sobral LGS. Bioremediation of oil-contaminated soils: Emphasis on the use of bioreactors. Environmental Technology Series, STA - Environmental Technology Centre 37, Cetem. 2006;61.
2. Cerqueira VS, Costa JA. Biodegradation of toluene and fish oil impacted soils using chemical and biological surfactants. New Chemistry. 2009;32nº:2.
3. Lima ASE, Alegre RM. Evaluation of Emulsifier Stability of Biosurfactant Produced by *Saccharomyces lipolytica* CCT-0913. Braz. Arch. Biol. Technol. 2009;52(2):285-290.
4. Deleu M, Paquot M. From renewable vegetables resources to microorganisms: New trends in surfactants Compters Rendus. Chim. 2004;7:641-646.
5. Luna JM, Sarubbo L, Takaki GMC. A New Biosurfactant Produced by *Candida glabrata* UCP 1002: Characteristics of Stability and Application in Oil Recovery. Braz. Arch. Biol. Technol. 2009;52(4):785-793.
6. Mukherjee S, Das P, Sen R. Towards commercial production of microbial surfactants. Trends Biotechnol. 2006;24:509-515.
7. Castiglioni GL, Bertolin TE, Costa JAV. Produção de biossurfactante por *Aspergillus fumigatus* utilizando resíduos agroindustriais como substrato. Quim. Nova. 2009;32(2):292-295.
8. Araújo HWC, Ceballos BSO, Campos-Takaki GM. Biosurfactant production by *Chromobacterium prodigiosum* (*Serratia marcescens*), In: Current Research Topics in Apllied Microbiology and Microbial Biotchnology, 2nd, *S*evilha-Spain. 2009;676-680.

9. Kuyukina MS, Ivshina IB, Philp JC, Christofi N, Dunbar SA, Ritchkova MI. Recovery of *Rhodococcus* biosurfactants using methyl tertiary-butyl the extraction. J Microbiol Met 2001;46:109-120.
10. Cirigliano MC, Carman GM. Isolation of a bioemulsifier from *Candida lipolytica*. Appl environm Microbiol. 1984;48:747-750.
11. Cooper DG, Goldenberg BG. Surface active agents from two *Bacillus* species Appl. Environ. Microbiol. 1987;53:224–229.
12. Navon-Venezia S, Zosim Z, Gottlieb A, Legmann R, Carmeli S, Ron EZ, Rosenberg Alasan E. A new bioemulsifier from *Acinetobacter radioresistens*. Applied and Environmental Microbiology. 2007;61(9):3240-3244.
13. Manocha Ms, San-Blas G, Centeno S. Lipid composition of *Paracoccidioides brasilienses:* Possible correlation with virulence of different strains. J Gen Microbiol 1980;117:147–154.
14. Costa GAN. Biotechnological production of surfactant agroindustrial residue in Bacillus subtilis, characterization and application. Campinas, Dissertation - (Master in Food Science), Faculty of Food Engineering, State University of Campinas - Unicamp. 2005;87.
15. Pacwa-Płociniczak M, Płaza GA, Piotrowska-Seget Z, Cameotra SS. Environmental Applications of Biosurfactants: Recent Advances. Int. J. Mol. Sci. 2011;12:633-654. DOI:10.3390/ijms12010633
16. Ghurye GL, Vipulanandan C, Wilson RCA. Pratical approach to biosurfactant production using nonaseptic fermentation of mixed cultures. Biotecnol. Bioeng. 1994;44:661-666.
17. Cao XH, Liao Zy, Wang Cl, Yang WY, Lu MF. Evaluation of a lipopeptidebiosurfactant from *Acillusnato* TK – 1 as a potencial source of anti-adhesive, antimicrobial and antitumor activies. Braz J. Microbiol. 2009;2(40):373-379.
18. Deka MG, Das K. Effect of biosurfactant from two strains of *Pseudomonas* on germination seedlings of *Cicerarietinum*and *Phaseolus mungoroxb*. African J. Biotechnol. 2009;3(28):350-356,
19. Cunha CD, Rosário MD, Leite SGF, Rosado AS. *Serratia* sp SVGG16: A promising biosurfactant producter isolated from tropical soil during growth ethanol-blended gasoline. Proc. Biochem. 2004;39:2277-2282.
20. Silva TAL, Araújo HWC, Tambourgi EB, Silva CAA, Takaki GMC. Biotechnological potential of a new strain of Pseudomonas fluorescens biosurfactant production using oil as substrate. Exacta, Sao Paulo. 20097;1:31-37.
21. Matsuura ABJ. Production and characterization of biosurfactants aimed at industrial and application in bioremediation processes. Thesis: Faculty of Food Engineering, State University of Campinas; 2004.
22. Kim PI, Bai H, Bai D, Chae H, Chung S, Kim Y, Park R, Chi YT. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. J. Appl. Microbiol. Purification and characterization of a lipopeptide produced by Bacillus thuringiensis CMB26. J. Appl. 2004;97:942-949.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=538&id=11&aid=4980