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Production of biosurfactant by *Bacillus subtilis* UCP 0999 using cassava wastewater (CWW) and waste frying oil (WFO) as renewable substrates

Produção de biossurfactante por *Bacillus subtilis* UCP 0999 usando manipueira e óleo residual de fritura como substratos renováveis

Producción de biosurfactante por *Bacillus subtilis* UCP 0999 utilizando agua residual de yuca y aceite de fritura residual como sustratos renovables

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Abstract

This study focused on the low-cost production of biosurfactant by *Bacillus subtilis* UCP 0999, using cassava wastewater (CWW) and waste frying oil (WFO) as renewable substrates. A 2^2 full-factorial design (FFD) was employed to investigate the effects of concentrations of agro-industrial wastes in surface tension (ST) and emulsification index (EI_{24}). According to the results, the minimum value of ST (33.2 mN/m) and the maximum value of EI_{24} (95%) was obtained at central point of the FFD, using 5% CWW and 2% WFO. Statistical analyses demonstrated the significative influence of both substrates in ST and EI_{24}. The biosurfactant showed 2.67 g/L yield and anionic and lipopeptide nature by Zeta potential and Fourier transform infrared (FT-IR) spectroscopy, respectively. In addition, promising applications of biosurfactant as biodispersant and viscosity reducing agent were demonstrated, suggesting its potential use in petroleum industry or bioremediation of hydrophobic pollutants.

Keywords: Agro-industrial substrates; Anionic tensioactive; Surface tension; Emulsification index.

Resumo

Este estudo teve como foco a produção de baixo custo de biossurfactante por *Bacillus subtilis* UCP 0999, utilizando manipueira e óleo residual de fritura como substratos renováveis. Um planejamento fatorial completo 2^2 foi empregado para investigar os efeitos das concentrações dos resíduos agroindustriais na tensão superficial (TS) e no índice de emulsificação (IE_{24}). De acordo com os resultados, o valor mínimo da TS (33,2 mN/m) e o valor máximo de IE_{24} (95%) foram obtidos no ponto central do planejamento fatorial, utilizando 5% de manipueira e 2% de óleo residual de fritura As análises estatísticas demostraram a influência significativa de ambos os substratos sobre a TS e IE_{24}. O biossurfactante apresentou rendimento de 2,67 g/L e natureza aniônica e lipopeptídica por potencial Zeta e espectroscopia de infravermelho com transformada de Fourier (FT-IR), respectivamente. Além disso, foram demonstradas aplicações promissoras do biossurfactante como biodispersante e agente redutor de viscosidade, sugerindo seu potencial uso na indústria do petróleo ou na biorremediação de poluentes hidrofóbicos.

Palavras-chave: Substratos agroindustriais; Tensoativo aniônico; Tensão superficial; Índice de emulsificação.

Resumen
Este estudio se centró en la producción de bajo costo de biosurfactante por *Bacillus subtilis* UCP 0999, utilizando agua residual de yuca y aceite de fritura residual como sustratos renovables. Se empleó un diseño factorial completo 2^2 para investigar los efectos de las concentraciones de los residuos agroindustriales en la tensión superficial (TS) y el índice de emulsificación (IE_{24}). De acuerdo con los resultados, el valor mínimo de TS (33,2 mN/m) y el valor máximo de EI_{24} (95%) se obtuvo en el punto central del diseño experimental, utilizando 5% de agua residual de yuca y 2% de aceite de fritura residual. Los análisis estadísticos demostraron la influencia significativa de ambos sustratos en TS y IE_{24}. El biosurfactante mostró un rendimiento de 2.67 g/L y naturaleza aniónica y lipopeptídica por potencial Zeta e espectroscopía de infrarrojo por transformada de Fourier (FT-IR), respectivamente. Además, se demostraron aplicaciones prometedoras del biosurfactante como biodispersante y agente reductor de la viscosidad, lo que sugiere su uso potencial en la industria petrolera o en la biorremediación de contaminantes hidrofóbicos.
Palabras clave: Sustratos agroindustriales; Tensioactivo aniónico; Tensión superficial; Índice de emulsificación.

1. Introduction

Surfactant molecules are mainly characterized by reducing surface tension between immiscible systems. From the structural point of view, they exhibit both a hydrophobic tail with one or several hydrocarbon chains (saturated or unsaturated fatty acids), and a hydrophilic head with one or several polar groups (such as carbohydrates, peptides and amino acids) (Alwadani & Fatehi, 2018; Samsu, et al., 2021). Furthermore, they are classified in three main groups: nonionic, anionic and cationic surfactants (Cheng, et al., 2020; Lichinga, et al., 2022).

Anionic surfactants are known to be effective at removing soil, clay, dirt and oily stains. They are generally effective at removing soil but are sensitive to the presence of multivalent ions present in hard water. Anionic surfactants ionize in the presence of water and become negatively charged, they then bind to positively charged particles such as clay. In addition, they are based on structures of quaternary ammonium molecules with alkyl chains attached to a positively charged nitrogen atom (Cheng, et al., 2020).

Due to the environmental problems caused by synthetic surfactants, the search for microbial surfactants has increased, due to the properties of these biomolecules: biodegradability, selectivity, nontoxicity, stability in a wide range of pH, temperature and salinity (Devda, et al., 2021). These advantages over classic surfactants, makes the demand for biosurfactants increase worldwide. An annual increase in the compound rate of 5.6% was expected between 2018 and 2027 (Gaur, et al., 2022). However, due to the Covid-19 (SARS-Cov-2) pandemic, the detergent industries have increased production demand in order to reduce the spread of the virus. This was proven by the 200% increase in sales in Italy in March 2020 (Collins, et al., 2022). Biosurfactants are able to interact with the plasma membrane of the virus, causing damage to the microorganism (Smith, et al., 2020; Daverey & Dutta, 2021).

However, traditional industrial production of biosurfactant via microbial synthesis remains a challenge due to the high cost is the use of synthetic nutritional raw materials (Prajapati, et al., 2021; Gaur, et al., 2022). Thus, an alternative approach is related to the use of renewable carbon and nitrogen sources from agro-industrial waste and by-products. For instance, waste frying oil (WFO) and cassava wastewater (CWW), a by-product of cassava (*Manihot esculenta*) processing for flour production, are among the suitable medium components to produce biosurfactant (Maia, et al., 2018; Ickofa, et al., 2020). Hence, the aim of this study was to produce a biosurfactant by *Bacillus subtilis* UCP 0999 using CWW and WFO as alternative and low-cost substrates.

2. Methodology

2.1 Microorganisms and preparation of inoculum

Bacillus subtilis UCP 0999 was kindly provided by the Culture Collection UCP (Universidade Católica de Pernambuco), Catholic University of Pernambuco, Recife-PE, Brazil, and registered in World Federation for Culture

Collections (WFCC). The microorganism was maintained on test tubes containing nutrient agar (peptone 5.0 g/L, meat extract 3.0 g/L and agar 18 g/L, pH 7), at 5°C (Maia, et al., 2018). For preparation of inoculum, *B. subtilis* was transferred from nutrient agar to nutrient broth (peptone 5.0 g/L, meat extract 3.0 g/L, pH 7), and incubated for 12 h at 30°C and 150 rpm.

2.2 Agro-industrial substrates

In this study, waste frying oil (WFO) was obtained from a local market and cassava wastewater (CWW) was kindly supplied by a flour house of the municipality of Pombos-PE, Brazil.

2.3 Production biosurfactant

Production of biosurfactant was carried out in previously autoclaved 250 ml Erlenmeyer flasks containing different concentrations of CWW and WFO, according to a 2^2 full-factorial design (FFD) (Table 1). Then, they were inoculated with 5% of the inoculum containing 10^8 colony-forming units (CFU)/ml and incubated for 96 h under orbital shaking at 150 rpm and 30°C. Subsequently, the cultures were centrifuged at 4000 *g* and filtered for separation of the cell-free supernatants, which were used to determine the ST and EI_{24}. The data obtained from the experiments were subjected to statistical analysis by Statistica® software, version 8.0 (StatSoft Inc., USA), using the concentrations of CWW and WFO as independent variables and ST and EI_{24} as response variables. The significance of the results was tested at $p < 0.05$ level.

Table 1. Levels of the variables for production of biosurfactant by *Bacillus subtilis* UCP 0999 using a 2^2 full-factorial design.

Variables	**Levels**		
	Low (-1)	**Central (0)**	**High (+1)**
Cassava wastewater - CWW (%, *v/v*)	2.0	5.0	8.0
Waste frying oil - WFO (%, *v/v*)	1.0	2.0	3.0

Source: Authors.

2.4 Determination of surface tension

ST was measured on cell-free metabolic liquids, using an automatic tensiometer (model Sigma 70, KSV Instruments Ltd., Helsinki, Finland) and the Du Noüy ring method, at room temperature (Kuyukina, et al., 2001). Measurements were performed in triplicate and surface tension of distilled water was used as control.

2.5 Determination of emulsification index

To determine the EI_{24} of the biosurfactant, 1 ml of the cell-free metabolic liquid and 2 ml of burned engine oil were insert in test tubes and vortexed during 2 min. The EI_{24} was determined after 24 h as the percentage of the height of the emulsion layer divided by the total height of the liquid column (Cooper & Goldenberg, 1987). Triplicates were performed for each sample.

2.6 Microscopic analysis of emulsions

The microscopic analysis of emulsion formed by biosurfactant in the best condition of FFD were carried out using a light microscope (Olympus BX50) with an increase of 40x and 100x. The diameter of the droplets obtained from the emulsion were observed and measured and the images were photographed in a digital camera.

2.7 Isolation of the biosurfactant

The isolation of the biosurfactant produced by *B. subtilis* was performed according to Falode, et al., (2017) from the cell-free supernatant using the ethanol precipitation method. The yield was determined by gravimetry and expressed in g/L from the crude biosurfactant washed twice with distilled water.

2.8 Preliminary characterization of the biosurfactant

To investigate the ionic charge of the biosurfactant, 100 mg were solubilized in distilled water. The Zeta potentiometer ZM3-D-G, Zeta Meter System 3.0+, was used determined the ionic character of the biosurfactant from the direct images recorded in a Zeta Meter video, San Francisco, CA, USA (Maia, et al., 2018).

The predominant functional groups of the biosurfactant were identified by Fourier transform infrared (FT-IR) spectroscopic analysis, using an attenuated total reflection (ATR) accessory consisting of a mixed “diamond/ZnSe” crystal. Infrared spectrum was recorded on a Shimadzu equipment, IR-TRACER 100, within the wavelength range of 500–4000 cm^{-1}.

2.9 Determination of dispersing capacity

The ability of the biosurfactant to disperse burned engine oil was investigated based on the appearance of a clear zone after the addition of the metabolic liquid containing the biosurfactant, indicating the dispersing capacity (Techaoei, et al., 2007). The diameter of the clear zone was measured as the oil displacement area (ODA) (Equation 1) and the results were expressed in cm^{-1}.

$$ODA = 3.14 \times r^2 \qquad \text{(Eq. 1)}$$

2.10 Determination of viscosity

The viscosity of hydrophobic compounds (WFO and burned engine oil) were investigated in test tubes containing 6 ml of the respective oils and 2 ml of the cell-free metabolic liquid containing the biosurfactant. The tubes were vortexed for 1 min and the viscosity was measured at 25ºC in automatic viscometer (Brookfield Middleboro, Middleborough, MA, USA; TC 500). The results were expressed in centipoise (cP).

3. Results and Discussion

3.1 Production of biosurfactant by *Bacillus subtilis* UCP 0999

The main condition to consider a microorganism as biosurfactant producer is the reduction of the surface tension to 40 mN/m or less (Nitschke & Pastore, 2004; Shah, et al., 2019). In current study, the surface tension values varied from 38.5 to 33.2 mN/m in all conditions of FFD, indicating the promising production of biosurfactant by *B. subtilis* UCP 0999 using CCW and WFO. However, the lower surface tension value (33.2 mN/m) was obtained in the central point (Table 2). This result was similar to those previously obtained by *B. subtilis* B20 using date molasses (Al-Bahry, et al., 2013) and *B. subtilis* ICA56 using cheese whey and sunflower oil (de França, et al., 2015). Recently, Felix et al. (2019) reported the production of biosurfactant by *B. subtilis* in medium containing clarified cashew apple juice, confirming the suitability of the utilization of agro-industrial wastes and by-products as alternatives substrates for this purpose.

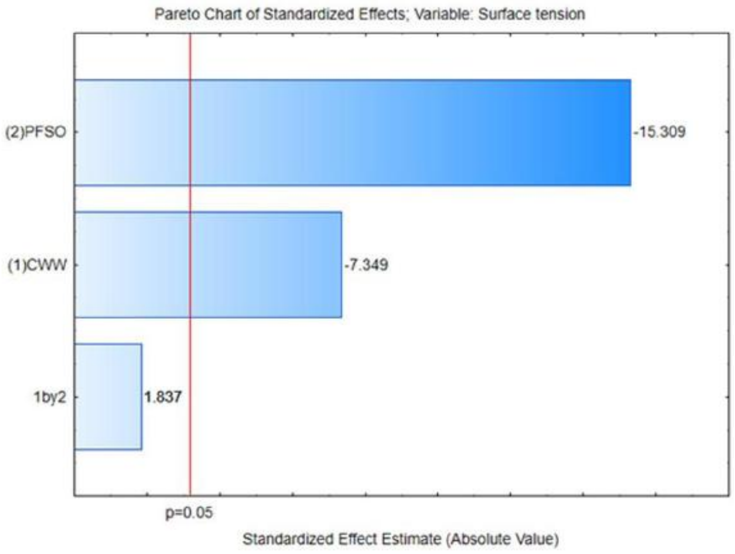
According to the literature, the medium composition, such as carbon and nitrogen sources strongly influences the production process of microbial surfactants (Silva, et al., 2018; Sun, et al., 2021). In this sense, Figure 1 illustrates the Pareto chart with the effect of CWW and WFO as independent variables on the surface tension, with a confidence level of 95%. According to the graphic, the input variables had a significant and negative effect on the surface tension (i.e., on the decrease of the surface tension in the cell free metabolic liquid). This means that the increase in the concentrations of CWW or WFO leads to a lower surface tension, suggesting the production of biosurfactant in the culture medium.

Table 2. Factorial design applied to the production of biosurfactant by *Bacillus subtilis* UCP 0999.

Assays	Production medium		ST	EI_{24}
	CWW (%)	WFO (%)	(mN/m)	(%)
1	2.0	1.0	38.5	83.2
2	8.0	1.0	37.0	63.3
3	2.0	3.0	35.7	87.8
4	8.0	3.0	34.8	90.0
5	5.0	2.0	33.6	94.1
6	5.0	2.0	33.4	94.3
7	5.0	2.0	33.4	94.1
8	5.0	2.0	33.2	94.1

Source: Authors.

Figure 1. Pareto chart of standardized effects of CWW (1) and WFO (2) on the surface tension for the 2^2 full-factorial design.



Source: Authors.

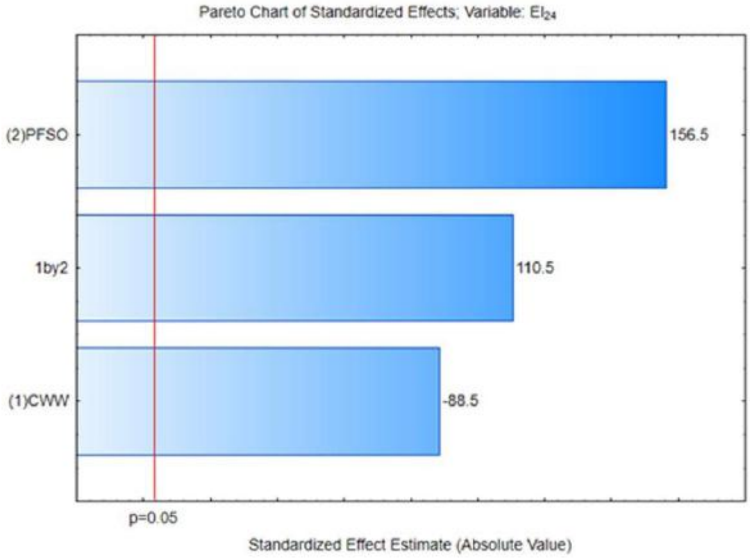
Previously, the production of biosurfactant by *B. subtilis* strains using CWW have been described in the literature (Nitschke & Pastore, 2004; 2006; Barros, et al., 2008; de Andrade, et al., 2016). In addition, various researches demonstrated the effectiveness of CWW and WFO as suitable substrates for microbial biosurfactant production (Montero-Rodríguez, et al., 2015; Araújo, et al., 2019; Ferreira, et al., 2020).

3.2 Emulsification index (EI_{24}) of the biosurfactant

Regarding the EI_{24}, the biosurfactant produced by *B. subtilis* showed the ability to maintain more than 60% of the original emulsion volume 24 h after its formation, for all conditions of FFD (Table 2). The maximum EI_{24} (94.3%) using burned engine oil was obtained in the central point. In this sense, this property allows biosurfactants to solubilize poorly-soluble substrates, which consequently increase their suitability in the cosmetics, food, pharmaceutical and petroleum industries (Uzoigwe, et al., 2015; Maia, et al., 2018; Meena, et al., 2021).

According to the Pareto chart showed in Figure 2, both substrates (CWW and WFO) and its interaction significantly influenced the EI_{24} in this study. However, CWW and its interaction with WFO showed a positive effect on the EI_{24}, indicating that the increase in the CWW concentration, as well as the simultaneous increase or decrease of both substrates, leads to an increase in the EI_{24}.

Figure 2. Pareto chart of standardized effects of CWW (1) and WFO (2) on the emulsification index for the 2^2 full-factorial design.

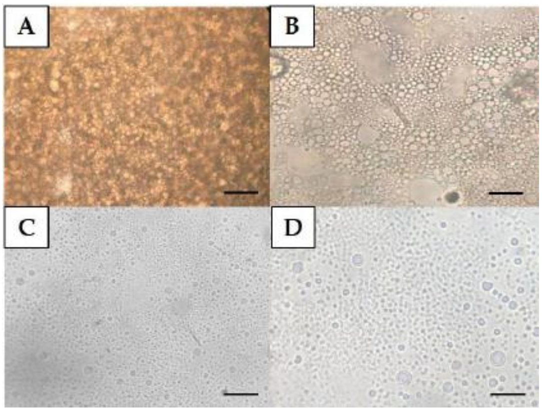


Source: Authors.

3.3 Characterization of emulsion droplets by light microscopy

Emulsions were formed using burned engine oil and WFO as hydrophobic substrates in the best condition of production (5 % of CWW and 2 % WFO) of the FFD. The emulsion formed with burned engine oil consisted small and stable droplets (size 0.5–1.0 µm) (Figure 3A), small areas with flocculation were observed at 100x magnification (Figure 3B). Figure 3C shows emulsion using WFO and small droplets (0.5-1.5 µm), and scattered, as show in figure 3D. Both emulsions show to be monodisperse, indicating stability of the emulsion against coalescence and were of water-in-oil-type emulsions.

Figure 3. Microscopic analysis of the droplets formed by the biosurfactant produced by *Bacillus subtilis* UCP 0999 with burned engine oil and waste frying oil (WFO). (A) Drops of emulsion with burned engine oil (40x); (B) Flocculation present in the emulsion with burned engine oil (100x). (C) Drops of emulsion with WFO (40x). (D) Drops dispersed in the emulsion with WFO. Bars: A and C = 50 µm; B and D = 10 µm.



Source: Authors.

Maia et al. (2018) described the presence of drops less than 0.5 µm of size and drops reaching an average size of 20 µm, in emulsion formed by the bioemulsifier produced by *B. subtilis* UCP 0146. In contrast, the bioemulsifier produced by the yeast *Candida lipolytica* UCP 0988 using burned motor oil also showed stability, however the average size of small and large drops was 0.1–5 µm (Souza, et al., 2016). In the same study, they observed that the synthetic surfactant sodium dodecyl sulfate (SDS) showed 100% of EI_{24} with average size of small and large drops of 2.5–12 µm.

3.4 Yield of biosurfactant

The biosurfactant produced at central point of the FFD (5% CWW and 2% WFO) was isolated using ethanol and a yield of 2.67 g/L was obtained. In this context, several researches described the production of biosurfactant by *B. subtilis* strains using various agro-industrial substrates and recovery techniques, according to Table 3.

Table 3. Production of biosurfactant by different *Bacillus subtilis* strains using agro-industrial substrates and recovery techniques.

Micro-organism	Agro-industrial substrate	Recovery technique	Yield (g/L)	Reference
B. subtilis ATCC 6633	Whey powder	Acid precipitation and extraction with solvents	0.18-0.24	Cagri-Mehmetoglu, et al. (2012)
B. subtilis B20	Date molasses	Acid precipitation	2.29	Al-Bahry, et al., (2013)
B. subtilis ICA56	Glycerol	Acid precipitation	1.29	De França, et al., (2015)
B. subtilis N1	Olive mill waste	Extraction liquid-liquid and acid precipitation	0.003	Ramirez, et al., (2015)
B. subtilis LB5a	CWW	Acid precipitation and extraction with solvents	0.3-3.0	de Andrade, et al., (2016)
B. subtilis KP7	Carrot peels extract	Acid precipitation and extraction with solvents	0.141	Paraszkiewicz, et al., (2018)
B. subtilis UFPEDA 438	Sugarcane molasses	Acid precipitation and extraction with solvents	0.2	Rocha, et al., (2021)
B. subtilis UCP 0999	CWW and WFO	Extraction with ethanol	2.67	Present study

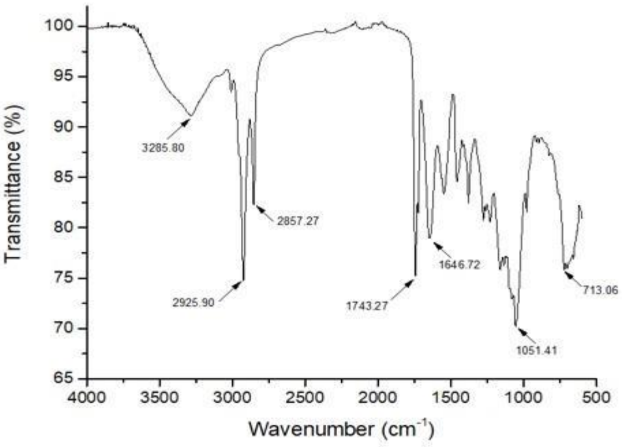
Source: Authors.

3.5 Preliminary characterization of the biosurfactant

The zeta potential determines the ionic charge of the particle and defines the stability of colloidal suspensions and emulsions. Thus, higher values of Zeta potential (30 mV) (either positive or negative) indicate a stable system (Ghazala, et al., 2017). According to the analyses using a Zeta Potential Meta 3.0+, the biosurfactant produced by *B. subtilis* showed stability and an anionic character (– 50.32 mV).

In addition, the chemical functional groups of the biosurfactant produced by *B. subtilis* UCP 0999 were investigated using the FTIR spectroscopic analysis. Figure 4 shows the infrared spectrum obtained, where an absorbance band was observed at 3285.80 cm^{-1} corresponding to a hydroxyl group (−OH); bands at 2926.90 and 2857.27 cm^{-1} for methyl and methylene groups ($-CH_2$ and $-CH_3$); and absorption band at 1646.72 cm^{-1} indicative of an amide moiety ($-CONH^-$). The functional groups identified in the present study are very similar to those previously reported by Das & Kumar (2019) and Jaiswal, et al. (2019). Hence, the biosurfactant produced by *B. subtilis* UCP 0999 might belong to lipopeptide group. Lipopeptide biosurfactants present in their composition heptapeptides and fatty acid chains and have been applied in bioremediation, in addition to having antiviral activity (Santos, et al., 2018; de França, et al., 2021).

Figure 4. FTIR spectrum of biosurfactant produced by *Bacillus subtilis* UCP 0999.



Source: Authors.

3.6 Dispersing capacity of biosurfactant

Dispersing agents are mixed additives to various products to form a uniform mixture and prevent from setting and clumping. They are formed by chemical surfactants and solvents and are widely used in varied industrial applications such as cleaning products, oil & gas, paints & coatings, construction, pulp & paper, agricultural, and others (Song, et al., 2013). However, chemical dispersant usage may cause some degree of environmental harm due to toxicity and non-biodegradability of some compound of its formulation. Consequently, there is a growing demand for environmentally friendly and cost-effective dispersants and biosurfactants are considered as promising candidates for such dispersant formulations (Almeda, et al., 2014; Shah, et al., 2019)

In this context, the dispersion effectiveness determined in this study demonstrated a good capacity for dispersion (ODA 25.12 cm^2) of the biosurfactant produced by *B. subtilis* UCP 0999. Similar results were previously reported by biosurfactants produced by *B. safensis* J2 (Das & Kumar, 2019) and *Bacillus sp* (Ram, et al., 2019).

3.7 Viscosity of biosurfactant

In this study, the effect of the biosurfactant of *B. subtilis* UCP 0999 on viscosity was investigated. According with the results, the biosurfactant reduced the viscosity of WFO from 119.2 to 62.5 cP and burned engine oil from 140.7 to 72.3 cP. Earlier study carried out by (Maia, et al., 2018) reported similar results by bioemulsifier produced by *B. subtilis* UCP 0146. This ability to reduce viscosity of hydrophobic compounds is promising for use in the petroleum industry and other environmental activities, such as the recovery of crude oil, the cleaning of reservoirs and the transportation of petroleum and derivatives (Andrade, et al., 2014; Ferreira, et al., 2020).

4. Conclusion

The promising future of biosurfactants depends fundamentally on the use of low-cost and widely available raw materials to achieve lower production costs. In this context, our study addressed to the utilization of CWW and WFO as alternative substrates to produce biosurfactant by *B. subtilis* UCP 0999. Biosurfactant produced showed efficient reduction of surface tension and distinctive emulsification properties. The biomolecule was preliminary characterized as anionic and lipopeptide nature and it showed good ability to reduce the viscosity of hydrophobic substrates as well as, to disperse burned motor oil in water. Therefore, this biosurfactant could be employed in petroleum industry in management of oil spills and oil recovery operations, for the development of sustainable environment.

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