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An Efficient Producing-Bioemulsifier by *Bacillus Subtilis* UCP 0146 Isolated from Mangrove Sediments

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Abstract: In this work was investigated the potential of *Bacillus subtilis* UCP 0146 in the bioconversion of the medium containing 100% of cassava flour wastewater to obtain bioemulsifier. The evaluation of the production was carried out by the emulsification index (IE_{24}) and surface tension (TS). The ionic charge, stability (temperature, salinity and pH measured by IE_{24} and viscosity), ability to remove and disperse oil and textile dye were investigated. *B.subtilis* produced an anionic bioemulsifier in the medium containing 100% of cassava wastewater in condition 4 of the factorial design (9% of the inoculum, at 35 °C and agitation of 100 rpm) with surface tension of 39mN/m, IE_{24} of 95.2 % and yield 2.69 $g.L^{-1}$. Stability at different pH (2-8), temperatures (0-120ºC) and NaCl, dispersed (55.83 cm^2-ODA) and reduced the viscosity of the burned engine oil (90.5 cP) , removed 94.4% petroleum and demonstrated efficiency in methylene blue removal (62.2%). The bioemulsifier and its synthesis from bacteria and also emphases on the role of surfactants in oil remediation.

Keywords: *Bacillus subtilis*; bioemulsifier; cassava wastewater; removal pollutant; methylene blue dye.

1. Introduction

The Bioemulsifiers are amphiphilic compounds, with high molecular weight, that have high response to surface tension. They have an hydrophobic portion (amino acids, mono /disaccharide peptides, polysaccharides) and hydrophilic (saturated or unsaturated fatty acids), allowing the interaction with fluids of different polarities (oil/water and water/oil), forming microemulsions, providing detergency, solubilizing and emulsifying action [1- 3].

A variety of microorganisms are producers of biomulsifiers, some have higher production capacity such as bacteria, and yeasts and to a lesser extent filamentous fungi. Regarding bacteria, there is the *Bacillus* species, in particular *B. subtilis* which has ability to produce lipopeptide, degrading long chain alkanes and reducing the viscosity of hydrocarbon [4-8]. In recent years, bioemulsifiers have been cited as leading biomolecules in the therapeutic and biomedical sector, as well as in agriculture, pharmacological products, dermatology, food and cosmetic industry and bioremediation [9-11].

The use in the petrochemical industry occurs as a result of the bioemulsifiers being considered better than the synthetic chemicals due to the characteristics of biodegradability, low toxicity and production from renewable sources [12- 18].

The use of agroindustrial residues as a low-cost raw material for the microbial production of bioemulsifiers is a well explored strategy in the field of biotechnology, in order to reducing production costs and practicing current environmental policies [19-24].

Thus, cassava wastewater is the main effluent from the cassava press due to the industrial production of flour. The chemical composition is basically constituted by carbohydrates, nitrogen and several mineral salts, characterizing itself as a supplement with significant potential [25-33].

The main objective this work was the bioemulsifier production by *Bacillus subtilis* UCP 0146 applying ecofriendly methology using as substrate cassava wastewater, in order to improve understanding the stability of the emulsion properties and applicability of this biomolecule.

2. Materials and Methods

2.1 Microorganism and Cultural Condition

The microorganism used was *Bacillus subtilis* UCP 0146 isolated of mangrove sediments (Rio Formoso- PE). It is mantained the Culture Collection UCP (Universidade Católica de Pernambuco)-Catholic University of Pernambuco, Recife-PE, Brazil, which is registered in World Federation for Culture Collection (WFCC). The bacterium was maintained in Nutrient agar(peptone 5.0 $g.L^{-1}$; meat extract 3.0 $g.L^{-1}$and agar 18 $g.L^{-1}$), at 5ºC, and was transferred for Nutrient broth (peptone 5.0 $g.L^{-1}$andmeat extract 3.0 $g.L^{-1}$).

2.2 Substrates

Cassava wastewater, a by-product of cassava (*Manihot esculenta*) processsing for flour production, was kindly supplied by the flour house of the municipality of Pombos -PE, Brazil. Methylene blue dye (molecular mass -333.6 g/mol) - (Sigma-Aldrich).

2.3 Production of Bioemulsifier

The inoculum was prepared by transferring *B.subtillis* cells from the solid medium to the nutrient broth, maintained at 30°C for 12h at 150rpm. Then, 5% of the inoculum containing 10^8 CFU/mL were transferred to Erlenmeyer flasks of 250 mL capacity with the bioemulsifier production medium, containing 100% cassava wastewater as described by Nitschke and Pastore [33]. The flasks were kept under 150rpm orbital shaking, for 96 h at 30°C. After this time, the samples were centrifuged at 4000 rpm and filtered for separation of the cell-free metabolic liquid. The experiments were performed in triplicate.

2.4 Full Factorial Design

The effects and interactions between inoculum size, temperature and agitation speed for bioemulsifier production were investigated using a 2^3 factorial design, as shown in Table 1. STATISTICA software version 6.0 of StatSoft® was used for statistical analysis of the results.

Table 1. Levels of the variables studied in 2^3 full factorial design for bioemulsifier production by *Bacillus subtilis* UCP 0146

	Levels		
Variables	**-1**	**0**	**+1**
Inoculum10^8 CFU/mL (v/v)	1	5	9
Temperature (ºC)	25	30	35
Orbital speed (rpm)	100	150	200

2.5 Determination of the pH

The pH of the bioemulsifier production medium was determined in the metabolic liquid in Orion potentiometer (Model 310) (Orion Research Inc., Cambridge, MA, USA).

2.6 Determination of Surface Tension (ST)

The surface tension was determined oncell-free metabolic liquid using tensiometer model Sigma 70 (KSV Instruments Ltd., Finland) by the Du Nouyring method at room temperature (± 28°C) reported by Kuyukina et al.[34].

2.7 Determination of Emulsification Index (EI_{24})

The emulsification index of cell-free metabolic liquid was analyzed according to Cooper and Goldenberg, [35]. The hydrophobic substrates used were vegetable oils (soybean, corn, canola, olive and waste soybean oil) and petro-derivatives (diesel and burned engine oil). After 24 h, the emulsification index (EI_{24}) was determinated and expressed in percentage. In addition, the shelf life was evaluated by emulsification index and viscosity for 150 days.

2.8 Optical Microscopic Analysis of Emulsions

The analysis of emulsions was carried out after adding 2 mL of hydrophobic substrates and 1 mL of cell-free metabolic liquid collected at 24, 48, 72 and 96h in test tubes and then, they were vortexed at high speed for 1 min. The emulsions were observed in optical microscope with an increase of 40x and a digital camera was used to capture the images.

2.9 Determination of Viscosity

The effect of the bioemulsifier on the viscosity of different oils (soybean, corn, waste soybean and burned motor oil) was investigated in test tubes containing 6 mL of the respective oils and 2 mL of 1% bioemulsifier solution (*w*/*v*).Then the tubes were vortexed for 1 min and the viscosity measured at 25ºC in an automatic viscometer (Brookfield (Middleboro, MA, USA; TC 500). The anionic surfactant sodium dodecyl sulfate (SDS) and commercial detergent were used as controls. The viscosity results were expressed in centipoise (cP) and percentage (%).

2.10 Determination of the stability

The stability of the bioemulsifier was evaluated in the cell-free metabolic liquid submitted to different pH (2, 4, 6, 8, 10, 12 and 14), NaCl concentrations (0, 2, 4, 6, 8, 10, 12 and 14%) and temperatures (0, 5, 70, 100 and 120°C) for 10 min.

2.11 Extraction and yield of the bioemulsifier

Bioemulsifier of *B. subtilis* was extracted from cell-free metabolic liquid using the ethanol precipitation method according to Nahato et al. [36].After extraction, the crude bioemulsifier was washed twice with lyophilized distilled water and the yield expressed in $g.L^{-1}$.

2.12 Determination of Ionic Charge

The ionic charge of the bioemulsifier was investigated using 100 mg of the solubilized biomolecule in 5 mL of distilled water. The ionic character was determined in Zeta potentiometer ZM3-D-G, Zeta Meter System 3.0+, and the direct images recorded in Zeta Meter video, San Francisco, CA., USA.

2.13 Application of the Bioemulsifier in Oil Spreading TestandDye Removal in Water

The ability of the bioemulsifier to disperse burned motor oil was investigated using fifty milliliters of distilled water were added to a large Petri dish (15 cm in diameter) followed by adding 20 μL of burned motor oil to the water surface and 10 μL of culture broth supernatant. A clear halo was visible under light. The area of this circle was measured and calculated to determine the oil displacement area (ODA) using the following equation: The appearance of a clear zone after the

addition of the metabolic liquid containing the bioemulsifier indicates the dispersing capacity of the bioemulsifier. The clear zone diameter was measured as the oil displacement area (ODA) and the results expressed in cm^{-1} Techaoei et al. [37].

The bioemulsifier produced by *B. subtillis* was tested for its potential to remove the methylene blue dye (molecular mass -333.6 g/mol) according to the methodology proposed by Asku et al. [38], modified by the use of the bioemulsifier to replace the chemical surfactant Sodium Dodecyl Sulfate (SDS).The experiments were carried out in 250 mL Erlenmeyer flasks containing methylene blue solution prepared from a standard solution ($1g.L^{-1}$) and the crude bioemulsifier ($1g.L^{-1}$), suspended in distilled water. The dye solution without bioemulsifier was used as a negative control. The experiments were performed in triplicate. The flasks containing methylene blue solution and bioemulsifier were maintained in orbital shaking at 150rpm for 48 h with removal of 5mL aliquots for determination of the biosorption kinetics of dye by the bioemulsifier. All samples were centrifuged (4000 rpm) for 5 min and the supernatant analyzed in a spectrophotometer at wavelength 663 nm. The biosorption efficiency was calculated according to the following equation 2:

$$\% \, Decolorization = \frac{A - B}{A} x \, 100 \qquad \text{Eq .(2)}$$

where:
A-Initial absorbance
B-Absorbance of decolorized solution

2.14 Application in the Removal of Oil in Marine soil

The ability of the bioemulsifier to remove oil in marine soil was investigated using 20 g of marine soil collected from the beach (Recife-PE), impregnating with 5 mL of burned motor oil and transferred to a 250 mL Erlenmeyer flask. Then, 30mL of the cell-free metabolic liquid containing the bioemulsifier was added and the flasks were kept under orbital shaking at 150 rpm and 28°C for 48 h, according to the methodology of Nitschke and Pastore [4]. Removal of the oil was determined by washing the marine soil with hexane and the results were expressed in percentage.

3. Results

3.1 Production of Bioemulsifier by Bacillus subtilis UCP 0146 in Medium Containing Cassava Wastewater

The production of Bioemulsifier by *Bacillus subtilis* in medium formulated with cassava flour wastewater (100%) reduced the surface tension from 72 to 39mN/m and resulted in 95% of emulsification using motor burned oil (Table 2).

Table 2. Surface tension and emulsification index (EI_{24}) for different hydrophobic substrates obtained by bioemulsifier produced from *Bacillus subtilis* UCP 0146in medium formulated with cassava wastewater

Surface tension (mN/m)	Hydrophobic substrates	Emulsification index (EI_{24}) %
38.2	Soybean oil	50.0
38.0	Corn oil	65.0
38.2	Canola oil	50.0
38.4	Olive oil	90.0
38.7	Wastes soybean oil	50.0
37.8	Kerosene	40.0

37.2	Diesel	-
39.0	Burned engine oil	95.0
37.4	Olive oil	90.0

From the identification of the occurrence of the production of the bioemulsifier by *Bacillus subtilis*, a complete factorial design was performed (Table 1) to evaluate the effect of temperature, agitation and inoculum volume in bioemulsifier production. The results showed that the bioemulsifier produced in condition 4 of the factorial design (temperature 35ºC, 100rpm and inoculum of 9%) maintained a high emulsifying capacity (95.2%) after 150 days (IE_{150}) using motor burnt oil as a hydrophobic substrate (Table 3). The bioemulsifier produced in condition 6 of the factorial design resulted in values of IE_{24} of 95% similar the condition 4 (95.2%). However did not maintain stability during 150 days (IE_{150}) with the motor burned oil, thus being chosen the condition 4 of the planning.

Table 3. Results of factorial design of 2^3 to evaluate the influence of temperature, agitation and inoculum volume in production of bioemulsifier by *Bacillus subtilis* UCP 0146 evaluated during 150 days by the emulsification index EI 24 (%) and EI 150(%)

Assay	Burned Motor Oil		Soybean Oil		Waste Soybeanoil		Corn Oil		Canola Oil	
	EI_{24} (%)	EI_{150} (%)	EI_{24} (%)	EI_{150} (%)	EI_{24} (%)	EI_{150} (%)	EI_{24} (%)	EI_{150} (%)	EI_{24} (%)	EI_{150} (%)
1	85.0	65.0	20.0	4.0	10.0	5.0	15.0	11.0	15.0	10.0
2	95.0	75.0	1.0	45.0	25.0	5.0	10.0	10.0	30.0	10.0
3	75.0	25.0	10.0	10.0	45.0	10.0	10.0	10.0	15.0	8.0
4	**95.2**	**95.0**	**10.0**	**5.0**	**15.0**	**10.0**	**10.0**	**25.0**	**20.0**	**15.0**
5	95.0	80.0	50.0	5.0	50.0	10.0	25.0	15.0	35.0	15.0
6	95.0	65.0	50.0	5.0	50.0	5.0	65.0	7.0	50.0	7.0
7	87.0	73.0	5.0	0.0	35.0	5.0	13.6	10.0	5.0	10.0
8	90.0	75.0	5.0	5.0	40.0	5.0	8.7	8.0	5.0	5.0
9	93.3	86.0	10.0	5.0	26.1	20.0	15.0	15.0	8.7	5.0
10	87.5	60.0	16.7	15.0	56.5	25.0	10.0	15.0	10.0	5.0
11	87.5	75.0	45.0	10.0	87.0	25.0	10.0	15.0	15.0	10.0
12	92.0	85.0	43.5	10.0	90.9	25.0	18.2	15.0	10.0	10.0

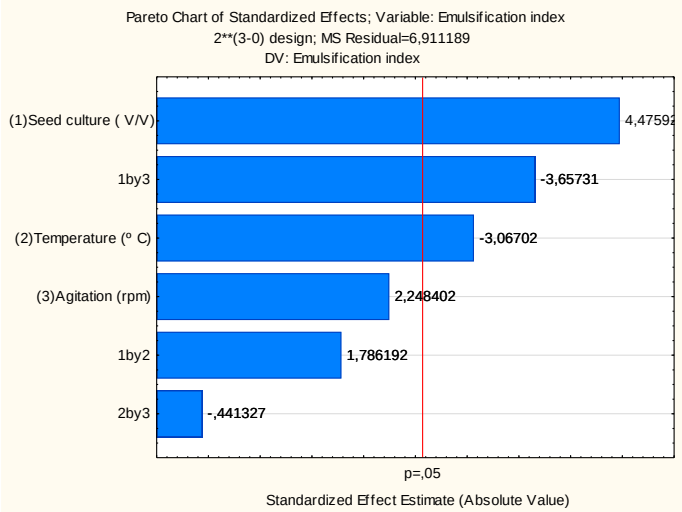
Levels : Inoculum: -1 (1) , 0 (5) , 1 (9);
Temperature (ºC): -1 (25) ,0 (30) , 1 (35);
Orbital speed (rpm): -1 (100) , 0 (150) , 1 (200)

In the selected condition 4 of the factorial design (temperature 35 °C, 100 rpm and 9% inoculum) the crude bioemulsifier yield was 2.69 $g.L^{-1}$. The cell-free metabolic liquid containing the bioemulsifier was able to form a mixture with the burned motor oil and reduced the viscosity of this oil from 170 to 90.5Cp after 24h. This assay was followed for 150 days to identify the ability of the bioemulsifier to keep stable the viscosity of the emulsions formed. The results showed that there were few variations in viscosity during 150h, resulting in values ranging from 68.8Cp to 90.5Cp. However, it was confirmed that the metabolic liquid cultured at 60 h was the best to maintain the viscosity and stability of the emulsions with values of 88.9Cp after 150h.

3.2 Determination of Influence of Inoculum size, Temperature and Agitation on bioemulsifier production by Bacillus subtilis

The effect of the independent variable volume of the inoculum, temperature and speed about the emulsification index (IE_{24}) using burned engine oil as hydrophobic substrate is represented in the Pareto Diagram (Figure 1) with a confidence level of 95%. According to this diagram the increase in inoculum volume exercised a statistically positive effect about increase of IE_{24}. In addition, it was also observed that the interaction of the inoculum volume variable and the agitation variable exercised a statistically negative effect statistically effect on the increase of IE_{24}.

Figure 1. Pareto chart for determinations of the influence of inoculum size, temperature and agitation on bioemulsifier production by *Bacillus subtilis*.



3.3 Characterization of Emulsion Droplets Using Burned Engine Oil

The emulsions formed by the bioemulsifier using burned engine oil were measured by optical microscopy (Figure 2), and characterized according the main properties and emulsification index and viscosity showed in Table 4. The results showed the formation of water-in-oil type emulsions, in condition 4 of the factorial design (9% inoculum, cultivation at 35ºC and agitation of 100 rpm), in medium containing 100% cassava wastewater. The cell-free metabolic liquid cultured at 24h showed the presence of large and thermodynamic unstable droplets, with globose and heterogeneous appearance and EI_{24} of 80% (Figure 2A).

Figure 2B shows the metabolic liquid cultured at 48 h where no significant changes occurred, but flocculation of the droplets and greater free space were observed. In Figure 2C appears the metabolic liquid cultured at 72 h with the formation of droplets with smaller, larger, homogeneous and stable droplets with an EI_{24} of 90%. However, the formation of droplets with smaller diameter (0.3 μm), globose, homogeneous, stable and with a high emulsification index (90%) occurred in the metabolic liquid cultured at 96 h (Figure 2D). Similar characteristics of stability, size and type of emulsions formed by the chemical surfactant Sodium Dodecyl Sulfate (SDS) were mentioned by (Souza et al., 2016)and are compatible with those observed in this study using the metabolic liquid of *B. subtilis* after 96 h of cultivation in cassava wastewater (Figure 2D).

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Figure 2. Microscopic observations of the droplets formed from bioemulsifier: (A) Metabolic liquid at 24h; (B) Metabolic liquid at 48 h; (C) Metabolic liquid at 72h (D) Metabolic liquid at 96h.

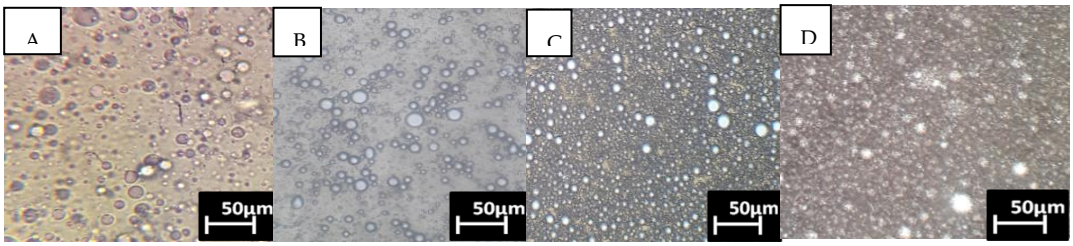

Table 4. Evaluation of stability of bioemulsifier after exposition in different pH, concentrations of NaCl and temperatures by viscosity determinations

Time (h)	Characteristics of emulsions	Diameter of drops (µm)	Emulsification index EI_{24} (%)	Viscosity cp	Thermodynamics tability
24	Big, globose and heterogeneous droplets	1-30	80	90.5	Unstable
48	Big, globose and heterogeneous drops	1-30	84	87.2	Unstable
72	Small, compact and homogeneous drops	0.5-20	90	47.0	Stable
96	Small, compact and homogeneous drops	0.3-20	92	48.0	Stable
*SDS (control)	Small and homogeneous drops	2.5-12	100	24.1	Stable

3.4 Stability of the bioemulsifier by determination of viscosity and emulsification index

The *B. subtilis* bioemulsifier was able to maintain high emulsifying capacity with IE_{24} values above 80% after abrupt pH variations (with exception of pH 14)(Figure 3A). In relation to high concentrations of NaCl (Figure 3B), it was shown that the emulsions with motor oil remained stable in all tested concentrations of NaCl, with values around 90%. The thermal stability of the bioemulsifier (Figure 3C) was performed between 0 and 120ºC, revealing that the emulsions with burned motor oil, with a decrease of 10% at 120ºC.

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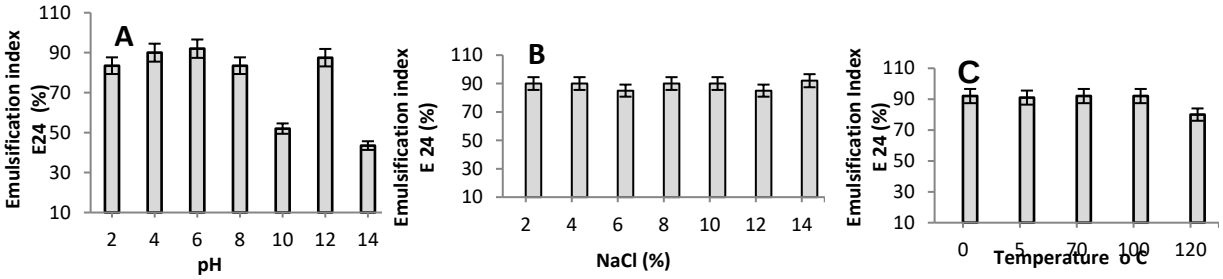
Figure 3. Stability of the bioemulsifier produced by *Bacillus subtilis* UCP 0146 according to emulsification index (EI_{24}): pH (A), NaCl (B), and temperature (C)



The stability of the bioemulsifier according to the viscosity were also investigated and it was demonstrated that the maximum reduction of the viscositiy of the emulsions (10-20Cp) occurred after the change in the pH of the cell-free metabolic liquid, except at pH 4 (Table5).

Table 5. Characteristics and properties of the bioemulsifier produced by *Bacillus subtilis* UCP 0146

Assay	**Viscosity Determination (cP)/Condition**						
pH	**2**	**4**	**6**	**8**	**10**	**12**	**14**
cP	8.20	11.00	17.00	8.30	13.50	10.30	5.40
NaCl (%)	**2**	**4**	**6**	**8**	**10**	**12**	**14**
cp	20.50	79.40	13.10	7.86	38.40	30.20	30.70
Temperature(^{0}C)	**0**	**5**	**70**	**100**	**120**		
cP	15.10	12.00	12.40	16.30	7.86		

Significant emulsifying activity, stability over a wide range of pH, extreme temperatures and high salt concentrations, in addition to reducing oil viscosity indicates that the bioemulsifier is suitable for use in the petroleum industry and other environmental activities, such as recovery of oil, cleaning of reservoirs, and transportation of crude oil.

3.5 Efficiency of the Bioemulsifier in petroderivate Dispersing and Pollutants Removal

The anionic bioemulsifier obtained by *B. subtilis* in medium containing 100% cassava wastewater, under condition 4 of the FFD, was investigated as to the biosorption potential of the cationic dye methylene blue present in water. The results showed that the efficiency of biosorption was promising because the biomulsifier was able to remove 62.2% of the dye in aqueous solution after 12 h (Figure 4) when compared to the control synthetic emulsifier.

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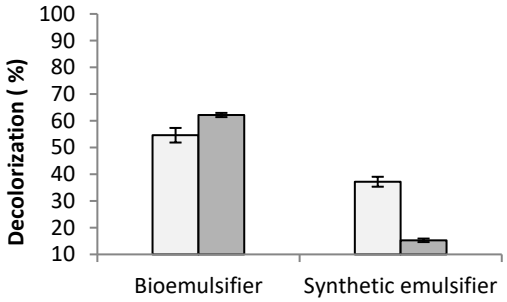
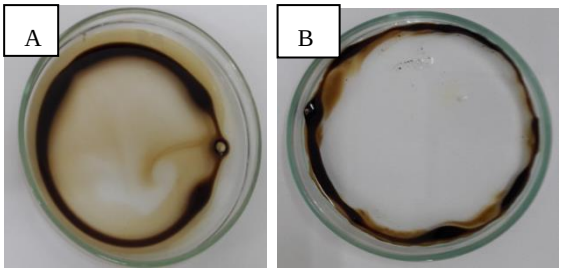
Figure 4.Methylene blue dye removal by bioemulsifier produced from *Bacillus subtilis* during the time 1 and 12h



The bioemulsifier produced by *B. subtilis* in the best condition of the Full Factorial Design (Assay 4) was applied in the dispersion and removal of burned engine oil present in water and marine soil, respectively. The dispersion efficiency measured as the oil displacement area (ODA) demonstrated that the bioemulsifier exhibits excellent dispersant capacity (55.38 cm^2 ODA) after having been compared with dispersion by the commercial detergent as shown in Figure 5.

Figure 5. Dispersion of burned engine oil in water added bioemulsifier produced by *Bacillus subtilis*: (A) Commercial detergent as Control and (B) Bioemulsifier.



On the other hand, the bioemulsifier of *B. subtilis* was also able to demonstrate a significant capacity to act as a petroderivative removal, after detecting the removal of 94.4% of the burned engine oil impregnated in marine soil.

4. Discussion

The results obtained in this work suggest that the biomolecule produced by *B. subtilis* has characteristics of a bioemulsifier. According with Rahman, et al. [39] the bioemulsifiers are characterized by not causing significant changes in the reduction of surface tension between liquids and high emulsifying activity.

According to this diagram the increase in inoculum volume exercised a statistically positive effect about increase of IE_{24}. In addition, it was also observed that the interaction of the inoculum volume variable and the agitation variable exercised a statistically negative effect statistically effect on the increase of IE_{24}.

According to Willumsen, Chiistian and Ruzicka [40], the main characteristic of emulsifying agents is their ability to maintain stability of substances with different degrees of polarity represented by emulsification values (EI_{24}) above 50% after 24 h. Presented similar results [40,42], obtaining significant values throughout the pH range, except when the biosurfactant ws exposed do pH 12. Sarrubbo et al. [43] reported that pH extremes denature protein components by increasing ionization.

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The biosurfactant produced by *C. lipolytica* grown in corn steep liquor and waste soybean oil also obtained emulsion activity with the motor oil burned unchanged up to 10% NaCl, lost activity at 12% NaCl concentration [40-45].The bioemulsifier produced by *C. lipolytica* in medium supplemented with corn steep liquor and waste soybean oil did not obtain emulsifying activity in values higher than 5% NaCl [3].

Studies by Santos et al.[42] showed stability at low and high temperatures, exhibiting a 20% (73%) reduction in emulsification index at 120 °C. Studies conducted by Regina and Silva, [14] and Souza et al.[3], maintained emulsions formed in 65% and 80% motor oil, respectively, when exposing the bioemulsifier at 100 ° C.

According to Wei et al. [46], biosurfactants capable of reducing the viscosity of oils are suitable for use in the petroleum industry, since the low viscosity facilitates the considerable removal of the oil. As the study by Regina and Silva [14] added the bioemulsifier to motor oil obtained viscosity increase 148.9 cP to 210.7 cP.

The removal of cationic dyes by anionic agents is explained by the fact that they are compounds of opposite charges that enter into equilibrium in aqueous solution and forming the pairs of hydrophobic ions, or more complexes associated, or even aggregates between the cationic dye and the anionic bioemulsifier in solution [40-47].

In the study of Andrade at al. [14], the bioemulsifier obtained from the fermentation of *Cunninghamella echinulata* in waste soybean oil and corn steep liquor showed and oil dispersion area of 37.36 cm^2. The study conducted by Souza et al.[3] produced a bioemulsifier by *Candida lipolytica* with dispersibility of 45.34 cm^2.

The biosurfactant produced by *C. lipolytica* UCP 0988 investigated by Santos et al.[42], removed 92.3% of the burned engine oil. In the study by Andrade et al. [38], the biosurfactant produced by *Candida glabrata* removed by the washing method 95.7% of burned engine oil.

5. Conclusion

The utilization of cassava wastewater for bioemulsifier production by *Bacillus subtilis* UCP 0146 is truly significant as eco-friendly methodology and it is available, regionally significant and inexpensive waste material generated from food industry. The bioemulsifier produced in this study is a biomolecule showed important properties with higher biotechnological potential for bioremediation in pollutants removal and dye decolorization.

Bioemulsifier advantages is production from cost-free by-products, and have advantages of use of the waste for minimization of In the other hand, bioemulsifier was able to form stable emulsion for up to five months, suggesting great potential for pharmaceutical and cosmetic industrial applications, as well as in bioremedition process. Moreover, the *Bacillus subtilis* UCP 0146 showed potential for use recalcitrant waste as an alternate carbon source for bioemulsifier production would provide an advantage for industrial production.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, G.M.C.T. and R.F.S.A.; Methodology, P.C.V.S.M.; Software, M.A.C.L., T.A.L.S. and V.P.S; Validation, P.C.V.S.M, M.A.C.L and V.P.S.; Formal Analysis, G.M.C.T., M.A.C.L. and A.S.F.; Investigation, P.C.V.S.M ; Resources, P.C.V.S.M and T.A.L.S.; Data Curation, G.M.C.T., P.C.V.S.M, A.S.F. and M.A.C.L; Writing-Original Draft Preparation, P.C.V.S.M.; Writing-Review & Editing, R.F.S.A.; Visualization, P.C.V.S.M., R.F.S.A and G.M.C.T.; Supervision, G.M.C.T.; Project Administration, G.M.C.T.; Funding Acquisition, G.M.C.T;” please turn to the CRediT taxonomy for the term explanation.

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