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Application of a Biosurfactant Produced by *Bacillus cereus* UCP 1615 from Waste Frying Oil as an Emulsifier in a Cookie Formulation

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Abstract: Biosurfactants have attracted increasing interest from the food industry due to their emulsifying, foaming and solubilizing properties. However, the industrial use of microbial biosurfactants has been hampered by the high production costs related mainly to the use of expensive substrates. The search for low-cost alternative substrates is one of the strategies adopted to overcome this problem. In the present study, a biosurfactant produced by *Bacillus cereus* UCP1615 by fermentation in a medium supplemented with waste frying soybean oil as a low-cost substrate was evaluated as a bioemulsifier for the production of cookies. The biosurfactant was evaluated for its emulsifying capacity against different vegetable oils, antioxidant activity and toxicity, demonstrating favorable results for use in food. In particular, it showed satisfactory antioxidant activity at the tested concentrations and no cytotoxicity to the L929 (mouse fibroblast) and Vero (monkey kidney epithelial) cell lines using the MTT assay. The biosurfactant was then added at different concentrations (0.25%, 0.5% and 1%) to a standard cookie dough formulation to evaluate the physicochemical characteristics of the product. Cookies formulated with the biosurfactant exhibited similar energy and physical characteristics to those obtained with the standard formulation but with a lower moisture content. The biosurfactant also ensured a good preservation of the cookie texture after 45 days of storage. These results suggest that the biosurfactant has a potential application as a green emulsifier in accordance with the demands of the current market for biocompatible products.

Keywords: biosurfactant; bioemulsifier; waste frying oil; *Bacillus cereus*; food additives; cookie

1. Introduction

Globalization and the growth of the population have promoted the expansion of the production of, and investments in, complex food supply dynamics. However, food safety issues arise such as the origin and properties of products and components added to food. Most of these substances, including thickeners, stabilizers and emulsifiers, are important additives for the quality of food [1] because they help maintain or even improve their appearance, freshness, flavor, texture and safety [2].

The search for "green" ingredients has intensified in the food industry thanks to the progress of studies and the increase in competition in the sector as well as the growing interest of consumers for natural ingredients over synthetic additives [3]. This interest is mainly associated with the growing demand for natural, organic and vegan food [4].

Biosurfactants are promising products obtained from biological sources whose attractiveness is due to their biodegradability, specific bioactivity, sustainable production and low toxicity [5,6]. These features give biosurfactants considerable potential for practical applications particularly in the food, cosmetic, healthcare, biomedical and pharmaceutical sectors [5].

The literature describes improvements in the texture, volume and conservation of baked goods due to the addition of biosurfactants. Researchers reported improvements in the viscosity of food products when using microbial emulsifiers, the efficient emulsification of fat from meat products, enhanced solubilization of aromas and a greater stability of salad dressings [1]. Biosurfactants are also effective in solubilizing vegetable oils, stabilizing fats during cooking processes and improving the organoleptic properties of bread. Biomolecules can be used in ice cream formulations, muffins (as an ingredient to replace baking powder and eggs), cookies and salad dressings. The use of microbial emulsifiers was also shown to reduce the use of currently marketed emulsifiers in farinaceous food and to improve their rheology [1,2].

Among the different types of biosurfactants explored, lipopeptides and glycolipids stand out due to their desirable properties for application in the food industry such as antibacterial activity against a variety of species, antioxidant activity and low cytotoxicity. The lipopeptides produced by bacteria of the genus *Bacillus* are examples of microbial biosurfactants obtained by fermentation [7,8] whose main characteristics are an emulsification capacity and a reduction of surface tension along with antioxidant, antiadhesive, antibiofilm, antibacterial, antifungal, antitumor and antiviral properties [6,9].

However, the industrial production of biosurfactants faces great challenges due to the high costs of microbial cultivation and their recovery [10]. One of the solutions to make the industrial production of these biomolecules economically feasible consists of the use of agro-industrial co-products as substrates for the fermentation process, given that the substrate accounts for up to 50% of the final manufacturing cost [7]. The food industry generates waste products that often contain high concentrations of carbohydrates, lipids and proteins, which makes these co-products attractive candidates for fermentation processes [11]. Among such substances, waste cooking oil—which is produced in the kitchens of homes, restaurants and industries—is considered to be hazardous to the environment and human health; therefore, it should be collected to reduce the environmental impact of its improper disposal [12].

A 2019 report on the prospects for the commodities market by the Organization for Economic Cooperation and Development (OECD) and the Food and Agriculture Organization (FAO) of the United Nations states that approximately 210 million tons of vegetable oils are produced and consumed by humans every year. Therefore, the estimated annual global production of waste cooking oil is around 42 million tons [13]. The use of agro-industrial waste products or renewable raw materials in fermentation processes to produce biosurfactants is in line with green chemistry and is an important tool for sustainable innovation, which meets the demands of the current market [14].

Therefore, the aims of the present study were (a) to investigate the use of a biosurfactant produced by *Bacillus cereus* UCP 1615 as an additive in a cookie formulation, (b) to analyze the nutritional benefits of its addition, (c) to check its non-toxicity, (d) to determine its antioxidant potential and (e) to evaluate its effects on the physicochemical properties as well as the texture of the product.

2. Materials and Methods

2.1. Microorganism and the Production of the Biosurfactant

The bacterium *Bacillus cereus* UCP 1615 obtained from the culture bank of the Catholic University of Pernambuco was used as the biosurfactant-producing microorganism. This strain was previously isolated from environmental samples of water contaminated with petroleum byproducts spilled from ships (port area) in the Atlantic Ocean in the state of Pernambuco, Brazil. As described by Durval et al. [15], the bacterium was cultured by adding a 2% cell suspension (optical density of 0.7 at a wavelength of 600 nm corresponding to a 24 h inoculum of 10^7 colony-forming units/L) to a 500 mL flask containing 100 mL of a mineral salt medium (0.087% K_2HPO_4, 0.65% trisaminomethane, 0.02% KCl, 0.06% $MgSO_4 \cdot 7H2O$, 0.01% NaCl and 0.005% yeast extract) supplemented with 2% waste frying soybean oil and 0.12% peptone with the pH adjusted to 7.0 ± 0.2. After culturing for 48 h at 28 °C and 250 rpm, the samples were withdrawn to determine the concentration of the biosurfactant.

2.2. Isolation of the Biosurfactant

The biosurfactant was extracted after centrifugation of the fermentation broth at 5000× *g* for 30 min to remove the cells. A 6.0 M HCl solution was added to the supernatant to adjust the pH to 2.0, followed by the addition of a 2:1 (*v*/*v*) solution of $CHCl_3/CH_3OH$. After vigorous shaking by hand for 15 min and a phase separation, the organic phase was removed and the operation was repeated two more times. The organic phases were pooled and put on a rotary evaporator under a vacuum at 40 °C until the complete evaporation of the solvents and the obtention of the isolated biosurfactant [15]. The extraction procedure allowed the isolation of the biomolecule from the fermented broth and, at the same time, the suppression of any live cells still present in it.

2.3. Evaluation of the Biosurfactant Cytotoxic Potential

The biosurfactant cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) method [16,17]. The L929 (mouse fibroblast) cells and the Vero (renal epithelial) cells from the African green monkey were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% inactivated fetal bovine serum and a 1% antibiotic (penicillin and streptomycin) solution. The cells were kept in a chamber at 37 °C in an atmosphere enriched with 5% CO_2 and 95% humidity.

The cells were plated (10^5 cells/mL) in 96-well plates and incubated for 24 h. Next, 10 µL of the biosurfactant solutions was added to the wells at a final concentration of 3.12 to 400 µg/mL. After a further incubation for 72 h, 25 µL of the MTT solution (5 mg/mL) was added, followed by an incubation of 3 h. The culture with the MTT was then aspirated and 100 µL of dimethyl sulfoxide was added to each well. The absorbance was measured in a microplate reader at a wavelength of 560 nm. The experiments were conducted in triplicate and the percentage of inhibition was calculated with the aid of the demo version of GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA).

An intensity scale was used for the determination of toxicity. Samples with 95 to 100% inhibitory activity were considered to be highly toxic, those with 70 to 90% inhibitory activity were considered to be moderately toxic and those with inhibitory activity less than 50% were considered to be non-toxic [18].

2.4. Antioxidant Activity

2.4.1. Total Antioxidant Capacity

The total antioxidant capacity (TAC) was determined using the phosphomolybdenum method [19], which is based on the reduction of Mo^{6+} to Mo^{5+} by the sample and the subsequent formation of a greenish phosphate/Mo^{5+} complex. Tubes containing the samples and reagents (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 100 °C for 90 min. The absorbance of each solution was measured at wavelength of 695 nm against a blank. The TAC was expressed in relation to a

solution of ascorbic acid at a concentration of 1 mg/mL assumed to be 100%. All assays were carried out in triplicate.

2.4.2. DPPH Scavenging Activity

The antioxidant activity of the biosurfactant was also evaluated using the free radical sequestration method based on hydrogen donation using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) [20]. The measurements were performed in triplicate and the inhibition activity was calculated based on the percentage of DPPH$^{\bullet}$ scavenged. The vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and butylated hydroxytoluene (BTH) were used as standards. The percentage of inhibition (I%) was calculated using the equation:

$$I\% = \frac{A_c - A_s}{A_c} \times 100 \quad (1)$$

in which Ac is the absorbance of the control and As is the absorbance of the sample.

2.4.3. ABTS Scavenging Activity

The determination of the antioxidant activity of a biosurfactant using the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay is based on the generation of the cationic chromophore radical obtained from the oxidation of ABTS by potassium persulfate [20]. The measurements were performed in triplicate and the inhibition activity was calculated based on the percentage of ABTS removed. Trolox and BTH were used as standards. The percentage of inhibition (I%) was calculated as described above for the DPPH scavenging activity.

2.5. Emulsification Activity

The emulsification activity of the biosurfactant was determined following the method described by Gaur et al. [21]. Vegetable oils from soybeans, corn, canola and peanuts were added at a 1:1 proportion (*v*/*v*) to an aqueous biosurfactant solution at concentrations of 10, 5.0 and 2.5 mg/mL in glass tubes and the content was vortexed for 2 min at a frequency of 50 Hz. After 24 h, the emulsification index (E_{24}) was determined according to the equation:

$$E_{24} = \frac{h_e}{h_t} \times 100 \quad (2)$$

in which h_e is the height of the emulsion layer and h_t is the total height of the mixture.

All samples were stored at room temperature.

2.6. Cookie Formulation and Preparation

The biosurfactant was tested in the standard cookie formulation described by Ribeiro et al. [22] (Table 1). The extract containing the biosurfactant was used in this formulation as an additive at three different concentrations (1%, 0.5% and 0.25%) in relation to the total dough for the analysis of the physical and physicochemical properties.

Table 1. Composition of the cookie dough.

Ingredient	Standard Formulation (%)
White wheat flour	47.0
Margarine	20.0
Sugar	15.0
Vanilla extract	3.0
Baking powder	1.0
Pasteurized egg white	40.0
Pasteurized egg yolk	4.0
Biosurfactant	0.0

The dough was also prepared following the method described by Ribeiro et al. [22]. The ingredients were purchased from local establishments and blended in a mixer (Turbomix Duo MX21, Arno Ciranda, Florianópolis, SC, Brazil) for 7 min. The dough was then spread on a polyethylene cutting board and shaped into circular forms with the aid of stainless steel molds with a 50 mm diameter to standardize the cookies. The specimens were placed in an oven for 5 min at 150 °C after which the temperature was increased to 180 °C for an additional 15 min. The cookies were then cooled, weighed, packed in vacuum-sealed plastic bags and stored at room temperature for 24 h.

2.7. Physicochemical Properties and the Energy Value of the Cookies

The weight, diameter, thickness and spread factor of the cookies were determined with and without the biosurfactant [23,24]. The total diameter was determined by using four randomly selected specimens placed side by side. The cookies were then turned 90° and the diameter was measured again. The final diameter was expressed as the mean of the two measurements divided by four. The thickness was determined by stacking four cookies and expressed as the total height divided by four. The spread factor was determined by dividing the diameter by the height.

The physicochemical properties of the cookies were determined using the analysis methods of the Association of Official Analytical Chemists [25]. The moisture content was determined gravimetrically considering the mass loss from the specimens submitted to heating in an oven at 105 °C until a constant weight was reached. The concentration of total protein was calculated using the Kjeldahl method, which consists of the acid digestion of organic matter followed by distillation, the quantitative determination of nitrogen by titration and multiplication of the obtained value by a factor of 6.5. The gravimetric method was used for the determination of the fixed mineral residue (ash) based on the loss of mass from the specimens submitted to incineration at 550 °C. The Bligh and Dyer [26] extraction method was employed to quantify the lipids. The energy value was determined by the sum of the values of carbohydrates, lipids and proteins multiplied by 4, 9 and 4, respectively [27].

2.8. Texture Profile Analysis

The texture profile analysis involved the determination of the hardness, cohesiveness, adhesiveness and springiness of the cookie dough with and without the biosurfactant after baking. For this purpose, a CT3 texture analyzer (Brookfield, Middleboro, MA, USA) was used with a 245 N load cell. The specimens were compressed at a constant velocity of 1 mm/s using a 60 mm-wide polymethacrylate plate. A second compression was performed after a 5 s interval and the hardness was defined as the force of half of the height on the specimen during the first compression. The cohesion was defined as the ratio between the compression work in the second compression cycle and the compression work in the first cycle. The sponginess was calculated using the relative height of the remaining specimen when the initial force was registered during the second compression [28].

2.9. Statistical Analysis

The data were analyzed statistically using the one-way procedure in Statistica (version 7.0) (StatSoft. Inc, Tulsa, OK, USA), followed by a linear one-way analysis of variance (ANOVA). The results were expressed as mean ± standard deviation (SD) determined from the triplicate experiments. The differences were examined using the Tukey post hoc test with a 95% confidence level, i.e., a significant level (p) of 0.05.

3. Results and Discussion

3.1. Cytotoxicity of the Biosurfactant

Cytotoxicity tests were among the first in vitro bioassays used to predict the toxicity of substances and are performed in laboratories throughout the world to classify compounds and evaluate safety [29]. The biosurfactant produced by *Bacillus cereus* UCP 1615 was

submitted to the MTT assay to monitor the response of the cells in the cultures and determine the viability of the biosurfactant for human consumption.

The results of the cytotoxicity tests regarding the viability of the L929 (mouse fibroblast) cells and the Vero (renal epithelial) cells from the African green monkey exposed to different concentrations of the biosurfactant are displayed in Table 2.

Table 2. Viability (percentage) of L929 and Vero cell lines after contact with the biosurfactant from *Bacillus cereus* UCP 1615 at different concentrations (data expressed as mean ± SD of the triplicate determinations).

Concentration (μg/mL)	Cell Viability (%)	
	L929 Line	Vero Line
Control	99.99 ± 3.33	100.03 ± 2.80
3.12	111.41 ± 5.18	102.66 ± 5.79
6.25	103.00 ± 5.42	103.51 ± 5.28
12.5	103.80 ± 2.56	101.25 ± 4.96
25	92.89 ± 2.56	98.13 ± 3.56
50	92.17 ± 2.49	94.84 ± 5.60
100	98.61 ± 0.56	86.05 ± 5.21
200	104.30 ± 2.09	74.77 ± 6.40
400	53.89 ± 1.80	12.13 ± 0.55

The viability of the L929 cells was 54% when submitted to the highest concentration of the biosurfactant (400 μg/mL) but above 93% when submitted to concentrations between 3.12 and 200 μg/mL. The viability of the Vero cells was 12.13, 74.77 and 86.05% when exposed to concentrations of 400, 200 and 100 μg/mL, respectively, but above 95% when exposed to concentrations between 3.12 and 50 μg/mL (Table 2). Substances that enable an 80% or higher cell viability rate are considered to be without cytotoxic activity [30].

The MTT results revealed that the biosurfactant may have a potential application in food as it did not exhibit cytotoxicity to either cell line at concentrations of up to 100 μg/mL, equivalent to 0.1 g/L. Moreover, the viability of the L929 cell line was 100% at the relatively high concentration of 200 μg/mL. These results are compatible with those reported by Ribeiro et al. [22] for the biosurfactant produced by *Saccharomyces cerevisiae* incorporated into a cookie formulation, which exhibited no toxicity to the L929 and RAW 264.7 (mouse macrophage) cell lines.

The use of the biosurfactant produced by *Candida bombicola* URM 3718 in a cupcake formulation was successful after the determination of its toxicity to the L929 and Vero cell lines at concentrations up to 50 μg/mL [31]. The survival rate of the BHK-21 cell line (kidney cells from hamster pups) was 63% after exposure to a biosurfactant produced by *Bacillus cereus* MMC at a concentration of 10^4 μg/mL [32]. The biosurfactant produced by *Lactobacillus helveticus* also exhibited no cytotoxicity to the L929 cell line at concentrations of up to 25 × 10^3 μg/mL [33].

3.2. Antioxidant Activity of the Biosurfactant

Oxidation can occur during the processing and/or storage of food, resulting in the deterioration of their nutritional value, color, flavor, texture and safety. The most effective, convenient and economical method employed to control oxidation is the use of antioxidants [34]. The food industry uses antioxidants to stabilize lipids in food, which are the most sensitive compounds to the oxidation process [35]. In addition to the preservation of food, antioxidants are also used in fields related to health and wellbeing due to their capacity to protect the body from oxidative damage.

The results of the total antioxidant capacity (TAC) as well as those of scavenging the DPPH$^{\bullet}$ radical and the ABTS cation radical (ABTS$^{\bullet+}$) by the biosurfactant are presented in Table 3.

Table 3. Total antioxidant capacity (TAC) and oxidative inhibition based on the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging capacity of the biosurfactant produced by *Bacillus cereus* UCP 1615 at different concentrations.

Biosurfactant Concentration (mg/mL)	TAC (%)	DPPH$^{\bullet}$ (I%)	ABTS$^{\bullet+}$ (%)
40.00	476.43 ± 12.34	28.45 ± 3.24	36.67 ± 4.23
20.00	353.46 ± 10.45	19.34 ± 5.34	25.62 ± 3.52
10.00	218.25 ± 14.37	11.23 ± 3.28	18.24 ± 4.23
5.00	147.56 ± 17.45	4.34 ± 1.35	10.23 ± 3.60
2.50	98.35 ± 8.56	2.14 ± 1.11	7.24 ± 2.49
1.25	49.56 ± 4.03	0.35 ± 0.04	4.65 ± 0.97
0.62	27.45 ± 3.15	0.00 ± 0.00	3.35 ± 1.38
0.32	15.34 ± 7.36	0.00 ± 0.00	2.84 ± 1.87

The biosurfactant showed promising results in terms of reducing the phosphomolybdenum complex when comparing its percentage of the total antioxidant capacity (TAC) with that of ascorbic acid at a concentration of 1 mg/mL. The addition of the biosurfactant at the lowest concentration (0.32 mg/mL) led to a TAC of 15.34%; it exceeded 100% with concentrations above 2.5 mg/mL and reached 476.43% at the highest concentration (40 mg/mL). A linear increase in the TAC with the increase in biosurfactant concentration was observed.

One percent (equivalent to 10 mg/mL) was the maximum concentration of the biosurfactant tested in the present study for application in the cookie formulation. The TAC at this concentration was 218.25%, demonstrating that the biosurfactant had potential regarding protection from oxidation in food. The total antioxidant activity in this study was consistent with the indices reported for biosurfactants from *Candida bombicola* [31] and *Saccharomyces cerevisiae* [22], which were also evaluated for use in food.

In the assessment of the oxidative inhibition in terms of the DPPH$^{\bullet}$ scavenging capacity of the biosurfactant, the results were low even at the highest concentration tested (40 mg/mL), preventing only 28.45% of oxidation (Table 3). In the assessment of the antioxidant activity based on ABTS$^{\bullet+}$ scavenging, the index achieved at the highest biosurfactant concentration was 36.67%. These results show that the biosurfactant under investigation did not have sufficient antioxidant potential to serve as the only antioxidant agent in a formulation.

3.3. Emulsification Activity

Stability is an important indicator when determining the commercial value of food products with water-in-oil emulsions. However, these emulsions are thermodynamically unstable due to the large interfacial area of the dispersed phase [36]. Their structural organization and amphiphilic nature make biosurfactants excellent emulsifiers acting at the oil–water interface, promoting the thermodynamic stability of unstable systems. Moreover, the characteristics of biosurfactants enable these natural compounds to interact with carbohydrates and proteins in food products [37].

The choice of vegetable oils in the emulsification tests was based on their importance and use in the food industry. Soybean oil stands out in terms of production and consumption whereas the other oils were selected due to their specific beneficial and functional properties for human consumption. In particular, peanut oil has a high vitamin E content, canola oil has a low content of saturated fatty acids and contains omega 3, and corn oil has essential acids and is considered to be of a high quality [38]. The emulsifying capacity of the biosurfactant produced by *B. cereus* at different concentrations against the selected vegetable oils is displayed in Table 4 in terms of the emulsification index (E_{24}).

Table 4. Emulsification index (E_{24}) of the biosurfactant produced by *B. cereus* UCP 1615 for different vegetable oils (data expressed as mean ± SD of the triplicate determinations). Data are expressed in %.

Biosurfactant Concentration (mg/mL)	Vegetable Oil			
	Corn	Soybean	Peanut	Canola
10.0	64.5 ± 1.1	56.0 ± 1.0	68.1 ± 0.0	65.8 ± 1.5
5.0	65.9 ± 1.4	54.0 ± 0.0	62.7 ± 1.7	64.4 ± 1.6
2.5	42.1 ± 1.1	47.7 ± 1.3	53.5 ± 1.5	36.0 ± 2.0

The results indicated that the biosurfactant was able to ensure a satisfactory emulsification of all the oils studied. As expected, the increase in the concentration of the biosurfactant led to an E_{24} increase. The best results were achieved at concentrations of 5.0 and 10.0 mg/mL, with E_{24} values ranging from 54 to 68%.

Few studies have investigated the capacity of biosurfactants produced by the genus *Bacillus* to emulsify vegetable oils or the application of these natural compounds as bioemulsifiers in food products. A study involving a biosurfactant produced by *Bacillus subtilis* ICA56 reported E_{24} > 50% for soybean oil [39]. Studies involving bioemulsifiers produced by *Candida albicans* reported E_{24} values around 50% for peanut, mustard, olive and soybean oils [21]. The bioemulsifier from *Candida utilis* showed indices around 30% for corn and sunflower oil under different conditions of pH and salinity [40]. The biosurfactant produced by *C. bombicola* achieved indices of 56% for corn oil, 51% for soybean oil, 69% for peanut oil and 50% for canola oil [31]. Thus, the present results were consistent with the findings described in the literature.

3.4. Characterization of the Cookies

The concentrations of the biosurfactant chosen for this study were defined based on the maximum concentrations recommended for most of the emulsifying additives authorized by both the Brazilian Health Vigilance Agency (Agência Nacional de Vigilância Sanitária, ANVISA) and the US Food and Drug Administration [41,42]. Figure 1 illustrates the cookies before and after baking, and the mean values of their physical properties (weight, diameter, thickness and spread factor) are gathered in Table 5.

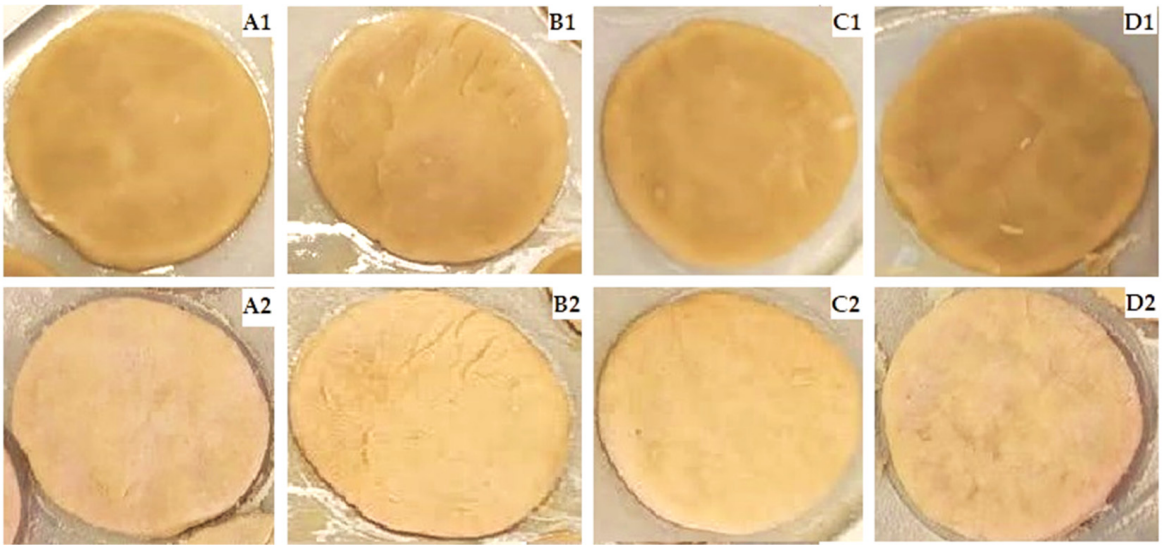

Figure 1. Cookies before (**1**) and after baking (**2**). (**A**): standard formulation; (**B**): formulation with 1% biosurfactant; (**C**): formulation with 0.5% biosurfactant; (**D**): formulation with 0.25% biosurfactant.

Table 5. Physical properties of the cookies after baking for standard formulation, formulation A (1% biosurfactant), formulation B (0.5% biosurfactant) and formulation C (0.25% biosurfactant).

Formulation	Weight (g)	Diameter (mm)	Height (mm)	Spread Factor
Standard	5.85 ± 0.02^{a}	48.66 ± 0.31^{a}	6.42 ± 0.15^{a}	7.59 ± 0.19^{a}
A	4.88 ± 0.25^{b}	48.87 ± 0.40^{a}	5.79 ± 0.14^{b}	8.44 ± 0.19^{b}
B	5.87 ± 0.53^{a}	49.11 ± 0.16^{a}	6.08 ± 0.04^{c}	8.08 ± 0.06^{c}
C	5.37 ± 0.14^{ba}	50.18 ± 0.09^{b}	6.50 ± 0.16^{da}	7.72 ± 0.18^{da}

$^{a, b, c, d}$: Different letters in same column denote statistically significant differences ($p \leq 0.05$, Tukey test). The values for each property were compared statistically taking the standard formulation as a reference.

To complement the ANOVA results, the Tukey test was used to evaluate statistically significant differences ($p \leq 0.05$) between the standard formulation and the formulations containing different concentrations of the biosurfactant with regard to weight, diameter, height and spread factor.

The increase in the concentration of the biosurfactant led to a linear increase in the spread factor. In addition to the benefits regarding the dough homogenization, the addition of the biosurfactant produced by *B. cereus* UCP 1615 promoted an increase in the quantity of lipids in the cookies due to the presence of fatty acids in its non-polar portion, which is a biochemical characteristic of the biomolecule previously described by Durval et al. [15]. This was reflected in the greater spread rate (increase in diameter) during cooking, which was likely related to the increase in the mobility of the system as the lipid fraction melted. It is a well-known fact that the spread rate exerts a direct influence on the diameter and height. Indeed, significant differences in height were found among the formulations as a higher spread factor led to a shorter height.

The physicochemical composition and energy value of the baked cookies are listed in Table 6. This study was necessary to determine whether the addition of the biosurfactant maintained the pre-established standards of identity and quality of the cookie.

Table 6. Physicochemical composition and energy value of cookies prepared with standard formulation, formulation A (1% biosurfactant), formulation B (0.5% biosurfactant) and formulation C (0.25% biosurfactant) (data expressed as mean ± SD of triplicate determinations).

Variable	Standard Formulation	Formulation A	Formulation B	Formulation C
Moisture (%)	6.35 ± 0.04^{a}	5.44 ± 0.06^{ba}	4.87 ± 0.06^{cb}	5.87 ± 1.24^{cba}
Ash (%)	1.60 ± 0.08^{a}	1.92 ± 0.07^{b}	1.74 ± 0.17^{cb}	1.66 ± 0.01^{ca}
Lipids (%)	11.10 ± 0.13^{a}	13.83 ± 0.82^{b}	11.69 ± 0.69^{a}	11.47 ± 0.31^{a}
Proteins (%)	1.07 ± 0.00^{a}	1.02 ± 0.06^{b}	1.07 ± 0.00^{a}	1.07 ± 0.00^{a}
Carbohydrates (%)	87.83 ± 0.13^{a}	85.14 ± 0.88^{b}	87.74 ± 0.69^{a}	87.47 ± 0.31^{a}
Energy Value (cal)	455.52 ± 0.67^{a}	469.17 ± 4.10^{b}	455.97 ± 3.46^{a}	457.33 ± 1.56^{a}

$^{a, b, c}$: Different letters on same line denote statistically significant differences ($p \leq 0.05$, Tukey test). The values obtained for each physicochemical variable in the different formulations were compared statistically taking the standard formulation as a reference.

A 24% reduction in moisture was found in formulation B containing 0.5% biosurfactant compared with the standard formulation. Thus, the use of the biosurfactant was quite promising as the reduction in moisture minimizes the proliferation of microorganisms, thereby enhancing the durability of the product. On the other hand, the ash content only differed (around 20%) when the highest concentration of the biosurfactant was used.

Seventy-eight percent of the fatty acids in the composition of the biosurfactant were essential and unsaturated fatty acids [15], which suggests that its addition should not compromise the nutritional aspects of the cookie. The biochemical composition of the biosurfactant investigated in this study had a direct impact on the quantity of lipids in the

cookies with a statistically significant increase in formulation A containing 1% biosurfactant compared with the other formulations, promoting a proportional increase in the caloric value of the cookies. A cupcake and cookie containing a glycolipid biosurfactant also presented a caloric value increase [22,31]. Conversely, the protein content was unaffected, which may be related to the low protein moiety of the biosurfactant.

3.5. Texture Profile

Studies on the texture profile of food emerged due to the need for a better understanding of human sensorial sensitivity in relation to food. Over time, such studies have acquired a greater relevance due to the need for the presence of functional ingredients and the emergence of innovative technologies that improve texture to ensure quality and satisfy the preferences of consumers [43].

Table 7 shows the results of the texture profile analysis of the cookies after the addition of the biosurfactant. The Tukey test revealed that the addition of the biosurfactant did not cause any significant change in most of the variables analyzed (hardness, cohesiveness and sponginess).

Table 7. Texture profile analysis of the dough 24 h and 45 days after baking for standard formulation, formulation A (1% biosurfactant), formulation B (0.5% biosurfactant) and formulation C (0.25% biosurfactant) (data expressed as mean ± SD of triplicate determinations).

Formulation	Hardness (N)		Cohesiveness (mm)		Sponginess (mm)	
	24 h	45 days	24 h	45 days	24 h	45 days
Standard	2133.70 ± 570.75 ^{a}	2440.30 ± 94.80 ^{a}	0.34 ± 0.03 ^{a}	0.49 ± 0.03 ^{a*}	0.40 ± 0.00 ^{a}	0.60 ± 0.00 ^{a}
A	2827.70 ± 82.59 ^{a}	1882.00 ± 63.32 ^{a*}	0.31 ± 0.04 ^{ba}	0.42 ± 0.06 ^{a}	0.63 ± 0.12 ^{b}	0.63 ± 0.06 ^{a}
B	4557.00 ± 566.77 ^{b}	2310.00 ± 763.42 ^{a*}	0.45 ± 0.03 ^{ca}	0.52 ± 0.02 ^{a*}	0.60 ± 0.00 ^{cb}	0.57 ± 0.06 ^{a}
C	3234.70 ± 236.90 ^{a}	1969.30 ± 278.44 ^{a*}	0.49 ± 0.07 ^{c}	0.42 ± 0.06 ^{a}	0.63 ± 0.06 ^{cb}	0.63 ± 0.06 ^{a}

$^{a, b, c}$: Different letters in same column denote statistically significant differences ($p \leq 0.05$, Tukey test). The values for each texture component in the different formulations were compared statistically taking the standard formulation as a reference. * Asterisk on same line denotes a significant difference after storage ($p \leq 0.05$, Tukey test).

The similarities among the results for the different formulations are important for the maintenance of the typical characteristics of a cookie; the bioemulsifier proved to be effective in this sense, having led to an improvement in the properties of the cookies in comparison with those of the standard formulation. A considerable difference in hardness was found for the cookie made with formulation B containing 0.5% biosurfactant especially in comparison with the standard formulation, which suggested an increase in crispness when using this biosurfactant concentration. The significant increases in cohesiveness and sponginess can be expected to enhance the chewability.

Zouari et al. [28] reported that the addition of the biosurfactant produced by *B. subtilis* at a concentration of 0.1% to a cookie formulation promoted a significant improvement in the texture profile of the dough; moreover, the action of this bioemulsifier was more pronounced than that of a commercial emulsifier (glycerol monostearate). Kiran et al. [44], who incorporated a lipopeptide at a concentration of 0.75% to a muffin formulation, found an improvement in the final softness due to the increase in sponginess and cohesiveness as well as a reduction in hardness.

The literature offers other reports on the potential of microbial biosurfactants [1,3,45] but with few examples of applications in the formulation of products for human consumption. Such examples include the addition of microbial bioemulsifiers to the formulations of mayonnaises [46], cupcakes [31] and cookies [22].

The biosurfactant at the lowest concentration (formulation C) did not promote a significant improvement in the texture profile compared with the standard formulation. However, cohesiveness was maintained after 45 days of storage, which did not occur with the standard formulation. Campos et al. [40] found that the use of a bioemulsifier produced by *C. utilis* at a concentration of 0.7% offered a greater stability and hardness to a salad

dressing formulation after 30 days of storage; the product was considered a good emulsifier compared with commercial products such as guar gum and carboxymethylcellulose.

In the production of food, the useful life of emulsions during long-term storage should be considered to ensure the consistent quality of the product. Studies have shown that the oily phase composition and the type of emulsifier exert significant effects on the long-term stability and sensorial properties such as spreadability, viscosity and appearance [36].

As the biosurfactant investigated herein had no significant negative effect from the statistical standpoint on the texture profile of the cookie, it could be considered to be a potential ingredient for the food industry. A simple assessment of aroma, flavor, color and texture revealed no significant differences between the formulations containing the biosurfactant and the standard formulation. However, sensory assessments are needed and the biosurfactant should be added to other formulations of flour-based products to determine whether it can be incorporated into other food without compromising the desired characteristics. Such investigations could expand the applications of this biosurfactant.

4. Conclusions

The present findings demonstrate that the biosurfactant produced by *Bacillus cereus* UCP 1615 grown in a medium containing waste frying oil has the potential for use as a bioemulsifier in food systems because it has been shown to be an effective emulsifying agent for various vegetable oils. The addition of the biosurfactant did not drastically affect the final product as the biosurfactant-containing formulations showed energetic and physical characteristics similar to those of the standard formulation, indicating the feasibility of applying this biomolecule in the formulation of cookies. The biosurfactant was non-toxic, which suggested its safe use, and had a considerable antioxidant activity. The biosurfactant demonstrated promising results as an ingredient for a flour-based product in terms of the physical, physicochemical and textural properties of the cookies formulated. The biosurfactant also ensured a good preservation of the cookies. Based on the results obtained in this study, the bacterial surfactant could be tested in other products as a green additive in the food industry. However, further studies are needed to enhance the economic viability of the production of this microbial surfactant on an industrial scale for its use as an emulsifier in food.

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