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Unveiling the Potential of Biosurfactant Produced by *Pseudomonas aeruginosa* from Amazonian Agro-Industrial Waste as Stabilizing Agent and Former of Antibacterial Nanoemulsions

Desvendando o Potencial do Biossurfactante Produzido por *Pseudomonas aeruginosa* a Partir de Resíduos Agroindustriais da Amazônia como Agente Estabilizador e Formador de Nanoemulsões Antibacterianas

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Abstract

Copaiba (*Copaifera* L.) and andiroba (*Carapa guianensis* Aubl.) are native tropical trees of the Amazon, extensively utilized for their oils, which possess diverse nutritional, economic, and pharmacological applications. These oils exhibit antimicrobial properties against various bacteria, including key pathogens implicated in bovine mastitis, a prevalent inflammatory condition affecting dairy cattle's mammary glands. However, their hydrophobic characteristics pose challenges in formulating effective therapeutic agents. Furthermore, inadequate disposal of by-products from andiroba processing results in severe economic, social, and environmental repercussions. This study explores the feasibility of utilizing residual biomass from andiroba fruit processing to produce a rhamnolipid biosurfactant by the bacterium *Pseudomonas aeruginosa*, designated as BSAW. The extraction yield of BSAW was approximately 5 mg/mL, exhibiting an emulsifying activity of 60% and a surface tension reduction to 28 mN/m. At concentrations as low as 2.5 mg/mL, BSAW exhibited antibacterial activity against ATCC strains of *Staphylococcus aureus* and *Streptococcus agalactiae*, as well as clinical isolates of *Escherichia coli*, coagulase-positive and coagulase-negative *Staphylococcus aureus*, *Klebsiella* spp., and *Corynebacterium bovis*. Additionally, BSAW serves as a stabilizing agent for an oil-water nanoemulsion containing copaiba oil (NEBSAW) through high-energy methods, enhancing antimicrobial activity at concentrations below 0.02 mg/mL. This study underscores the potential of rhamnolipid biosurfactants as eco-friendly alternatives for nanoemulsion formulations and highlights the importance of regional biodiversity in driving innovative and environmentally responsible solutions. Future recommendations include optimizing extraction methodologies and investigating additional applications of BSAW to address unsustainable development practices.

Keywords: Natural Resources. Rhamnolipid. Bovine Mastitis.

Resumo

Copaíba (*Copaifera* L.) e andiroba (*Carapa guianensis* Aubl.) são árvores tropicais nativas da Amazônia, amplamente reconhecidas por seus óleos, que possuem aplicações significativas nas áreas nutricional, econômica e farmacológica. Os óleos extraídos dessas plantas demonstram propriedades antimicrobianas contra uma variedade de bactérias, incluindo patógenos relevantes associados à mastite bovina, uma condição inflamatória que afeta as glândulas mamárias do gado leiteiro. Entretanto, as características hidrofóbicas desses óleos limitam sua eficácia como agentes terapêuticos. Além disso, o descarte inadequado dos subprodutos gerados no processamento da andiroba acarreta sérias repercussões econômicas, sociais e ambientais. O presente estudo investigou a viabilidade do aproveitamento da biomassa residual oriunda do processamento do fruto da andiroba para a produção de um biossurfactante ramnolipídico pela bactéria *Pseudomonas aeruginosa*, designado BSAW. O rendimento da extração de BSAW foi de aproximadamente 5 mg/mL, apresentando atividade emulsificante de 60% e promovendo redução da tensão superficial para 28 mN/m. Em concentrações iniciais de 2,5 mg/mL, BSAW exibiu atividade antibacteriana contra cepas ATCC de *Staphylococcus aureus* e *Streptococcus agalactiae*, e isolados clínicos de *Escherichia coli*, *S. aureus* coagulase-positivo e coagulase-negativo, *Klebsiella* spp. e *Corynebacterium bovis*. Adicionalmente, BSAW revelou-se eficaz como um agente estabilizador de uma nanoemulsão óleo-água contendo óleo de copaíba (NEBSAW), quando produzida por métodos de alta energia, potencializando o efeito antimicrobiano em concentrações inferiores a 0,02 mg/mL. Este estudo ressalta o potencial dos biossurfactantes ramnolipídicos como alternativas ecologicamente sustentáveis para formulações de nanoemulsões, evidenciando a importância da biodiversidade regional na promoção de soluções inovadoras e ambientalmente responsáveis. Recomenda-se, para trabalhos futuros, a otimização das metodologias de extração e a exploração de aplicações adicionais de BSAW na mitigação dos impactos de práticas de desenvolvimento insustentáveis.

Palavras-chave: Recursos Naturais. Ramnolipídio. Mastite Bovina.

1 Introduction

Brazil stands out for its abundant environmental assets, encompassing six distinct biomes, with particular emphasis on the Amazon region (Valério Filho et al., 2022). Within the vast Amazonian plant's biodiversity, *Carapa guianensis* Aubl., popularly known as andiroba, a term derived from the Tupi-Guarani language (an indigenous language of Brazil) meaning "bitter taste", stands out. This canopy tree, belonging to the Meliaceae family, is native to the Amazon region and occurs in the wetland forests of South and Central America, especially in the Amazon River basin (Dias et al., 2023).

Other plant richness, referred to as "copaibeiras" or "pau d'óleo" in the vernacular, copaiba trees belong to the *Copaifera* L. genus of the Leguminosae family, native to Latin America and West Africa, with a significant presence in the Amazon and Central-West regions of Brazil (Veiga Junior; Pinto, 2002). Notably, copaiba trees produce an oil comprising resinous acids and volatile compounds that exhibit bactericidal and bacteriostatic effects against various bacteria, including those causing bovine mastitis Oliveira et al., 2020), a prevalent inflammation of the bovine mammary gland parenchyma that significantly impacts dairy herds worldwide (Morales-Ubaldo et al., 2023). Bovine mastitis incurs annual losses exceeding 35 billion dollars, with damages estimated to exceed 40 billion dollars worldwide (Neculai-Valeanu; Ariton, 2022).

The complexity of the bovine mastitis aetiology involves the etiological agents, the host, and the environment, with over 140 potentially pathogenic microorganisms, predominantly bacteria, identified as etiological agents of contagious and environmental mastitis. While *Streptococcus agalactiae, Staphylococcus aureus*, and *Mycoplasma bovis* are associated with contagious mastitis, environmental mastitis is primarily attributed to *Escherichia coli*, *Klebsiella* spp., *Streptococcus dysgalactiae*, and *Streptococcus uberis* (Ashraf; Imran, 2020; Cheng; Hanm 2020). Various advancements in management practices to mitigate losses related to dairy production have been recorded, emphasizing the potential of vegetable oils in controlling bovine mastitis (Oliveira et al., 2020).

Although the anti-mastitis potential of copaiba oil is well-documented, developing oil-based therapeutic formulations still encounters challenges due to its hydrophobic nature. Recent studies conducted by Garcia et al. (2022) detail the benefits of developing and utilizing nanoemulsified systems for delivering vegetable oils with antimicrobial properties. Nanoemulsified systems allow the administration of hydrophobic compounds in aqueous media, preventing the degradation of the active ingredient, and favouring its controlled release and bioavailability. Despite the potential toxicity and environmental persistence of chemical surfactants, they play a crucial role in nanoemulsified system development, reducing the surface and interfacial tensions of immiscible compounds (Al-Sakkaf; Onaizi, 2024). In this scenario, biosurfactants emerge as promising alternatives to chemical surfactants in nanosystem development due to their biocompatibility, biodegradability, and stability against coalescence, and production from renewable sources (Qamar; Pacifico, 2023).

Rhamnolipids, in particular, are biosurfactants primarily produced by the bacterium *Pseudomonas aeruginosa*, arousing interest due to its physicochemical and biological properties (Santos et al, 2024), commercial competitiveness (estimated global market value for rhamnolipids is US$14.3 million by 2032) (GMI, 2024), and high production yield using alternative raw materials, like vegetable processing wastes (Mishra et al., 2021).

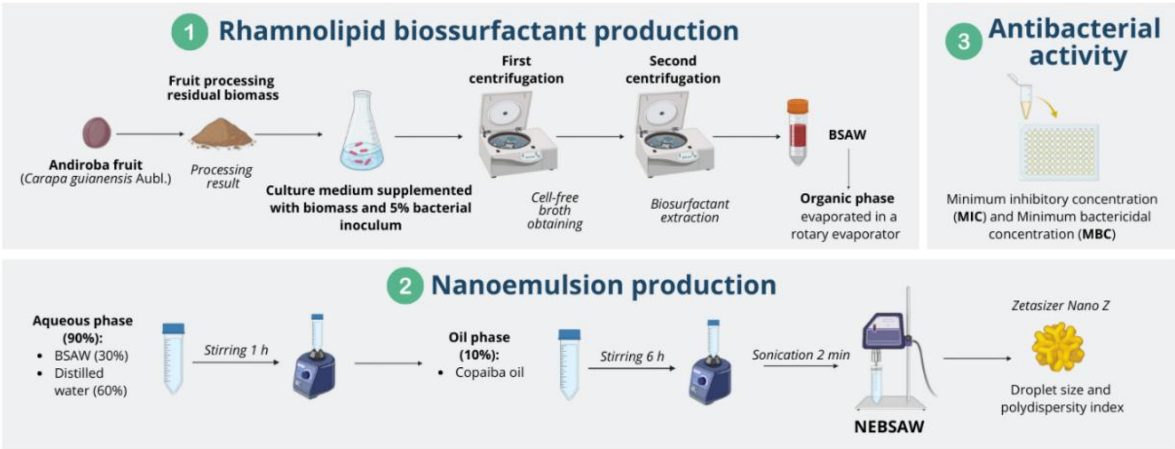
Producing rhamnolipid biosurfactants from residual plant biomass makes the process economically competitive and viable in the medium to long term, further strengthening the circular economy and reducing environmental impact from improper waste disposal (Eras-Munoz et al., 2022; Qamar; Pacifico, 2023). The use of biological-based products in Brazil, particularly those derived from plant sources, is a well-established practice (Bomfim et al., 2021; Sá et al., 2023) endorsed by public health policies such as the National Health Policy and the Medicinal Plants and Phytotherapy Policy (Brasil, 2006). Furthermore, the functionalization of natural resources has a long-standing tradition, given that around 40% of drugs approved by the Food and Drug Administration (FDA) are of natural origin, including plants (Newman; Cragg, 2020).

Thus, aiming to broaden the complementary options for managing bacterial infections, this research investigated the potential of using andiroba residues in producing rhamnolipid biosurfactants by *P. aeruginosa*. This biosurfactant was used as a stabilizing and nanoemulsion-forming agent containing copaiba oil, with antibacterial properties against bacteria causing bovine mastitis. This approach not only enhances cost-effectiveness but also enables efficient disposal of underutilized agro-industrial wastes in the growing market, considering the low economic value of residual biomass from andiroba fruit processing in the Amazonian production chain.

2 Material and Methods

Ethical approval was not required for this study since it did not involve human subjects or cell lines. The research focused on utilizing bacterial strains as the organism responsible for producing the biosurfactant of interest, thus obviating the need for ethical approval. Figure 1 presents a summary of the methodological approach employed in this research.

Figure 1 - Biosurfactant production and extraction process (1), nanoemulsion development (2) and antimicrobial activity test (3)



Source: research data.

2.1 Biological materials

The rhamnolipid biosurfactant-producing bacterium, identified as *Pseudomonas aeruginosa* BM02, was isolated from the superficial soil of a bauxite mine in Paragominas-PA, Brazil (Santos et al., 2024). BM02 is preserved in glycerol solution at –20 °C in the Bioassays and Bioprocesses Laboratory (L@βio) of Universidade Federal do Sul e Sudeste do Pará, Marabá-PA, Brazil, and was registered in the Brazilian System for Management of Genetic Heritage and Associated Traditional Knowledge (SisGen), under registration number A4DA401.

The residual biomass from andiroba (*Carapa guianensis* Aubl.) seed processing was provided by the Praialta and Piranheira Agro-Extractive Settlement Project, situated in the municipality of Nova Ipixuna-PA, Brazil. This project spans 27,344 hectares and accommodates 253 settled families. The use of residual andiroba biomass was registered in SisGen, under registration number A27D6F4. Copaiba (*Copaifera reticulata* Ducke) oil was purchased commercially from the company BellArome (Brazil).

2.2 Biosurfactant production

Biosurfactant production was conducted through liquid fermentation in Erlenmeyer flasks containing vegetable saline medium (VSM), with the following compounds: K_2HPO_4; KH_2PO_4; $(NH_4)_2SO_4$; $MgSO_4.7H_2O$; pH 7.0; and residual biomass from andiroba seed processing as the only source of carbon and energy, at ideal cultivation concentrations (g/L). This formulation is safeguarded by industrial secrecy, with an Invention Patent deposit at the National Institute of Industrial Property (INPI, Brazil), registered under BR1020240094492.

A 5% bacterial inoculum (0.6-0.8 optical density at 600 nm; Bel V-M5 Visible Spectrophotometer, Biovera) previously cultured in Luria Bertani broth (Kasvi) was incubated in an orbital shaker (model SL-22, Solab) at 180 rpm and 30 °C for 9 days. After fermentation, the culture was centrifuged (model SL-700, Solab) at 4500 rpm for 15 minutes at 25 °C to obtain a cell-free broth, which was then acidified to pH 2.0 using hydrochloric acid (6 N). In a second centrifugation step (4000 rpm, 4 °C, 10 minutes), the biosurfactant was extracted with a chloroform-methanol solution (3:1, v/v). The hydroalcoholic phase was discarded, and the organic phase was evaporated using a rotary evaporator (model LGI-52CS-1, Scientific) and dried in a circulated air oven until a constant weight was achieved (Camilios-Neto et al., 2011). The biosurfactant obtained was designated as BSAW, standing for biosurfactant from andiroba wastes.

2.3 Physicochemical characterization of the biosurfactant

The emulsifying activity was carried out in test tubes containing 2 mL of BSAW (10 mg/mL) with an equal volume of commercial mineral oil (Cimed), followed by shaking for 2 minutes and resting for 24 hours. The emulsification index was determined by Das et al. (1998), with an emulsification index value of $\geq$ 50% considered active. Uninoculated VSM and 1% SDS (Dinâmica Química Contemporânea LTDA) were used as negative and positive controls, respectively. Surface and interfacial tension measurements were carried out using a tensiometer (DataPhysics, Oca15 plus) at 25 °C, employing the hanging drop method and an automatic video imaging system with the Oca 10/Oca 20 program (Song; Springer, 1996).

2.4 Nanoemulsion production

Oil/water (O/W) nanoemulsions were prepared using the ultrasonic emulsification method (Gurpret; Singh, 2018), following three steps: 1) preparation of the aqueous phase (90%), composed of the BSAW suspension (30% by weight) and distilled water (60% by weight), followed by vigorous stirring (magnetic stirrer model RT 15 P, IKA) for 1 hour; 2) preparation of oily phase (10%), composed of 1 mL of copaiba oil, added to the aqueous phase with subsequent vigorous stirring for 6 hours; and 3) final homogenization, using an ultrasonic cell disruptor (model Disruptor, Ultronique; Ultrasonic Frequency: 20kHz) with a macro tip at 20% for 2 minutes. The nanoemulsion obtained was named NEBSAW, standing for nanoemulsion of biosurfactant from andiroba waste.

2.5 Nanoemulsion characterization

NEBSAW was characterized by dynamic light scattering to determine droplet size and polydispersity index (PdI), utilizing the Zetasizer Nano Z (model nano S90, Malvern Panalytical), according to Kotta et al. (2021) recommendations. A dilution 1:20 (v/v) with deionized water was prepared, and aliquots (1 mL) were stored at 25 ± 2 ºC on two separate occasions (day 0 and day 7). Each measurement consisted of 10 runs of 10 seconds with 3-second intervals. The measurements were conducted at a detection angle of 173º, at 25 ± 2 ºC, and at a wavelength of 633 nm.

2.6 Antibacterial assays

The antibacterial activity of NEBSAW, BSAW and copaiba oil was evaluated by microdilution method, under the recommendations of the National Committee for Clinical Laboratory Standards (CLSI), against the ISO strains *Staphylococcus aureus* (ATCC 29213) and *Streptococcus agalactiae* (ATCC 13813) and the clinical isolates *Corynebacterium bovis* BER628, *Escherichia coli* M16, Coagulase-negative *Staphylococcus aureus* M24, Coagulase-positive *Staphylococcus aureus* M64, and *Klebsiella* sp. M35, obtained from the bacteriotheque of the Fish Bacteriology Laboratory at the Universidade Estadual de Londrina, Londrina, Paraná, Brazil.

The determination of minimum inhibitory concentration (MIC) was conducted in 96-well flat-bottom microplates (Kasvi), containing 100 µL of sterile Mueller-Hinton broth (MHB; Kasvi) (121 °C, 20 minutes). Serial dilutions were carried out individually with NEBSAW and BSAW at concentrations of 2.5 to 0.02 mg/mL, and with copaiba oil at concentrations of 10% to 0.08%. Then, 5µL of suspension bacteria was inoculated (1.5 x 10^8 bacteria/mL, adjusted spectrophotometrically using 0.5 McFarland scale). The plates were incubated at 37 °C for 22 hours, followed by the addition of 2,3,5-triphenyl tetrazolium chloride (4 µL; TTC, Sigma-Aldrich) revealing solution, and further incubation for 2 hours. Bacterial growth was indicated by a pinkish or reddish colour, while a negative result was indicated by the absence of colour change or the occurrence of a yellowish colour (CLSI, 2015) . MIC was defined as the lowest concentration capable of inhibiting bacterial growth. The antibiotic tetracycline (Prati-Donaduzzi) was used as a control.

The determination of minimum bactericidal concentration (MBC) was carried out considering the same concentrations of NEBSAW, BSAW and copaiba oil investigated in the MIC assays. 5 µL of the solution from the wells from the MIC assay were transferred to 96-well flat-bottom microplates containing 100 µL of sterile MHB (121 °C, 20 minutes). After a 22-hour incubation at 37 °C, TTC (5 µL) was added to the wells, and further incubation for 2 hours (CLSI, 2017). CBM was defined as the lowest concentration of substances capable of preventing the reestablishment of cell viability in MHB.

3 Results and Discussion

The bacterium *P. aeruginosa* BM02 produced a biosurfactant (BSAW) using the residual biomass from andiroba seed processing as a sole source of carbon and energy, with an extraction yield of 4.42 mg/mL. In previous studies, our group reported a yield of 2.28 mg/mL of biosurfactant produced by the same bacterial strain using commercial glycerol as the sole source of carbon and energy (Santos et al., 2024). These results demonstrate a clear improvement in the biosurfactant production yield when employing residual andiroba biomass as a raw material, opening discussions on the functionalization of these residues into biotechnologically active products.

The physicochemical properties of BSAW are presented in Table 1. Surfactant compounds, such as BSAW, are evaluated based on their emulsion stabilization capacity, considered promising when they maintain at least 50% of the original emulsion after 24 hours of formation (Pele et al., 2019). In the present study, the emulsifying activity of BSAW was approximately 60%, with maintenance and stability of the activity after 24 hours of emulsion formation. These results are like those described by Poonguzhali et al. (2022) for the biosurfactant produced by *P. aeruginosa* using rice water as substrate. Compared to values (54% to 60%) described for the emulsification index of biosurfactant produced by *P. aeruginosa* BM02 in glycerol as a substrate (Santos et al., 2024), our results confirm the competitiveness of residual biomass from andiroba seed processing as raw material renewable and accessible for biosurfactant production.

Table 1 - Physicochemical properties of biosurfactant produced by *Pseudomonas aeruginosa* BM02 (BSAW)

Compounds	Emulsification index (%)	Surface tension (mN/m)	Interfacial tension (mN/m)
BSAW	59.30 ± 1.00	28.52[1] ± 0.22	3.27[2] ± 0.10
VSM	0.00 ± 0.00	69.90 ± 0.74	17.00 ± 0.30
SDS	69.90 ± 1.10	NT	NT

BSAW: biosurfactant from andiroba wastes; VSM: vegetable saline medium (negative control); SDS: sodium dodecyl sulphate (negative control, 1%); NT: Not tested; [1]BSAW tested at a concentration of 10 mg/mL; [2]BSAW tested at concentration of 1.25 mg/mL. Results are expressed as mean ± standard deviation of three replicates (n = 3).
Source resource data.

BSAW demonstrated an effective ability to reduce the surface tension of the uninoculated VSM to approximately 28 mN/m, as shown in Table 1. This value is similar to that described by Deepika et al. (2016) for the biosurfactant produced by *P. aeruginosa* using Karanja oil as a raw material (30 mN/m). When comparing the efficacy of BSAW with the biosurfactant produced by *P. aeruginosa* BM02 in commercial glycerol as substrate (Santos et al., 2024) once again, we confirm the competitiveness of BSAW, observing similar values (~27 mN/m) for the ability to reduce the surface tension of the uninoculated culture medium. According to Saraç *et al.* (2022), the ability to reduce surface tension is directly related to the surfactant yield, with the surface tension of an aqueous medium decreasing as the biosurfactant yield increases. Furthermore, BSAW was also effective in reducing the interfacial tension of crude oil. Interfacial tension is a phenomenon that, at the molecular level, results from energy difference between molecules of two immiscible liquids and is crucial for understanding the role of surfactants in emulsion formation (Berry et al., 2015) . Understanding these behaviours is critical for the development of formulations with surfactant properties, and studies already explored the surfactant properties of microbial biosurfactants as potential agents in therapies against COVID-19 (Daverey et al., 2021), advanced oil recovery (Sarafzadeh et al., 2013), and breast cancer treatment (Santos et al., 2024).

These results are promising to use residual biomass from andiroba seed processing as raw material in biosurfactant production. In line with the discussions by Qamar and Pacifico (2023), this strategy is not only innovative from a biotechnological standpoint but also adds value to the production chain of Amazonian fruits, enabling economic benefits to traditional communities that use this biological material as a source of income. The issue of uncontrolled generation and improper disposal of industrial, agricultural, and domestic waste is closely associated with changes in lifestyles and rapid global urbanization, not limited to the Brazilian Amazon region (Koul; Yakoob; Shah, 2022). The Food and Agriculture Organization (FAO) estimates that agro-industrial waste production worldwide reaches 1.3 billion tons annually, compromising 28% of agricultural land on 1.4 billion hectares of fertile land. Reports from the FAO Regional Conference for the Near East (NERC, 2024) project an increase of 138 million tons in waste generation in urban areas by 2025. Given this scenario, the use of agro-industrial waste as a raw material in microbial biosurfactant production emerges as a viable and environmentally friendly solution for the reuse of these materials, while offering economic benefits to all involved in the process, aligning with the principles of the UN's 2030 Agenda for Sustainable Development.

It is pertinent to reinforce that the advantages of using agro-industrial waste in the production of microbial biosurfactants include cost reduction in the process, the availability of renewable inputs that are abundant and low-cost or of no market value, as well as the maintenance and/or improvement of the physicochemical properties of the biosurfactants (Dominguez Rivera et al., 2019). Recent advances in the use of agro-industrial waste as renewable raw materials for biosurfactant production serve as an ecological alternative to chemical surfactants (Sundaram et al., 2024). Encouraging results have been reported regarding the use of various agro-industrial wastes for bacterial biosurfactant production, such as mango residues by *Lactobacillus plantarum* (Sittisart; Gasaluck, 2022) and cassava residues by *P. aeruginosa* (Elakkiya et al., 2021). George and Jayachandran (2009) examined the use of banana, orange, carrot, and lemon peel wastes in biosurfactant production by *P. aeruginosa*. Jain et al. (2013) evaluated the biosurfactant production capacity by *Klebsiella* sp. using corn and potato peel powder. All these studies demonstrate the effectiveness of reusing agro-industrial waste in microbial biosurfactant production. The relevance of agro-industrial waste for biosurfactant production is related to the chemical composition of plant's biomass, composed of lipids, proteins, and carbohydrates, which serve as a carbon and energy source for bacterial growth and biosurfactant synthesis (Sundaram et al., 2024).

Among the biosurfactant-producing bacteria, the species *P. aeruginosa* stands out for its ability to produce biosurfactants belonging to the class of glycolipids, with rhamnolipids being the most well-known Zhao et al., 2021). Rhamnolipids are widely recognized for their physicochemical properties, low toxicity, and high biodegradability, making them versatile in various applications (Thakur et al., 2021; Santos et al., 2024). The structural characterization of the biosurfactant BSAW is protected by industrial secrecy due to the request for a Patent of Invention deposited at INPI (BR1020240094492l), but the analyses performed (data not shown) confirm that BSAW is a rhamnolipid biosurfactant.

The combination of biotechnological versatility and toxicological safety of rhamnolipid biosurfactants has driven research in the field of Nanotechnology, raising discussions about the potential use of biosurfactants produced by *P. aeruginosa*, such as BSAW, in formulating nanotechnological products. Surfactant compounds play a crucial role in the preparation of nanomaterials, preventing aggregation and increasing their stability (Miyazawa et al., 2021). However, the use of chemical surfactants raises concerns due to the risks of toxicity to human health and the environment (Johnson et al., 2021). This highlights the urgent need to employ more sustainable surfactants in the development of nanotechnological products and processes, as highlighted by Nitschke and Marangon (2022) In response to these challenges, rhamnolipid biosurfactants emerge as promising candidates to replace conventional chemical surfactants, as they have a similar amphiphilic nature and unique self-assembly properties and adsorption capacity at various interfaces (Jahan et al., 2020). By exploring the advantages of rhamnolipid biosurfactants, researchers can develop more innovative and sustainable methods for nanomaterial production, paving the way for safer and more sustainable nanobiotechnological applications in various areas.

In this study, BSAW was investigated as a nanoemulsion-forming and stabilizing agent using high-energy methods in its production. The results obtained revealed the formation of an O/W nanoemulsion with a milky cloudy appearance, named NEBSAW. As described in Table 2, after seven days the formation of NEBSAW, there was no variation in PdI, but an increase in droplet size was observed.

Table 2 - NEBSAW droplet size and polydispersity index

Analysis 1 (Day 0)		Analysis 2 (Day 7)	
Z-Ave (nm)	**Pdl**	**Z-Ave (nm)**	**Pdl**
89	0.4	108	0.4

Z-Ave: average particle size, in nanometers (nm); Pdl: Polydispersity Index.
Source: research data.

The use of 30% by weight of BSAW in the oil phase of NEBSAW, subjected to two minutes of sonication, resulted in nano-scale droplet size, demonstrating the formation of a nanoemulsion, as described by Ho *et al*. (2022) for dispersed systems with sizes between 20 and 500 nm. These researchers also highlight the amphiphilic nature of the emulsifiers in nanoemulsions, such as BSAW, which allows molecules to easily diffuse, absorb, and reorganize at the interfacial regions of the nanoemulsion, reducing interfacial tension and facilitating the formation of small, stable, and dispersed droplets.

Nanoemulsions can be produced through various methods, categorized based on the forces used to break the dispersed phase in high and low-energy emulsifications. In high-energy emulsification, droplets are broken by shear and turbulent forces generated mechanically, while in low-energy emulsification, the physical-chemical energy of diffusion and phase behaviour of the emulsion system is utilized to break the droplets (Chang; McClements, 2014). According to Singh et al. (2017), high-energy methods are more promising as they require smaller amounts of surfactants for droplet stabilization and due to the use of mechanical devices, such as ultrasonicators, which can create powerful disruptive forces to reduce droplet size.

Both the type of method (high energy) and the short sonication period (two minutes) seem to influence the nanometric size of the droplets in NEBSAW. The sonication period has a direct impact on this analysis, and studies exploring different sonication times in the production of nanoemulsions demonstrate the formation of droplets in various sizes, highlighting the importance of this parameter in obtaining nanoemulsions with desired characteristics. Asadinezhad et al. (2019) reported a droplet size of 63 nm using 10 minutes of sonication. Ochoa et al. (2016) obtained nanoemulsions with an average droplet size of 108 nm with 12 minutes of sonication. Mahdi Jafari, He and Bhandari (2006) produced nanoemulsions by sonication with a droplet size of 150 nm, observing that the emulsion droplet size decreased with increasing sonication time.

Additionally, the surfactant concentration also plays a crucial role in the production of nanoemulsions. Onaizi et al. (2021) demonstrated that dispersed systems prepared with different weight concentrations (0.1 to 4.0%) of a rhamnolipid biosurfactant presented nano-scale sizes (<200 nm), decreasing as the biosurfactant concentration increased. These data suggest that short sonication times and moderately elevated concentrations of the biosurfactant are promising in obtaining nanodispersed systems in nanometric sizes, and the results described in this research to obtain NEBSAW highlight this.

Nanoemulsions have been widely studied as potential carriers for biologically active compounds, especially hydrophobic ones (Shakeel et al., 2012) due to their low solubility in physiological fluids and consequently low *in vivo* bioavailability (Lipinski, 2002). The recognition of nanotechnological formulations by the FDA highlights their importance in improving various aspects of medical, food, and cosmetic products, increasing the bioavailability of drugs, improving food packaging, and preserving the cosmetics appearance (FDA, 2014). These advancements have encouraged the investigation of the antimicrobial potential of NEBSAW against bacteria causing bovine mastitis, and the results of the analyses are described in Table 3.

Table 3 - Antimicrobial activity of NEBSAW, BSAW and copaiba oil against bacterial strains causing bovine mastitis

Bacterial	**MIC**				**MBC**
	NEBSAW	**BSAW**	**CO**	**Tetracycline**	**NEBSAW**
Staphylococcus aureus (ATCC 29213)	2.5	>2.5	2.5	<0.02	2.5
Streptococcus agalactiae (ATCC 13813)	<0.02	2.5	<0.04	<0.02	<0.02
Escherichia coli (M16)	>2.5	>2.5	5.0	<0.02	>5.0
Coagulase-positive *Staphylococcus aureus* M64	2.5	>2.5	0.62	<0.02	2.5
Klebsiella spp. (M35)	0.16	>2.5	1.25	<0.02	0.16
Coagulase-negative *Staphylococcus aureus* M24	>2.5	>2.5	>5.0	<0.02	>5.0
Corynebacterium bovis (BER628)	1.25	2.5	5.0	<0.02	1.25

NEBSAW: nanoemulsion of biosurfactant from andiroba waste; BSAW: biosurfactant from andiroba wastes; OC: copaiba oil; MIC: minimum inhibitory concentration, expressed in mg/mL for NEBSAW, and expressed in % for CO; MBC: minimum bactericidal concentration of NEBSAW, expressed in mg/mL.
Source: research data.

The analyses of the results presented in Table 3 demonstrate the effectiveness of NEBSAW in inhibiting the growth of all investigated bacterial strains, with emphasis on the *S. agalactiae* (ATCC 13813), which proved to be particularly sensitive to the nanoemulsion, with MIC values below 0.02 mg/mL (the lowest concentration tested). The determination of ideal MIC values for natural formulations is a highly debated topic in the literature. According to Webster et al. (2008), satisfactory MIC values for more refined formulations, such as NEBSAW, should have MIC below 0.1 mg/mL. Based on this analysis and MIC values, NEBSAW shows great promise for the control of infections caused by *S. agalactiae*. The clinical isolate of *Klebsiella* spp. (M35) showed MIC values close to this ranking (MIC = 0.16 mg/mL). Because *Streptococcus aureus* is also recognized as one of the most common pathogens associated with bovine mastitis (Kabelitz et al., 2021), the MIC with an initial value of 2.5 mg/mL also considers and highlights the effectiveness of NEBSAW in combating this bacterium.

These results point to the need to consider the variability in the response of different bacterial strains to this treatment.

Copaiba oil is known for its broad-spectrum antibiotic effects, a property that has sparked interest in the treatment of bovine mastitis (Oliveira et al., 2020). In this research, *S. agalactiae* was also the most susceptible to copaiba oil, with MIC determined for values below 0.04% (the lowest concentration tested). The incorporation of copaiba oil into nanoemulsions has shown promising results in controlling bacteria causing bovine mastitis. Campanholi et al. (2023) developed a nanogel containing copaiba oil that, when administered to the dairy cows' teats, allowed the formation of a protective film capable of preventing infections. These results reinforce the potential of nanoemulsions containing copaiba oil as antimicrobial agents and strengthen the hypothesis that NEBSAW is a promising candidate for the bovine mastitis management.

In addition to acting as a bacteriostatic agent, NEBSAW also exhibits bactericidal properties against the investigated strains, with MBC determined for different concentrations of the nanoemulsion (Table 3). The analysis of the results suggests that the *S. agalactiae* strain and the clinical isolate *Klebsiella* spp. were more susceptible to NEBSAW. Although modest, the bactericidal activity of NEBSAW was also recorded for the clinical isolate *C. bovis*. According to the most consistent evidence observed in our studies, no studies on the bactericidal effects of nanoemulsions containing copaiba oil were found, making this work pioneering in the field. Among the available studies on the topic, Nie et al. (2023) describe that nanoemulsions containing black pepper essential oil exhibited bacteriostatic and bactericidal properties against *S. aureus* and *E. coli* at concentrations ranging from 12.5 to 25 mg/mL. The findings described in our research suggest that NEBSAW has bacteriostatic and bactericidal properties against the bacterial strains *S. agalactiae*, *Klebsiella* spp., and *C. bovis*. These results are encouraging in recommending NEBSAW as an effective option in combating infections caused by these bacteria.

Our results also support previous studies on the selectivity of nanoemulsions containing vegetable oils towards different bacteria, indicating a higher sensitivity to Gram-positive bacteria (Alam et al., 2023; Osanloo et al., 2022). In particular, the Gram-positive bacterium *S. agalactiae* commonly causes infections in dairy herds, with a prevalence of approximately 50% in South America and over 90% in China (Kabelitz et al., 2021). However, *S. agalactiae* also causes infections in other animals and humans (Wang et al. 2016). In Bulgaria, Gergova et al. (2021) describe the occurrence of multi-drug resistant and hypervirulent isolates of *S. agalactiae* in patients. In Brazil, Barros (2021) highlights a growing trend of macrolide and lincosamide resistance due to the spread of polyclonal strains of *Streptococcus*, where *S. agalactiae* represents the most clinically relevant *Streptococcus*, especially for neonatal infections. The issue is that there is no national notification system in these and other countries to monitor the prevalence of bacterial infections and antimicrobial resistance rates. Better communication between clinical and reference laboratories would also help reduce the incidence and antibiotic resistance cases and thus guide therapeutic strategies. These findings are significant as antibiotic-resistant Gram-positive pathogens are increasingly prevalent, necessitating a shift in antibiotic usage policies and the exploration of new effective molecules for treatment (Carcione et al., 2023). Among the available options, nanoemulsions containing vegetable oils show great promise.

According to Flanklyne, Mukherjee and Chandrasekaran (2016), vegetable oils act on the bacterial cell wall or membrane, leading to complete cellular disorganization, as evidenced by the presence of dense structures in the cell cytoplasm, irreversible loss of viability, increased cell membrane permeability, and partial solubilization of the cell membrane, resulting in bacteriostasis. While promising as antimicrobial agents, vegetable oils have some limitations that compromise their use in therapy, such as high volatility, low aqueous solubility, and potential for atypical skin reactions (Donsi; Ferrari, 2016). The use of nanoemulsions as carriers for hydrophobic antimicrobials, like vegetable oils, has been extensively researched due to their physicochemical properties that allow for better solubilization and stability of these substances, reducing their potential toxicity and extending the active ingredient shelf life (Minakshi et al., 2019). These findings highlight the biotechnological potential of NEBSAW, emphasizing the relevance of nanoformulations as a promising and effective approach to combating bacterial infections. The industrial relevance of these biomolecules is highlighted by the existence of numerous patent applications for the development of rhamnolipid-based antimicrobial products, intended for medicine and agriculture Nitschke; Marangon, 2022).

Our findings align with numerous studies on the use of natural resources for therapeutic purposes, including clinical ethnoveterinary studies conducted by Chishti et al. (1992) in Pakistan. The study confirms the therapeutic potential of plant-based formulations in the treatment of subclinical mastitis in 327 cows and 493 buffaloes. Islam and Kashem (1999), through numerous field visits, personal contacts, case studies, and group discussions confirm the importance of ethnoveterinary medicine by farmers and livestock breeders in Bangladesh. In Brazil, encouraging results were also presented by Souto et al. (2012), who, in addition to emphasizing the relevance of ethnoveterinary practices, suggest that the repertoire of chosen resources reflects the local accessibility/availability of resources. Therefore, management practices that value local biodiversity in the development of bioproducts for solving regional problems are still the best strategies.

In addition to its role in the formation and stabilization of NEBSAW, BSAW demonstrates the ability to contribute, albeit discreetly (CIM $\geq$ 2.5 mg/mL), to the antibacterial activity of NEBSAW, as evidenced in Table 3. This result is consistent with previous studies on the ability of rhamnolipid biosurfactants not only to assist in the encapsulation and stabilization of bioactive compounds but also to contribute to the antimicrobial properties of the formed nanoemulsion (Ganesan et al., 2023). Based on the results of this research, it can be inferred that BSAW performs a dual function: as a forming and stabilizing agent of the nanoemulsion, improving the encapsulation efficiency of the active compound, the size, and the physical stability of the nanodispersions, as well as an antimicrobial agent. These findings strengthen the hypothesis that BSAW has the potential to replace synthetic surfactants in the development of nanoemulsions and act as an active or synergistic antimicrobial agent, highlighting its promising role in the formulation of antimicrobial products.

The results of this research problematize the resilient management of Amazonian biological resources, which, according to (Albert et al., 2023) besides being the most promising option, promotes a sustainable bioeconomy in the Amazon, integrating local and international economies, prioritizing biodiversity and ecosystem services. Furthermore, the combination of valuing traditional community practices with modern resources, such as Nanotechnology, expands the possibilities for improving quality of life and reducing inter and intra-regional inequalities. However, achieving these changes requires revisions in legal, economic, and energy systems, both regionally and globally, allowing for decentralized governance with indigenous and rural communities (Ellwanger et al., 2020). Looking ahead, we hope that regulatory systems will work to streamline protocols for introducing new and safe management options for bovine mastitis, considering NEBSAW as a promising candidate for available therapeutic strategies. Furthermore, topical formulations for managing bovine mastitis are currently in the developmental stages, and it is anticipated that their effects on cattle herds under field conditions will be as promising as those described in this study.

4 Conclusion

The bacterium *Pseudomonas aeruginosa* BM02, isolated from a mining area in the Brazilian Amazon region, produced rhamnolipid biosurfactant, named BSAW, using the residual biomass from andiroba seed processing as the sole carbon and energy source. This biosurfactant exhibited promising yield indices and significant surface-active properties. BSAW proved effective in forming and stabilizing oil-in-water nanoemulsions using high-energy methods, resulting in the production of the NEBSAW nanoemulsion with bacteriostatic and bactericidal properties against various bacterial strains causing bovine mastitis. Among the investigated strains, *Streptococcus agalactiae*, *Klebsiella* spp., and *Corynebacterium bovis* showed particular sensitivity to the nanoemulsion. It was observed that BSAW collaborates with NEBSAW, enhancing the antimicrobial effect of the nanoemulsion, as well as improving the encapsulation efficiency of the active compound and the physical stability of the formed nanoemulsion. These results underscore the importance of integrating the valorisation of natural resources and traditional practices with Nanotechnology advancements for developing products that promote improvements in bioeconomy and the reduction of inter- and intra-regional inequalities.

Therefore, we advocate for the recommendation of NEBSAW as an effective option for managing bovine mastitis and the sustainable functionalization of Amazonian agro-industrial waste.

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