Evaluation of Biosurfactant Production by *Bacillus subtilis* Using Glycerol

Sergio Santos Silva Junior[1*], Arthur Gonçalves Dias Santos[1], Sthefany Ribeiro dos Santos[1], Ana Lucia Barbosa de Souza[1], Hendor Neves Ribeiro de Jesus[1], Tatiana Oliveira do Vale[1]

[1]SENAI CIMATEC University Center, Departament of Industrial Microbiology; Salvador, Bahia. Brazil

There is a constant concern about the environmental impacts due to the increase in industrial activity. The leading causes of these impacts come from chemicals, especially organic compounds such as hydrocarbons and their derivatives, which companies often use. In search of long-term solutions, biosurfactants have gained significant prominence regarding their cost-benefit. It is favorable when compared to surfactants of petroleum origin. In order to lower costs and reduce the impacts on the environment caused by industries, this work aims to evaluate the production of biosurfactants by *Bacillus subtilis* using glycerol in different concentrations.

Keywords: Biosurfactant. Industrial Waste. Glycerol. *Bacillus subtilis*.

Introduction

Developing eco-friendly and economically competitive substances has been gaining prominence for socio-environmental issues. It is necessary to invest in bioproducts to reduce the concerns and expenses associated with pollution prevention, waste management, and elimination or reduction of consumption of industrial products that are harmful to the environment. It is worth noting that agricultural or agro-industrial waste from various origins is an excellent sustainable raw material for various bioprocesses in producing bioenergy and other bioproducts. The need to convert the waste obtained into sustainable products is essential to minimize the need to use sources from oil. Initially, glycerol has stood out as a highly accessible raw material for manufacturing biomolecules of industrial importance due to its high consumption in biodiesel production. Glycerin is one of the main by-products linked to the biodiesel manufacturing chain. The biodiesel production process includes the combination of a triglyceride with short-chain alcohol (whether methanol or ethanol The companies involved in this industry assume that the increase in the availability of this co-product in the domestic market is related to the manufacture of biodiesel. For every 1,000L of biodiesel produced, approximately 100 kg of glycerol is generated; having said that, this material is of paramount importance since its excess production is harmful to the environment [1]. Surface tensions between immiscible liquids are minimized by creating a molecular layer that communicates between the interfaces of complex substances to homogenize. Biosurfactants undergo biotransformations of renewable materials, thus contributing little to adverse environmental effects and favoring the disuse of chemicals.

The production of biosurfactants faces many technical challenges that must be overcome to ensure an efficient and high-throughput process. The selection of the producing microorganism is a crucial step, requiring the choice of a species capable of synthesizing the desired molecules in significant quantities and with robustness to withstand adverse conditions. In addition, it is necessary to optimize cultivation conditions, such as pH, temperature, and carbon sources, to stimulate the synthesis of biosurfactants [2-4].

An approach that is successful for the production of biosurfactants, also known as surfactants of biological origin, is the use of glycerol as a substrate using bacteria as BS producers. The fact that agro-industrial residues are generated in tropical or temperate climate areas and are abundant makes them advantageous as an organic substrate for industrial fermentations. This study aimed to evaluate biosurfactants' production using glycerol

Received on 18 September 2023; revised 6 November 2023.
Address for correspondence: Sergio Santos Silva Junior. Avenida Orlando Gomes,1845, Piatã. Salvador, Bahia, Brazil. Zipcode: 42701-310. E-mail: jsilva.ss546@gmail.com.

J Bioeng. Tech. Health 2023;6(4):320-325

in different concentrations in the synthetic mineral medium by *B. subtilis*.

Materials and Methods

Cultivation of the Microorganism

For this work, the strain of *Bacillus subtilis* from the Federal University of Bahia (UFBA) was used in the laboratory of Microbiology and Bioprocesses Moacyr Durham de Moura-Costa, being used by the advisor Tatiana Vale in the doctoral thesis. The bacterium was isolated in waste from the oil and gas industry and produced water. The culture was maintained in the solid medium in plates with Tryptic Soy Broth (TSB) culture medium. The conservation of the microorganism is of great value because it prevents contamination, the loss of viability, and against possible mutations.

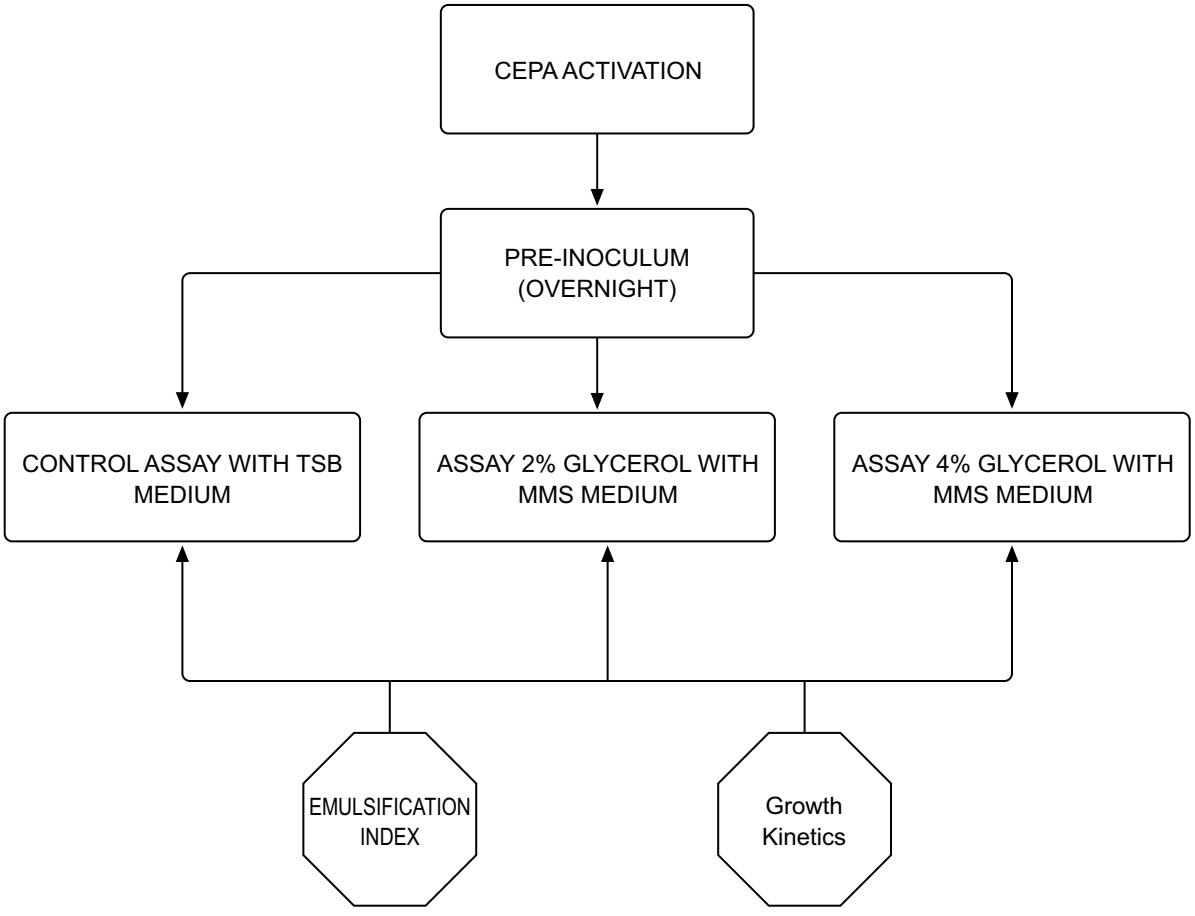
Flowchart and Organization of Bioassays (Figure 1)

The crops were developed in a TSB medium to determine the growth kinetics and maintenance of the strain. This culture underwent an activation (growth for 24 hours) and a pre-inoculum before the assays to ensure its adequate bacterial concentration for the assay. According to Vale, to maintain the conservation of the culture and its growth in ideal conditions, reaching a standard number of living and viable cells, a pre-inoculate (overnight) is performed using the activation previously performed containing 90mL ofmedium, 10mL of activation for 12-16 hours at 130 rpm and 37°C before the start of the assays [3].

Tests and Production of Biosurfactant

Simultaneous processes were carried out in duplicates of two different concentrations of

Figure 1. Flowchart of procedures performed until the development of the tests.



substrate-glycerol PA (20g/L and 40g/L) under 37ºC and 180 rpm for 72 hours on an agitator table. For each replicate was prepared in an Erlenmeyer of 250mL, 95 mL of synthetic mineral medium (MMS), proposed by Sant'Anna (2001), containing $Na_2HPO_4.12H_2O$ (4.2g/L); KH_2PO_4 (1g/L); Ca $(NO_3)_2.4H_2O$ (0.072g/L); (NH_4) The fermentation process for the production of biosurfactant was carried out with the aid of an agitating table, with pre-fixed agitation and keeping the temperature constant at 37°C.

Emulsion Index (Figure 2)

According to Alvarez and colleagues, the emulsifying activity is evaluated by mixing 2 mL of mineral oil and 2 mL of cell-free broth supernatant in a test tube. Each tube was shaken for 2 minutes and kept at 25°C for 24 hours. The index (E24%) was calculated using the formula shown below:

$$E24\% = (Hfe / Htotal) \times 100 \quad (1)$$

in which Hfe = Height of the emulsified phase, and Htotal = Total liquid height.

Aliquots were removed for cell growth analysis (verified through optical density) and E24% (Emulsification Index) in pre-established times. The tests were carried out to monitor the production of biosurfactants with different substrate concentrations under fixed process conditions. The emulsion index and optical density (600 nm) analyses were performed in duplicates throughout the test [5].

Results and Discussion

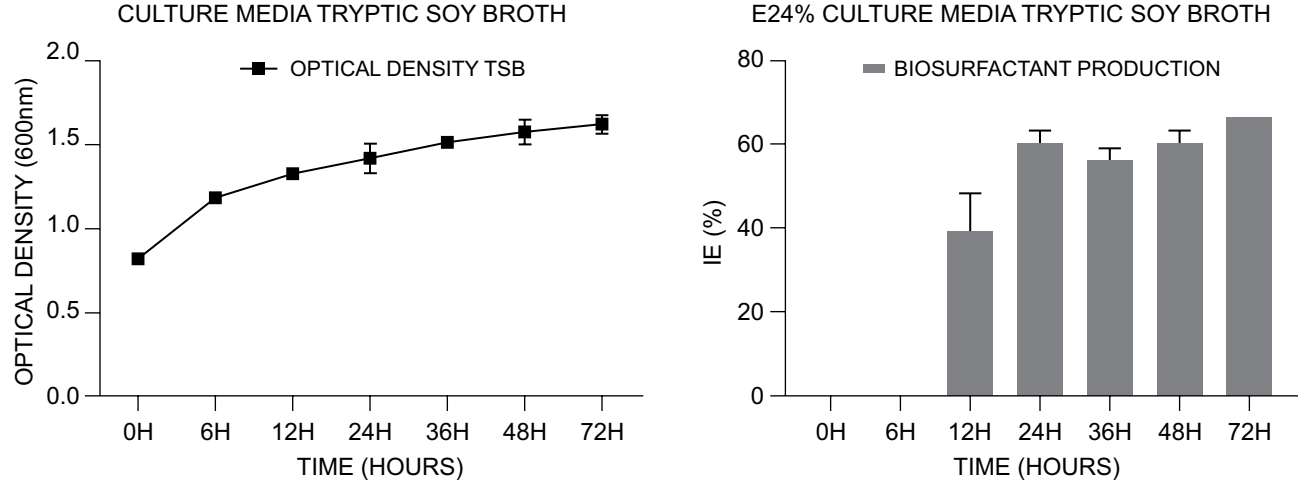
Tryptic Soy Broth Medium Test

Initially, a test was carried out in the TSB culture to recognize the bacterium's growth kinetics under ideal conditions. This trial was performed with 12 250 mL Erlenmeyers containing 95 mL of TSB and 5 mL of pre-inoculum. Each test point was collected in duplicate, representing its result as an average of the replicates.

The effectiveness of the produced biosurfactant can also be evaluated by its ability to emulsify mixtures of hydrocarbons in oil, resulting in a significant increase in the degradation of these organic compounds. Furthermore, the biosurfactant's capacity to influence the emulsions' stability or instability is a relevant indicator [6].

Based on these considerations, it is notable that this trial demonstrated the highest production of both biomass and biosurfactant. In a similar study, Vale associated *Bacillus subtilis* with the TSB synthetic culture medium as the most suitable commercially

Figure 2. Growth kinetics of the *Bacillus subtilis* bacterium in TSB culture medium and E24% emulsification index analysis of biosurfactant production with TSB medium.



producing biosurfactants [3]. These results reinforce the importance of the culture medium in obtaining high-efficiency biosurfactants and highlight the potential of this specific assay as a promising candidate for future industrial applications.

MMS Assay 2% Glycerol (Figure 3)

Therefore, a test was carried out with 12 Erlenmeyers containing 95 mL of MMS medium, 5 mL of pre-inoculum, and 1 mL of trace metal solution. Glycerol (20g/L) and yeast extract (5g/L) were added as carbon sources for the MMS.

In this specific assay, we observed slower bacterial growth. However, the production of biosurfactants occurred in quantities comparable to the use of the TSB medium as a reference. This phenomenon can be evidenced at the 24-hour and 72-hour time points, at which optical density (O.D.) values of 1.853 and 1.477 were obtained, together with emulsification rates (E24%) of 54.16% and 60.41 %, respectively. It is crucial to point out that the choice of the ideal culture medium for the production of biosurfactants by *B. subtilis* must be weighed in terms of economic efficiency, especially when compared to the use of an MMS medium. Sousa and colleagues also investigated biosurfactant production using the MMS medium, observing a gradual increase after 48 hours of incubation at 30°C under agitation at 180 rpm, culminating in a maximum peak after 70 hours of cultivation [7]. In an additional study, de Faria and colleagues performed an assay involving *B. subtilis* using a Tryptic Soy Agar (TSA) medium with glycerol as substrate. They obtained similar results in their optical density analyses (1022, 1036, 1044, and 1058) [8]. These findings reinforce the importance of choosing a suitable culture medium to produce biosurfactants by *B. subtilis* [8,9].

Assay in MMS 4% Glycerol

A trial was conducted with 12 Erlenmeyers containing 95 mL of MMS medium, 5 mL of pre-inoculum, and 1 mL of trace solution. Glycerol (40g/L) and yeast extract (5g/L) were added as carbon sources for the MMS.

In the present test, it is observed that the bacterial concentration reached significantly higher values in a substantially shorter period (24 hours) compared to the control test that used Tryptic Soy Broth (TSB) as a culture medium, where this bacterial growth led to 72 hours to complete. However, it is essential to highlight that the production of biosurfactants was significantly attenuated in the context of this experiment. It is essential to mention that Sousa and

Figure 3. Optical density analysis by spectrophotometry of bacterial growth in MMS 2% and E24% emulsification index analysis of biosurfactant production in MMS 2%.

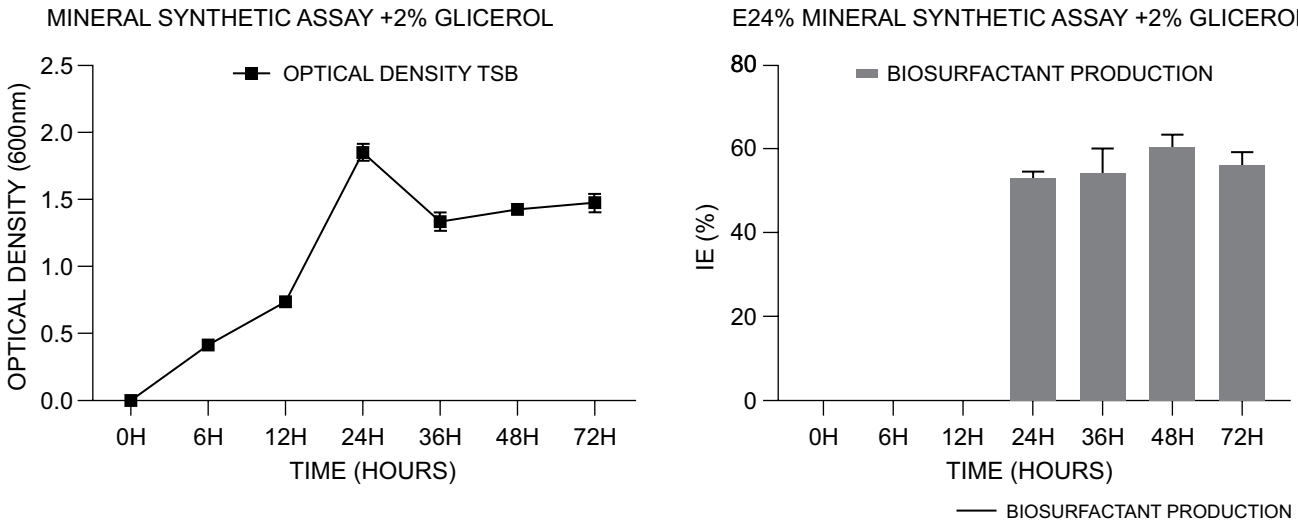
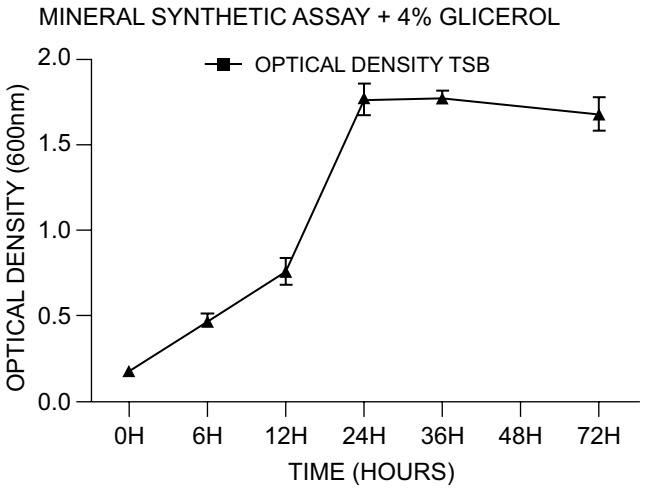
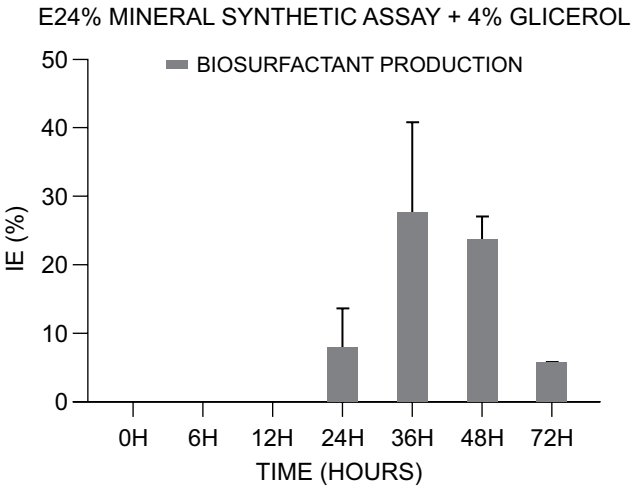


Figure 4. Optical density analysis by spectrophotometry of bacterial growth in MMS 4% and E24% emulsification index analysis of biosurfactant production in MMS 4%.





colleagues also elucidated the formation of surfactin through the application of glycerol in substrates containing *B. subtilis*, observing a maximum peak of 263.64 mg L^{-1} of surfactin after 30 hours of cultivation, corroborating our results—findings in this research context [7].

Conclusion

Among the two analysis tests carried out, it is notable that the test using glycerol at a concentration of 2% stood out significantly in terms of biosurfactant production when compared to the test using glycerol at 4%. On the other hand, the assay with 4% glycerol revealed a more robust bacterial development. It is essential to highlight that the test with TSB showed superior performance to the other tests due to its ideal intrinsic characteristics for the growth and production of biosurfactants. However, the test with 2% glycerol revealed that, when using a synthetic minimal medium (MMS) with glycerol as a carbon source, biosurfactant production reached comparable values in a substantially shorter period compared to the medium traditionally considered ideal for *Bacillus subtilis* cultures. This analysis provides many considerations, particularly concerning cost-effectiveness and lead time. Therefore, this study presents data of great relevance that can serve as a basis for future research, highlighting the need for more in-depth and refined investigations in this promising biotechnology field.

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