

Article

Lacticaseibacillus Biosurfactant Production from Cacao Pod Husk Enzymatic Hydrolysates

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Abstract

During cocoa processing, approximately ten times more cacao pod husk (CPH) waste is generated than cacao beans. Due to its high lignocellulosic content, CPH is an alternative feedstock for the production of fermentable sugars and bioproducts. In this study, CPH enzymatic hydrolysates were used as a carbon source to produce *Lacticaseibacillus* biosurfactants. CPH was subjected to alkaline pretreatment followed by enzymatic hydrolysis using the commercial enzyme cocktail Cellic Ctec2. The resulting hydrolysates were used to formulate culture media for growing *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus casei*. Cell growth and the activity of extracellular and cell-bound biosurfactants were evaluated. The highest glucose concentration in the hydrolysates (11.45 g/L) was achieved using 15% (*w/v*) solids loading of alkaline-pretreated CPH and an enzymatic load of 20 FPU/g CPH over 3 h. The maximum emulsification index (E24) was 60%, observed with the extracellular biosurfactant from *L. rhamnosus* cultured in CPH-based medium without supplementation. *L. casei* extracellular biosurfactants were effective at inhibiting *Pseudomonas aeruginosa* PA14 biofilm formation (39–45%) in CPH-based media supplemented with peptone, yeast extract, and both nutrients. These findings highlight the potential of CPH enzymatic hydrolysates as a sustainable carbon source for biosurfactant production with emulsification and antibiofilm activity, contributing to the valorization of cocoa agro-industrial waste.

Keywords: cacao pod husk; enzymatic hydrolysis; *Lacticaseibacillus*; biosurfactants; biofilm inhibition



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1. Introduction

Every year, thousands of tons of residual biomass are produced worldwide as a byproduct of agro-industrial activity. Agricultural waste is generally disposed of by burning, dumping, or unplanned landfill. It may lead to environmental pollution and health issues [1]. *Theobroma cacao* L. is used in the food industry to produce chocolate, butter, and cocoa powder, and in the pharmaceutical and cosmetic industries for its health benefits [2]. The cacao fruit consists of the bean, the bean husk, mucilaginous pulp, and the cacao pod husk (CPH) [3]. After cacao beans are extracted, the CPH is discarded as waste [2], and it is burned or piled along with other crops, allowing the proliferation of pests and insects [4]. To produce 1 ton of dry cocoa bean requires about 13 tons of cacao fruit, which generates

10 tons of wet CPH as waste [4]; annually, 20 million CPHs are produced worldwide [5]. The increase in the generation of residual biomass and the interest in reducing dependence on petroleum and its derivatives in industrial processes have sparked interest in the valorization of lignocellulosic material through biotechnological processes. CPH is mainly composed of lignin (14.6–26.38%), cellulose (24.24–35.0% *w/w*), and hemicellulose (8.72–11.0%) [3]. Cellulose and hemicellulose can be converted into fermentable sugars that, in turn, can be used for the growth of microorganisms of industrial interest and the production of value-added products [6].

Biosurfactants are amphipathic, surface-active compounds produced by microorganisms that have antimicrobial and antibiofilm properties. Their emulsifying activity enables applications in the pharmaceutical industry for drug delivery systems; in agriculture for soil nutrient enhancement and pest control [7]; in the food industry for ingredient mixing [8]; and in cosmetic and personal care products as a stabilizing agent [9]. Additionally, due to their interaction with hydrophobic substances, biosurfactants have improved microbial enhanced oil recovery [10] and shown positive results for the bioremediation of soils contaminated with oils.

The most extensively studied bacterial genera for biosurfactant production are *Pseudomonas*, *Acinetobacter*, *Bacillus*, and *Arthrobacter* [11]. However, many strains within these genera are pathogenic, which limits the direct application of their biosurfactants in food, pharmaceutical, and cosmetic products. Lactic acid bacteria are non-pathogenic microorganisms, which have Generally Recognized as Safe (GRAS) status, used in food production and food preservation [12]. They are also considered protective against various human pathologies due to their production of compounds such as biosurfactants. Biosurfactants from lactic acid bacteria have demonstrated antibiofilm [13], antioxidant [14], antimutagenic [15], and anticancer activity [16]. Although there are precedents of lactic acid bacteria biosurfactant production using hydrolysates from agro-industrial wastes, such as vineyard pruning waste [17,18], cheese whey [19,20], and corn and eucalyptus waste [21], there is no knowledge on the use of CPH hydrolysates for this same purpose.

Chemically and structurally, biosurfactants from lactic acid bacteria can vary between glycolipids and glycoproteins [22]. The type of carbon source influences the structure of the biosurfactant produced. When glucose from vineyard pruning waste hydrolysis was used as a carbon source, the biosurfactant produced by *Lactocaseibacillus paracasei* was a glycolipopeptide, whereas when lactose was used, the biosurfactant produced was a glycoprotein.

The aim of this study was to evaluate the production of extracellular and cell-bound biosurfactants from two *Lactocaseibacillus* strains using CPH hydrolysates as a carbon source. As a measure of surfactant activity, emulsification index (E24), antibacterial and antibiofilm activity, and surface tension were evaluated.

2. Materials and Methods

2.1. Cacao Pod Husk Characterization and Alkaline Pretreatment

CPH from *Theobroma cacao* L. variety CCN51 was collected from San Vicente de Chucurí, Colombia, washed, and sun-dried to a moisture content of 10%. Moisture was determined using an MA30 moisture analyzer (Sartorius, Göttingen, Germany). The dried CPHs were ground and sieved to obtain a particle size between 0.25 and 2.0 mm. Structural carbohydrates (cellulose, hemicellulose) and lignin content were determined through the Van Soest method [23]. Extractives were determined according to the National Renewable Energy Laboratory (NREL) protocol [24], and total ash content was measured by incineration at 500 °C according to AOAC standard 972.15 [25]. Alkaline pretreatment was performed with a 2% *w/v* NaOH aqueous solution at a solids loading of 14% *w/v*

CPH and 100 °C for 60 min [26] to remove extractives and disrupt the lignocellulosic matrix. The solid recovery (Equation (1)), cellulose retention (Equation (2)), hemicellulose retention (Equation (3)), and lignin retention (Equation (4)) were calculated according to the following equations:

$$\text{Solid recovery}(\%) = \frac{\text{Mass of recovered solid residue}}{\text{Mass of biomass before treatment}} \times 100\% \quad (1)$$

$$\text{Cellulose retention}(\%) = \frac{\text{Mass of cellulose in solid residue}}{\text{Mass of cellulose in biomass before treatment}} \times 100\% \quad (2)$$

$$\text{Hemicellulose retention}(\%) = \frac{\text{Mass of hemicellulose in solid residue}}{\text{Mass of hemicellulose in biomass before treatment}} \times 100\% \quad (3)$$

$$\text{Lignin retention}(\%) = \frac{\text{Mass of lignin in solid residue}}{\text{Mass of lignin in biomass before treatment}} \times 100\% \quad (4)$$

After alkaline pretreatment, the solid fraction was washed with potable water until reaching neutral pH, air-dried to a moisture content below 10%, and stored in vacuum-sealed plastic bags in a cool, dry place until enzymatic hydrolysis. Following enzymatic hydrolysis, the solids were recovered by vacuum filtration for surface characterization, while the hydrolysate was used as the carbon source for CPH-based culture media. Surface characterization of CPH before and after alkaline pretreatment and enzymatic hydrolysis was performed by Energy Dispersive X-ray Spectroscopy (EDS) on an EDAX APOLO X detector (AMETEK Materials Analysis Division, Mahwah, NJ, USA) with a resolution of 126.1 eV using the EDX Genesis Software V. 6.37 with gold-coated samples.

2.2. Enzymatic Hydrolysis

A commercial enzyme cocktail, Cellic[®]CTec2 (Sigma-Aldrich, St. Louis, MO, USA), was used for the enzymatic hydrolysis of the alkaline-pretreated CPH, and its activity was determined through the filter paper activity test [27]. To determine the hydrolysis conditions that yielded the highest glucose concentration, an enzyme–substrate ratio of 20 FPU/g CPH was used, and the effect of the solid load (5% and 15% *w/v* CPH) was evaluated. Hydrolysis tests were carried out in 250 mL Erlenmeyer flasks, adding the required solid load to 50 mL of citrate buffer (pH 4.8). The solids were left to stand for 4 h to absorb the buffer solution. After 4 h, the enzyme was added, and the Erlenmeyer flasks were incubated at 50 °C and 150 rpm for 168 h. Aliquots of 1 mL were taken, centrifuged, and filtered (0.45 µm), and total reducing sugars were determined by the 3,4-dinitrosalicylic acid (DNS) method [28]. The enzyme cocktail was inactivated by heating the sample to 100 °C for 5 min. Glucose quantification in the CPH enzymatic hydrolysate was performed by high-performance liquid chromatography (HPLC) on a Thermo Dionex Ultimate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) high-performance liquid chromatograph, equipped with a degasser, an autosampler, a refractive index (RI) detector, and a COREGEL 107H 7.8 × 300 mm (8 µm) column (Concise Separations, San Jose, CA, USA). The mobile phase was 8 mM H₂SO₄ in isocratic mode, and the injection volume was 20 µL. The glucose yield after enzymatic hydrolysis was calculated with Equation (5), where 1.11 refers to the conversion factor of cellulose to glucose [29]:

$$\text{Glucose yield} = \frac{\text{mass of glucose released}}{\text{Theoretical glucose in the alkaline pretreated CPH} \times 1.11} \times 100 \quad (5)$$

The highest glucose yield was obtained with 15% solids loading at 50 °C and 150 rpm over 3 h, and under those conditions, the CPH enzymatic hydrolysate was produced for the growth of *Lactocaseibacillus* cultures.

2.3. *Lactobacillus* Cultures and Biosurfactant Production

Lactocaseibacillus casei ATCC 393 and *Lactocaseibacillus rhamnosus* ATCC 7469 precultures were made in Man, Rogosa & Sharpe (MRS) medium [30] at 37 °C and 200 rpm for 18 h. Four culture media were formulated using the CPH enzymatic hydrolysate as a carbon source (adjusted by evaporation to a glucose concentration of 20 g/L) with varying nitrogen supplementation: (i) no supplementation, (ii) peptone at 10 g/L, (iii) yeast extract at 4 g/L, (iv) both peptone at 10 g/L and yeast extract at 4 g/L. The concentrations of glucose, peptone, and yeast extract in the culture media were selected based on their levels in the MRS medium, which is the standard culture medium for *Lactobacillus* strains [30]. Cultures were grown in 120 mL glass vials with a working volume of 50 mL and autoclaved for 15 min at 121 °C and 1 bar. Each vial was inoculated with precultures to achieve an initial optical density at 600 nm (OD_{600}) of 0.1. Incubation was carried out at 37 °C, pH 6.0, and 200 rpm for 72 h under aerobic conditions. Cultures were centrifuged at 8000 rpm and 4 °C for 10 min in an SL 8R centrifuge (Thermo Fisher Scientific, Waltham, MA, USA); cell-free supernatant (i.e., extracellular biosurfactant) was stored under refrigeration at 4 °C. To obtain cell-bound biosurfactants, cell pellets were washed three times with sterile Type 1 water and then resuspended in phosphate-buffered solution (PBS) (10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl, pH 7.0) in a 6:1 culture medium/buffer ratio [18]. The cells suspensions in PBS were incubated at 25 °C and 110 rpm for 24 h, then centrifuged, and the supernatant was stored at 4 °C (cell-bound biosurfactant). Therefore, four biosurfactant solutions were obtained: (i) *L. casei* extracellular biosurfactant, (ii) *L. rhamnosus* extracellular biosurfactant, (iii) *L. casei* cell-bound biosurfactant, (iv) *L. rhamnosus* cell-bound biosurfactant.

2.4. Emulsification Index (E24)

The emulsification index (E24) was determined in order to evaluate the emulsion-forming ability of the extracellular and cell-bound biosurfactants obtained as described in Section 2.3. A total of 2 mL of biosurfactant solution and 2 mL of toluene were added to test tubes, which were then vortexed for 2 min and left to stand for 24 h. The emulsification percentage was calculated using Equation (6). As a control, emulsions were made with water, uninoculated culture media, and PBS.

$$E24 = \frac{\text{Height of the emulsion } (H_e)}{\text{Total height } (H_T)} \times 100 \quad (6)$$

Total height (H_T) and emulsion height (H_e) were measured with a manual caliper. Figure 1 illustrates how the liquid column heights were measured.

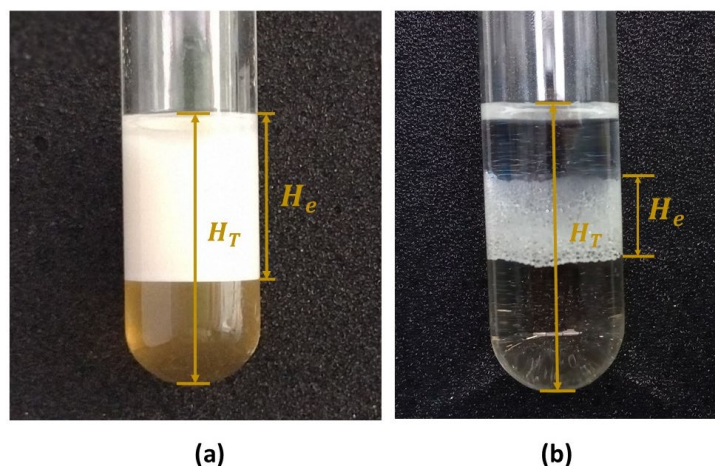


Figure 1. Biosurfactant–toluene emulsions: (a) Extracellular biosurfactant, (b) cell-bound biosurfactant.

2.5. Antibiofilm Activity

The antibacterial and antibiofilm activity of the biosurfactants were assayed in 96-well polystyrene plates. Wells were inoculated with overnight cultures of the pathogen *Pseudomonas aeruginosa* PA14 in Luria–Bertani (LB) medium at an initial OD₆₀₀ of 0.05. Biosurfactants (i.e., cell-bound and extracellular versions obtained in Section 2.3) from *L. casei* and *L. rhamnosus* produced in culture media based on CPH hydrolysate were added at a concentration of 5% of total volume; uninoculated LB medium was used as a negative control, and *P. aeruginosa* PA14 culture without biosurfactant was used as a positive control. PBS (5% volume) was used as a control to verify that it had no effect on PA14 growth or biofilm formation. The plate was incubated at 37 °C for 20 h quiescently. PA14 cell growth was evaluated as OD₆₀₀ using a Multiskan Go spectrophotometer (Thermo Scientific, Waltham, MA, USA). Biofilm formation was measured with crystal violet 0.1% as the absorbance measured at 570 nm (A₅₇₀) [31]. Each condition was evaluated in triplicate; ANOVA analysis was performed using the Tukey grouping method.

2.6. Surface Tension

Surface tension was determined using a Surface Tensiometer 20 (Fisher Scientific, Waltham, MA, USA) with a platinum ring. Each measurement was performed in triplicate for the biosurfactant that had the highest antibiofilm activity for each bacterial strain. Surface tension was measured with water, uninoculated culture media, and PBS as a control.

3. Results and Discussion

3.1. Cacao Pod Husk Characterization and Treatments

The mass compositions of the structural carbohydrates in raw CPH, alkaline-pretreated CPH, and enzymatic-treated CPH are presented in Table 1. Lignocellulose in raw CPH accounts for 43.3% on a dry basis. The extractives correspond to 38.4% and consist of inorganic matter, nitrogenous material, nonstructural sugars, waxes, chlorophyll, and minor components that are soluble in water or ethanol [24]. The ash content was 6.4%; the remaining 11.9% may be pectin, proteins, or lipids, which are not quantified by Van Soest analysis. In previous studies, pectin extracted from dried ripe CPH corresponded to 8–11%, and the method used to dry the CPH influences the yield of pectin extracted [32].

Table 1. Structural carbohydrate content of raw CPH, alkaline-pretreated CPH, and enzymatic-treated CPH.

Compound	Composition on a Dry Basis [%]		
	Raw CPH	Alkaline-Pretreated CPH	Enzymatic-Treated CPH After Alkaline Pretreatment
Cellulose	22.8	46.9	30.1
Hemicellulose	9.32	12.5	8.91
Lignin	11.2	22.6	21.5

After alkaline pretreatment, the percentage of solid recovery was 50%, the cellulose retention was 100%, the hemicellulose retention was 67.1%, and the lignin retention was 100%. SEM micrographs of CPH show that in raw CPH, the shell surface is compact and without porosity (Figure 2a). Even though cellulose and lignin were retained after alkaline pretreatment, cellulose microfibrils and pores with diameters of 1603–3732 µm are observed (Figure 2b), which increases the surface area available for the enzymatic complex during the subsequent enzymatic treatment.

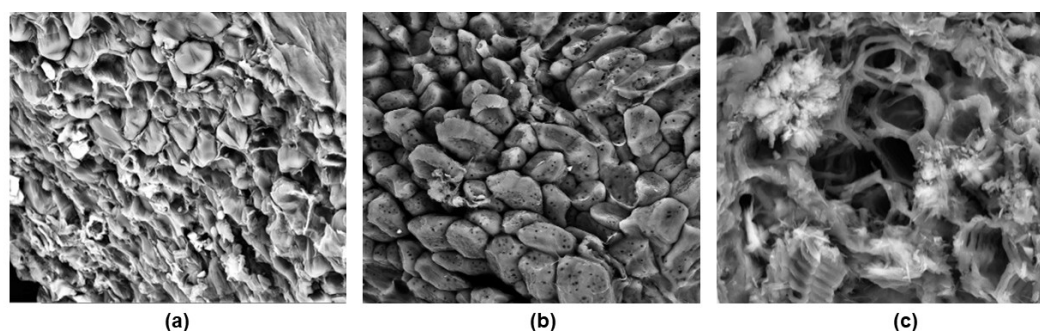


Figure 2. SEM micrographs of CPH at different stages of treatments, presented at a magnification of 100 $\times$. (a) Raw CPH; (b) alkaline-pretreated CPH with 2% NaOH, 100 $^{\circ}$ C; (c) enzymatic-treated CPH at 15% solids loading, 20 FPU/g.

The enzymatic activity of the Cellic[®]CTec2 complex (Sigma-Aldrich, St. Louis, MO, USA) was 108.99 FPU/mL. Figure 3 shows the release of glucose over 168 h of hydrolysis at 5% and 15% solids loading. At 5% solids loading, the highest glucose concentration in the hydrolysate was 6.37 g/L after 9 h of reaction and the glucose yield was 24.5%. Previously, enzymatic hydrolysis of hydrothermally pretreated CPH under the same solids loading and enzyme concentration produced hydrolysates with glucose concentrations from 7.5 g/L to 11.43 g/L; the lowest glucose concentration was obtained with pretreatment at 109.6 $^{\circ}$ C, and the highest glucose concentration at 180.4 $^{\circ}$ C. Therefore, the severity of the pretreatment increased the glucose recovery [33]. The alkaline pretreatment in the present study was applied at 100 $^{\circ}$ C with 2% NaOH, which correlates with a lower glucose concentration in the enzymatic CPH hydrolysate. At 15% solids loading, the maximum glucose concentration in the hydrolysate was 11.45 g/L after 3 h of reaction, and the glucose yield was 14.66%. The observed decrease in glucose release over time is most likely due to a reduction in enzyme activity, possibly caused by the presence of inhibitory compounds released during alkaline pretreatment or by non-productive enzyme adsorption onto residual lignin.

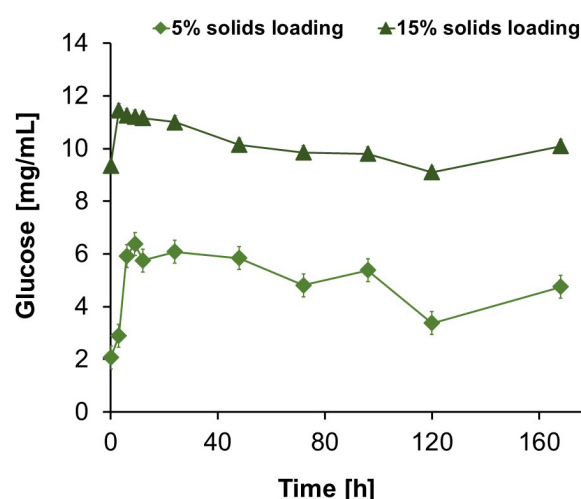


Figure 3. Glucose release curve over time during enzymatic hydrolysis of cocoa shell.

After enzymatic hydrolysis with 15% solids loading, the shell surface is fragmented, with a space between fibrils of 19.53–48.70 μ m (Figure 2c). The percentage of solid recovery was 65.6%, cellulose retention was 42.1%, hemicellulose retention was 46.7%, and lignin retention was 62.4%. These results indicate that the blend of cellulases, β -glucosidases, and hemicellulases partially released the fermentable sugars available in the structural carbohydrates; this may have occurred due to the lignin content remaining after the alkaline

pretreatment (22.6%), since lignin creates steric hindrance, and non-productive adsorption of the enzymes to the structural carbohydrates [34].

3.2. Growth in Hydrolysate-Based Culture Media

The growth of *L. casei* and *L. rhamnosus* in culture media based on CPH hydrolysates is shown in Figure 4. The highest biomass production was obtained for both strains in the culture medium including both supplements (peptone and yeast extract), and lower biomass production was observed in the culture medium with no supplementation (i.e., only enzymatic hydrolysate). The highest OD₆₀₀ value was 2.62 at 24 h of culture for *L. casei*, and 1.17 at 5 h for *L. rhamnosus*. Supplementation has a positive effect on biomass production, especially for *L. casei*. Compared to the medium with CPH hydrolysate with no supplementation, the addition of peptone increased the *L. casei* maximum OD₆₀₀ 4-fold, the addition of peptone increased the OD₆₀₀ 9-fold, and in the medium supplemented with both peptone and yeast extract, the OD₆₀₀ increased 12-fold. In contrast, for *L. rhamnosus*, the addition of peptone increased the maximum OD₆₀₀ 1-fold, while a 2-fold increase in OD₆₀₀ was observed in both the medium supplemented only with yeast extract and the medium supplemented with both peptone and yeast extract. Specific growth rates were calculated as the slope of the linear portion of the plot of Ln (OD₆₀₀) versus time during the exponential growth phase. *L. casei* had an average specific growth rate of 0.224 h^{−1} (R²: 0.972; *p*-value: 0.674) and *L. rhamnosus* of 0.194 h^{−1} (R²: 0.940; *p*-value: 0.674), with no significant differences observed between the different media formulations tested or between the type of *Lactobacillus* strain (*p*-value: 0.700).

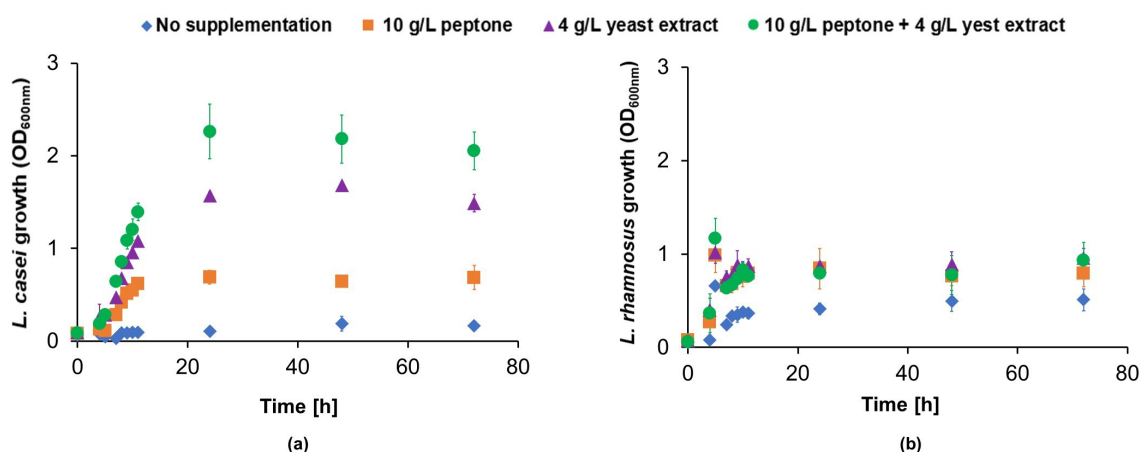


Figure 4. Growth curves of *Lactobacillus* in CPH hydrolysate-based culture media. (a) *Lactobacillus casei*; (b) *Lactobacillus rhamnosus*.

In the case of lactic acid bacteria, a decrease in pH in the culture medium is an indicator of cell growth. *L. casei* culture medium pH can decrease from 6.0 to 3.5 during growth [35]. In this study, the pH of the culture media decreased from 6.00 to 4.50 for *L. casei* and to 5.7 for *L. rhamnosus*. Cell growth of *L. rhamnosus* could have been affected by a lack of salts [36] or by the formation of bacterial growth inhibitors, like phenols and acids, during the alkaline pretreatment of the lignocellulosic material [37].

3.3. Evaluation of Surfactant Activity

3.3.1. Emulsification Index

The highest E24 observed for the *L. casei* extracellular biosurfactant was 31.25%, obtained in the CPH culture medium supplemented only with peptone (Figure 5a), and the highest emulsification index for the cell-bound biosurfactant was 36.66%, obtained in the

formulation supplemented only with yeast extract (Figure 5b). It was previously reported that peptone favored *L. paracasei* biosurfactant production, while yeast extract enhanced cell growth [38]. For *L. rhamnosus*, the highest %E24 was 60%, obtained for the extracellular biosurfactant produced in the culture medium with no supplementation (Figure 5c), and for the cell-bound biosurfactant, the highest %E24 was 51.7%, obtained in the CPH culture medium supplemented with peptone and yeast extract (Figure 5d). The emulsification index of 60% observed for *L. rhamnosus* in the non-supplemented medium may be attributed to the fact that biosurfactant production can be stimulated under nutrient-limited conditions, particularly in the absence of a nitrogen source [39]. Since for *L. rhamnosus* the final pH during cell growth was higher than for *L. casei*, it is likely that *L. rhamnosus* had a lower lactic acid production; therefore, there was less inhibition of biosurfactant production [40]. The E24 of the extracellular biosurfactants from *L. rhamnosus* obtained in this study is higher than the results reported previously (i.e., 33% for cultures in MRS broth and whey-based culture medium [41]). Since the ability to form and stabilize emulsions is a property of biosurfactants, the E24 results suggest that biosurfactants are present in the extracellular and cell-bound metabolites.

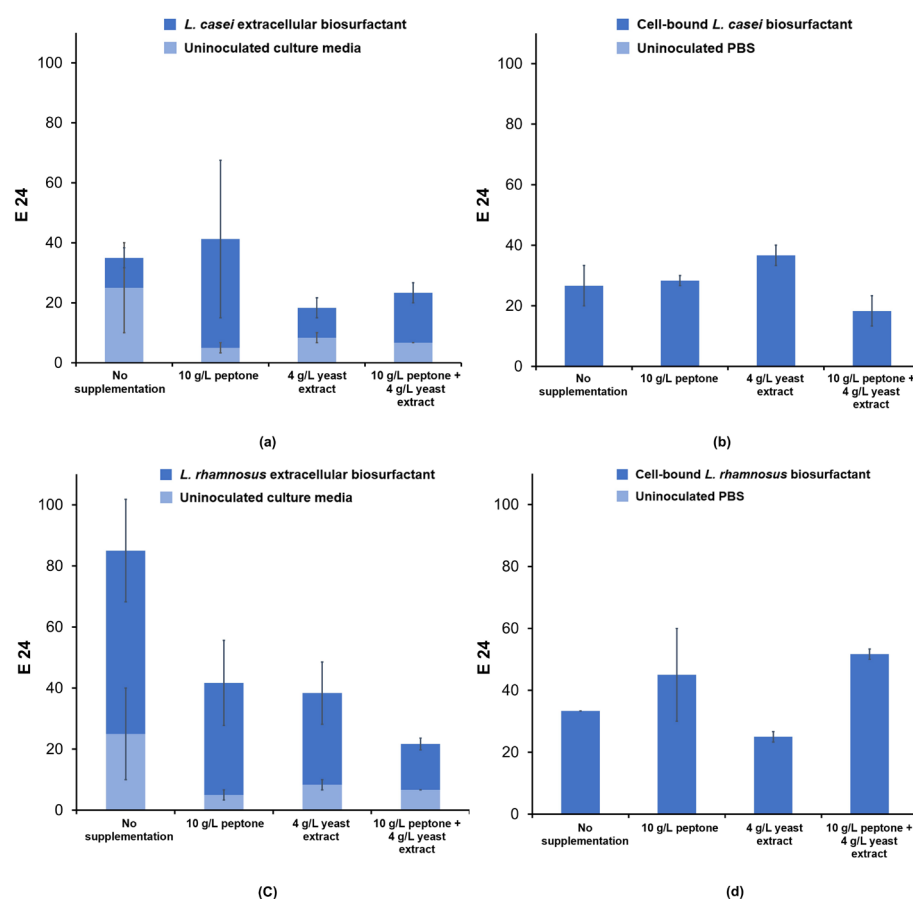


Figure 5. Emulsification index (E24) of the metabolites: (a) extracellular *L. casei*; (b) associated with *L. casei* cells; (c) extracellular *L. rhamnosus*; (d) associated with *L. rhamnosus* cells. Controls were formulated with uninoculated hydrolysate, and pure PBS was used to obtain the cell-associated metabolites.

3.3.2. Antibiofilm Activity

Figure 6 presents the results of biofilm formation by *P. aeruginosa* PA14 in LB medium in the presence of *Lactobacillus* biosurfactants. The biofilm formed by PA14 in the LB medium without biosurfactants was the positive control. For the extracellular biosurfactants, the corresponding CPH-based culture media without biosurfactants were added at 5% volume as controls to evaluate the possible inhibition of PA14 cell growth and biofilm formation by

the culture media used to produce the biosurfactants. Uninoculated PBS (i.e., 5% volume) was also used as a negative control, demonstrating that it does not inhibit PA14 growth (OD_{600nm} 0.75) or biofilm formation (A_{570nm} 1.18). The biosurfactants obtained in the formulations with CPH enzymatic hydrolysate did not inhibit growth (average OD_{600nm} 0.73); however, they did inhibit biofilm formation. There are significant differences between PA14 biofilm formation in LB medium compared with the biofilm formation in the presence of *L. casei* extracellular biosurfactants produced in all the CPH-based culture media with some supplementation. Biofilm formation decreased 44.6% in the medium supplemented only with peptone, 45.5% in the medium supplemented only with yeast extract, and 39.4% in the medium supplemented with both peptone and yeast extract (Figure 6a). *L. casei* cell-bound biosurfactants produced in culture medium supplemented only with peptone reduced biofilm formation by 25.5%, and the cell-bound biosurfactants produced in the culture medium supplemented only with yeast extract reduced biofilm formation by 19.5% (Figure 6b).

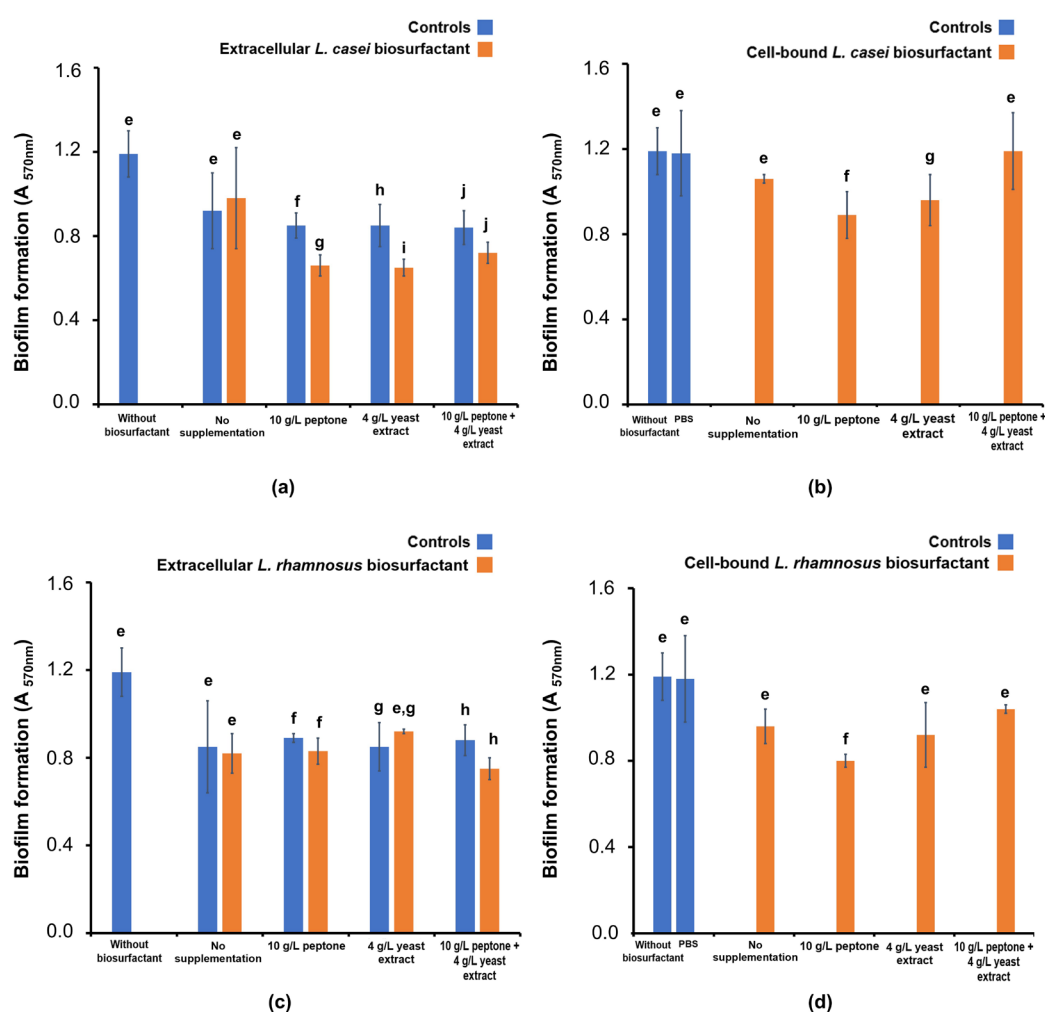


Figure 6. *Pseudomonas aeruginosa* PA14 biofilm formation in the presence of *Lactobacillus* biosurfactants produced in culture media based on enzymatic CPH hydrolysate. Equal letters over bars indicate no significant difference; different letters indicate a significant difference according to Tukey's test. (a) Extracellular *L. casei* biosurfactant; (b) cell-bound *L. casei* biosurfactant; (c) extracellular *L. rhamnosus* biosurfactant; (d) cell-bound *L. rhamnosus* biosurfactant. Controls correspond to PA14 in medium without biosurfactant, formulations with uninoculated hydrolysate, and pure PBS used to obtain cell-associated metabolites.

Similarly, extracellular *L. rhamnosus* biosurfactants produced in supplemented culture media significantly reduced PA14 biofilm formation. The inhibition percentage was 29.98% for the biosurfactant produced in the culture medium supplemented only with peptone, 22.9% for the culture medium supplemented only with yeast extract, and 37.2% for the biosurfactant produced in the culture medium supplemented with both peptone and yeast extract. The cell-bound *L. rhamnosus* biosurfactants produced in the culture medium supplemented only with peptone reduced PA14 biofilm formation by 33%.

Biosurfactants produced by *Lactobacillus* strains have been shown to inhibit the expression of genes associated with biofilm formation in pathogenic microorganisms [42]. It has been proposed that the interference between microbial adhesion and desorption processes on surfaces may be attributed to the adsorption of biosurfactants, which alters the hydrophobicity of the surface [43]. Previous studies have reported that biosurfactants produced by *Lactobacillus* strains with antibiofilm activity can be classified as glycoproteins [44], glycolipids [13], or glycolipopeptides [45]. In the case of *L. casei*, its biosurfactants have been suggested to be chemically similar to rhamnolipids [46] and exhibit over 70% antibiofilm activity against *P. aeruginosa* [13].

3.3.3. Surface Tension

Surface tension was determined for the biosurfactants exhibiting the highest emulsification index and greatest biofilm formation inhibition. These biosurfactants were obtained from culture media supplemented with peptone or yeast extract. There were no significant differences between the surface tension of the *L. casei* and *L. rhamnosus* biosurfactant solutions and the negative controls (Table S1). The average surface tension of the extracellular biosurfactant solutions was 60 ± 1 mN/m, while the uninoculated CPH-based culture medium had a surface tension of 58.7 ± 0.2 . Similarly, the cell-bound biosurfactant solutions had an average surface tension of 63 ± 3 , and the PBS has a surface tension of 74.6 ± 0.6 . These results suggest that the surface-active compounds produced by *L. casei* and *L. rhamnosus* may be high-molecular-weight bioemulsifiers that stabilize emulsions through steric and interfacial-film mechanisms rather than by lowering surface tension. This behavior is consistent with the higher E24 values observed in Figure 5 compared to the negative controls. *L. casei* and *L. rhamnosus* may produce macromolecules such as glycolipids, glycoproteins, or glycolipopeptides, since these chemical structures have been reported in biosurfactants produced by lactic acid bacteria [47].

It has been reported that *L. paracasei* reduces the surface tension of water to 41.8 mN/m [48], and *L. agilis* to 42.5 mN/m [44]. In the specific case of this study, the lack of reduction in surface tension may be influenced by the pH and salinity of the culture medium from which the biosurfactants were obtained, as these factors can affect the surface-active properties of the compounds [49]. To enhance surface tension reduction, it is recommended to extract both extracellular and cell-bound biosurfactants using appropriate purification methods. After centrifugation of the cultures and filtration of the supernatant through a 0.22 μ m membrane, biosurfactants can be precipitated by adjusting the pH to 2.0 with 6 N HCl and stored at 4 °C. The use of solvents facilitates the removal of impurities by dissociation; therefore, a mixture of ethyl acetate and methanol is recommended. Finally, purification can be carried out by reversed-phase high-performance liquid chromatography (RP-HPLC) [13].

4. Conclusions

Alkaline pretreatment of CPH with 2% NaOH at 100 °C for 1 h did not significantly remove cellulose or lignin, but reduced hemicellulose by approximately 33%, increasing porosity and surface accessibility for enzymatic hydrolysis. Enzymatic hydrolysis of the

pretreated biomass with Cellic CTec2 achieved the highest glucose release (11.45 g/L) after 3 h at 15% (*w/v*) solids loading and 20 FPU/g enzyme dosage. Beyond this time, sugar release stabilized, likely due to enzyme inhibition or adsorption onto residual lignin.

Culture media formulated with CPH enzymatic hydrolysates supported the growth of *Lactocaseibacillus rhamnosus* and *Lactocaseibacillus casei* and promoted biosurfactant production. *L. rhamnosus* biosurfactants exhibited the highest emulsifying capacity with an extracellular biosurfactant E24 of 60% and a cell-bound biosurfactant E24 of 51.7%. *L. casei* extracellular biosurfactants inhibited *Pseudomonas aeruginosa* PA14 biofilm formation by 39–45% in CPH-based media supplemented with peptone and/or yeast extract.

These results demonstrate that CPH enzymatic hydrolysates are a promising carbon source for *Lactocaseibacillus* cultivation and biosurfactant production. However, improved pretreatment methods are needed to enhance sugar recovery and enzyme efficiency. Further optimization of biosurfactant extraction and purification is also required to better assess and strengthen their surface-active and antibiofilm properties.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation11110641/s1>, Table S1: Surface tension values of *Lactocaseibacillus* biosurfactants obtained in the formulations with enzymatic hydrolysates of CPH that showed the greatest inhibition of biofilm formation.

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Abbreviations

The following abbreviations are used in this manuscript:

CPH Cacao Pod Husk
GRAS Generally Recognized as Safe

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