

Effect of incorporating an inert support for the production of poly-3-hydroxybutyrate (PHB) from cocoa bean shells by fermentation

Efecto de la incorporación de un soporte inerte para la producción de poli-3-hidroxibutirato (PHB) por fermentación a partir de cascarilla de cacao.

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Abstract

Poly(3-hydroxybutyrate) (PHB) can be used as substitute of non-biodegradable conventional plastics. In a context of circular economy, it is interesting the development of efficient fermentative technologies to produce these bioplastics from low-cost substrates, such as cocoa bean shell (CBS). The present research evaluates the beneficial effect of the presence of inert solid supports on PHB production by fermentation using *Bacillus firmus*. To this end, filtered CBS hydrolysates were employed as substrates for three different fermentations: (i) without solids, (ii) with polyester scouring sponge (PSS) and (iii) with basalt powder (BP). The best results were obtained when solid supports were added, with similar yields of around 36 mg of PHB/g of CBS in both cases. This value is ten times that obtained without solids. These results highlight the significant role that the presence of solids plays in microorganisms' metabolism, being essential for the production of PHB from CBS.

Keywords: *Bacillus firmus*, cocoa bean shell, hydrolysis, polyhydroxybutyrate (PHB), solid support.

Resumen

El poli(3-hidroxibutirato) (PHB) puede emplearse como sustituto de los plásticos convencionales no biodegradables. En un contexto de economía circular, resulta de gran interés el desarrollo de tecnologías fermentativas para producir estos bioplásticos a partir de sustratos como la cascarilla de cacao (CBS). Este trabajo evaluó el efecto de la presencia de un soporte sólido inerte en la producción de PHB utilizando *Bacillus firmus*. Se emplearon hidrolizados de CBS como sustrato para tres fermentaciones diferentes: (i) sin sólidos, (ii) con estropajo de polyester (PSS) y (iii) con polvo de basalto (BP). Los mejores resultados se obtuvieron en las fermentaciones en las que se añadió un soporte sólido, con rendimientos en torno a 36 mg de PHB/g CBS, valor aproximadamente 10 veces mayor que el obtenido sin sólidos. Estos resultados indican que la presencia de sólidos resulta esencial en el metabolismo de los microorganismos para la producción de PHB a partir de CBS.

Palabras clave: *Bacillus firmus*, cascarilla de cacao, hidrólisis, polihidroxibutirato (PHB), soporte sólido.

1. Introduction

Nowadays, the growing demand for plastic materials in every sector has led to an increase in plastic pollution, which is a major challenge in solid waste management (Mishra & Panda, 2023). It has been estimated that more than 400 million tons of plastics were produced worldwide in 2022, 40% of which come from the packaging sector (OECD, 2022; Statista Search Department, 2024). Globally, only 10% of these synthetic plastics are recycled, whereas more than 70% end up in landfills or are mismanaged, causing serious environmental threats, specifically in aquatic ecosystems (Saratale et al., 2021). In addition to environmental issues, plastic particles can provoke human health issues such as tissue damage, allergies or skin afflictions when inhaled or ingested (Arora et al., 2023).

Due to environmental concerns related to non-biodegradable plastic production and petroleum resources depletion, the interest in microbial-derived biodegradable polymers as a sustainable alternative is increasing (Briassoulis et al., 2021; Sohn et al., 2020). Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible bacterial polyesters, which can accumulate in the form of intracellular granules in response to stress conditions (Li & Wilkins, 2020; Sánchez et al., 2023a). Poly(3-hydroxybutyrate) (PHB), the most characteristic PHA, is a short chain length polymer accumulated as carbon and energy reserve by many microorganisms, including the genera *Bacillus*, *Pseudomonas*, *Cupriavidus*, *Azotobacter* and *Comamonas*, being *Bacillus* one of the main genera capable to produce PHB (Nath et al., 2024). Due to its high biodegradability (it can be completely degraded after 30 days), hydrophobicity, biocompatibility, and thermal and mechanical properties, PHB can be extensively applied in the food packaging industry and in the medical sector (specifically in tissue engineering) (Panda & Dash, 2023). In recent years, not only a wide range of bacteria species involved in the PHAs production have been studied, but also the use of different food wastes as substrates in its production has been addressed (Sirohi, 2021).

According to the Food Waste Index Report, more than 900 million tons of food waste were generated worldwide in 2021 throughout the food supply chain, from production to domestic consumption (Jaouhari et al., 2023). In this sense, the European Commission, through the Circular Economy Action Plan, has recently adopted the Sustainable Development Goals aimed at prioritizing residue prevention, recycling and valorization, considering waste disposal the most negative option. (Mariatti et al., 2021). The industrial scale-up of PHB production by microbial way is usually limited by the high cost of carbon sources, which accounts in some cases 50% of the total production expenditure (Saratale et al., 2021). Therefore, recently, there has been a growing interest in finding novel, cheap and easily available carbon sources. In this context, lignocellulosic biomass with proper treatment could be a promising renewable source to be employed as raw material for the production of bioplastics by fermentation. Cocoa bean shell (CBS) is the external part that covers the cocoa bean and is one of the main by-products derived from the chocolate industry. CBS is generally discarded as waste or used in low-value applications, i.e., as fertilizer or as animal food. This residue represents between 10-20% of the total cocoa bean weight and is mainly constituted by carbohydrates, phenolic compounds, dietary fibers, and fats (Sánchez et al., 2023b). In recent years, there has been a growing trend in using CBS to obtain value-added products with potential applications in the food, pharmaceutical and cosmetic sector, not only due to its interesting composition, but also because its valorization could be economically appealing (Okiyama et al., 2017).

Therefore, the aim of this work has been to evaluate the effect of inert supports (polyester scouring sponge and basalt powder) on PHB production by means of *Bacillus firmus* CECT 14, employing CBS hydrolysates as a carbon source.

2. Materials and methods

This work is an experimental research.

2.1.Raw material

CBS, which was obtained after roasting Forastero cocoa beans (Ivory Coast), was supplied by a local chocolate factory sited in Asturias (Spain).

2.2.Hydrolysis treatment

With the aim of obtaining a broth with a high content of fermentable sugars and considering previous works (Sánchez et al., 2023b, 2024), the following procedure was employed. Firstly, the raw material was milled in a blender (Braun 4041) to obtain 1-2 mm of particle size. A mixture of the milled CBS and 5% H₂SO₄ (Supelco, Bellefonte, PA, USA) (20% w/w) was introduced into a 1-L Pyrex bottle and hydrolyzed at 135°C and 2 bar in an autoclave (AES 110, Raypa, Spain) for 10 minutes. Once autoclaved, larger solids were removed with a sieve and the resulting liquid phase was filtered by a 20-μm cellulose sterile filter (Whatman™). The recovered supernatant was adjusted to pH 6-7 with 5M NaOH (Merck, Rahway, NJ, USA) and placed in a fermentation flask for inoculation.

2.3.Fermentation process

Prior to fermentation, two different supports were added to fermentation media in sterile conditions: polyester scouring sponge (PSS) and basalt powder (BP) (46.7% silica, 12.76% iron, 11.28% calcium, 9.5% magnesium, 0.4% phosphorus, 0.2% manganese, 0.02% copper and 0.02% zinc) (Cultivers, Spain). The preparation of polyester pieces followed the procedure described in Ruiz et al. (2015). The sponge was cut into cubes of approximately 1 cm side and 1 g of these fragments was added to the fermentation flask in sterile conditions. In the case of basalt powder, 36 g were added to the fermentation broth so that the solid content was similar to the substrate non-centrifuged. Figure 1 shows the support employed for the fermentation process. In addition, fermentation without solids and any support was carried out as a control. All fermentations were carried out at least in duplicate.

Broths were inoculated with *Bacillus firmus* CECT 14 supplied by CECT (Spanish Type Culture Collection) with an initial microbial load of 6×10^3 CFU/mL. Fermentations were conducted for 6 days at 37°C and 250 rpm and samples

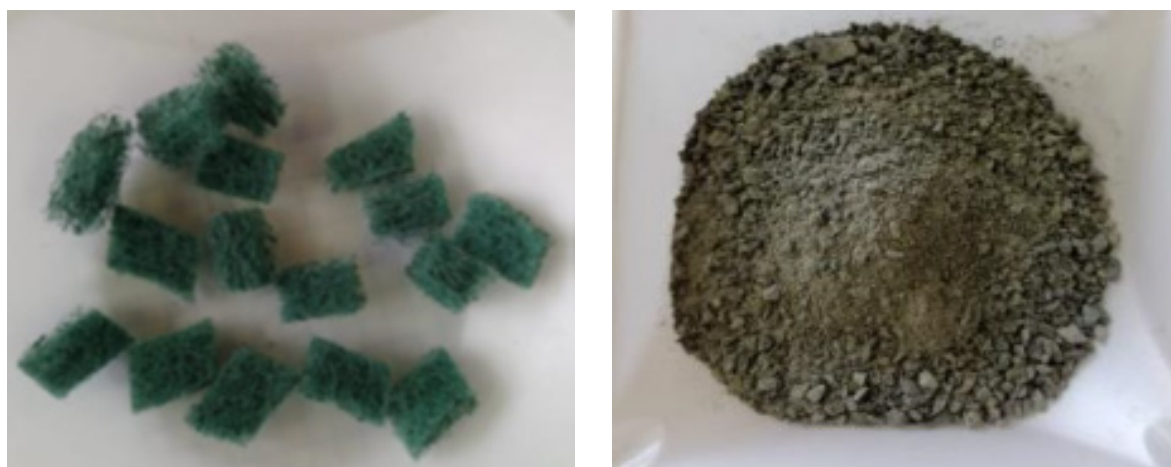


Figure 1: Supports employed in the fermentation process: polyester scouring sponge (left) and basalt powder (right).

were taken from the flasks periodically and centrifuged (Heraeus Multifuge X1 Centrifuge Series, Thermo Fisher Scientific, Waltham, MA, USA) at 10.000 rpm for 10 minutes. The pH of the supernatant was measured, and, after that, it was frozen until analysis of total carbohydrates and reducing sugars content. The pellet was also frozen until determination of PHB content. Additionally, microbial growth was followed by taking 1 g of sample, which was homogenized in a Stomacher (Stomacher 80 Biomaster, Worthing, West Sussex, UK) with 9 mL of 0.7% NaCl sterile solution. Serial dilutions were plated in triplicate onto nutrient broth agar medium and incubated at 30°C for 24 h before counting.

2.4. Analytical methods

All analyses were performed in triplicate and all reagents were supplied by Merck (Rahway, NJ, USA).

2.4.1. Total Carbohydrates

Total carbohydrates were quantified using the phenol-sulfuric acid method (Dubois et al., 1956) as described in Sánchez et al. (2022). For this assay, 1 mL of sample was mixed with 2.5 mL of 96% H₂SO₄ and 0.5 mL of 5% phenol solution and the mixture was incubated at room temperature for 1 h. Finally, the absorbance was measured at 492 nm using a spectrophotometer (DR/2500 HACH, CO, USA). Glucose was employed as the standard

2.4.2. Reducing sugars

The amount of total reducing sugars in the samples was measured as reported in Díaz et al. (2017), using an adaptation of the Dinitrosalicylic acid (DNS) method (Miller, 1959). For the analysis of reducing sugars, 0.5 mL of DNS reagent was added to 0.5 mL of sample and the mixture was incubated for 5 min in a water bath at 95°C. Then, samples were immediately cooled in an ice bath and the absorbance was measured employing a spectrophotometer (Thermo Scientific™ UV-Vis GENESYS™ 150, Waltham, MA, USA) at 540 nm. Glucose was employed as the standard.

2.4.3. Quantification of PHB

The extraction and quantification of PHB content in samples was carried out following the Law and Slepecky method (Law & Slepecky, 1960). Firstly, the pellet obtained in the fermentation was digested with 10 mL of 6-14% NaClO solution in a water bath at 37°C for 1 h. After that, the mixture was centrifuged for 30 minutes at 10.000 rpm (Heraeus Multifuge X1 Centrifuge Series, Thermo Fisher Scientific, Waltham, MA, USA) and the pellet was washed successively with distilled water, acetone, and ethanol. The supernatant was discarded, and the pellet was mixed with 10 mL of chloroform and the mixture was filtered through a 20 µm cellulose filter recovering the filtrate.

For the quantification of PHB content in samples, 100 µL of the filtrate was mixed with 10 mL of 96% H₂SO₄ and incubated at 95°C in a water bath. After 10 minutes, samples were cooled in an ice bath and the absorbance was measured employing a spectrophotometer (Thermo Scientific™ UV-Vis GENESYS™ 150, Waltham, MA, USA) at 235 nm. Crotonic acid was employed as standard.

2.4.4. Dry extract

To express the results on a dry weigh basis (dw) (w/w), the moisture content of CBS was determined gravimetrically. To this aim, 3 g of sample was weighed with sea sand in a stainless-steel capsule. The mixture was dried in an oven for 24 h at 105°C and, after cooling, was weighed again. The dried extract and moisture content were calculated considering the difference between the initial and final weight.

2.4.5. Statistical analysis

GraphPad Prism software (version 9.0; GraphPad Software Inc.) was used for statistical analysis. Results were expressed as average value ± standard deviation (SD). Analysis of variance (ANOVA) and Tukey's multiple comparisons tests were used for statistical analysis calculated with 95% confident interval ($p < 0.05$).

3. Results and discussion

It has been reported that the pretreatment of lignocellulose wastes is the first step to produce bacterial polyhydroxyalkanoates (Andler et al., 2021), consequently, with the aim to maximize the extraction of fermentable sugars, CBS was submitted to a hydrothermal treatment. In particular, PHB can be produced using different feedstocks, including agro-industrial wastes, as substrate (Sirohi et al., 2020). Concretely, the CBS hydrolysate obtained has a composition suitable for the production of PHB by fermentation (reducing sugars: 28.0 g/L, total nitrogen: 2.2 g/L and total phosphorus: 0.8 g/L) (Sánchez et al., 2023b). According to the literature, PHB is produced by microorganisms under restricted conditions of nitrogen and phosphorus and in the presence of an excess carbon source, for example, 22-30 g/L of carbon, 0.5 g/L of phosphorus and 2 g/L of nitrogen, similar values than those of the CBS hydrolysate (Hamdy et al., 2022). Ramos et al., (2023) studied the production of PHA from cocoa pod husk and reported a maximum PHA accumulation in a fermentation media with 20 g/L of glucose. Rebocho et al. (2019) employed a hydrolysate of apple pulp with a concentration of reducing sugars of 25.2 g/L for obtaining PHAs. In addition, Hamdy et al. (2022) evaluated the production of PHB by *Bacillus cereus* using different nitrogen sources and observed that the highest PHB concentration was achieved with 2.4 g/L of nitrogen in the initial medium. With respect to pH, Amulya et al. (2016) obtained the highest productivity of PHA under neutral conditions. The optimal pH for *Bacillus* growth is between 6.5 and 7, whereas for pH values below 5.8 a complete suppression of *Bacillus* growth was observed (Li & Wilkins, 2020). For these reasons, the initial pH of the medium employed in this work was initially adjusted to around 6.8.

Despite the apparent suitable characteristics for PHB production of the liquid medium obtained from hydrolyzing CBS, very low concentrations of PHB were obtained without solids (< 1 g/L). On the contrary, in previous works (Sánchez et al., 2023b), interesting PHB productivities were

achieved when the non-solubilized CBS was maintained in the media during fermentation. It has been demonstrated that the presence of solids in fermentation media can favor the formation of fermentation products due to the fact that these particles provide a solid surface for microorganism's attachment, which improves cell growth rate (Sánchez et al., 2023b; Bathgate, 2019).

3.1. Sugars consumption

The evolution of both total carbohydrates and reducing sugars were monitored during fermentation and results are shown in Figure 2. As can be appreciated, in the control the consumption of sugar was very low (approximately 5 g/L) compared to the results obtained in the broths with BP and PSS. In addition, the consumption took place just during the first 24 h of fermentation and significant differences ($p > 0.05$) were not observed between the sugar concentrations measured after 48 h. However, when a solid support was added, the concentration of sugars was reduced more noticeably. In both cases, with BP and PSS, a rapid consumption of sugars was observed during the first day of fermentation, decreasing onwards more slowly. The final concentrations of total carbohydrates and reducing sugars were very close for both fermentations, which means that, when solid particles were in the medium, the enzymes released by *B. firmus* could hydrolyze the dissolved complex carbohydrates into simple sugars. The total carbohydrates consumed at the final time (144 h) were 13.8 g/L and 14.6 g/L with BP and PSS, respectively (around 40%).

These results are in accordance with those reported in the literature for the production of PHB from other lignocellulosic residues. Pereira et al. (2021) studied the production of PHAs from apple pulp extracts by means of *Pseudomonas chlororaphis* and observed a maximum consumption of fermentable sugars of 15.2 g/L after 4 days of fermentation. In addition, Andler et al. (2024) evaluated the use of hydrolysates of grape residues

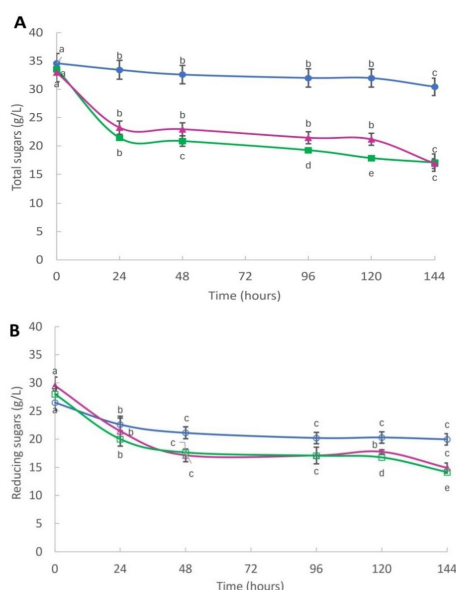


Figure 2: Evolution of (A) total carbohydrates (filled) and (B) total reducing (unfilled) sugars during fermentation for control (●, ○), BP (■, □) and PSS (▲, △), expressed in g/L. For each fermentation, the means followed by different letters indicates statistic differences ($p < 0.05$) by Tukey's test.

to produce PHB, using a suspended culture of *Azotobacter vinelandii*, and reported that over the course of fermentation, approximately 17 g/L of reducing sugars were consumed. Quintero-Silva et al. (2024) who studied the production of PHAs using cocoa mucilage as fermentation media, reported a consumption of 18 g/L of reducing sugars during the process and a maximum concentration of PHA of 2.3 g/L.

3.2. Microbial growth

As can be seen in Figure 3, a decrease in CFU counting in the control sample can be observed during the first 24 h of fermentation. In the fermentation with BP, the viability slightly increased during the first 48 h, decreasing drastically after this moment. On the contrary, in the broth with PSS a notable bacteria growth took place during the first 48 h of fermentation, which coincides with the fast consumption of sugars previously commented (Figure 2). Additionally, it is remarkable that sugar consumption was very similar for both BP and PSS broths, whereas the maximum bacterial concentration achieved was quite different, namely, 8.0×10^3 and 1.2×10^5

CFU/ml, respectively. No significant differences ($p > 0.05$) were observed in microbial viability after 48 h of fermentation in the control and PSS samples. Finally, it is noteworthy that the microbial growth behavior seems likely that some kinds of inhibitors were released during the fermentation process, since bacterial viability started to decrease when the concentration of reducing sugars was still high (Figure 2).

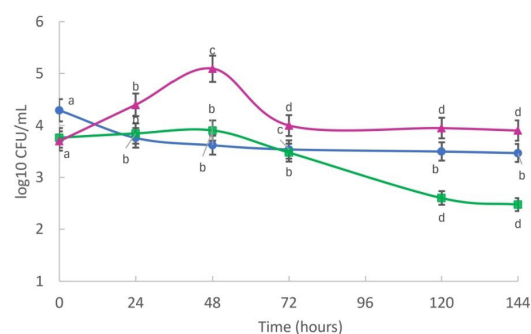


Figure 3: Evolution of microbial growth during fermentation process: control (●), BP (■) and PSS (▲). For each fermentation, the means followed by different letters means statistic differences ($p < 0.05$) by Tukey's test.

Results demonstrate that there is a clear relationship between the presence of a solid support in the fermentation media and bacterial growth. It has been reported that *Bacillus* genus can produce biofilms in response to stress, especially in presence of surfaces such as plastics, soil particles, and food (Viju et al., 2020). These structures enhance the metabolism and favor the survival of microorganisms under adverse conditions. Zhu et al. (2020) evaluated the effect of different culture parameters, i.e., pH, temperature, and carbon source, on the biofilm formation of *Bacillus pumilus*. They observed a stable biofilm structure on the surface of a solid medium (LB agar medium) and reported that a higher and more resistant biofilm was obtained at 37°C and pH 7, using glucose as carbon source, similar conditions than those employed here. Therefore, the differences observed when comparing fermentations with and without solids are due to the formation of bacterial biofilms on the surface of the solids, which protected the cells and increased the viability of *B. firmus*.

3.3. PHB production

The production of PHB by *B. firmus* during the fermentations without solids (control), with BP and with PSS, has been followed and results are shown in Figure 4.

As can be appreciated, notable differences in terms of PHB production were observed, depending on whether a solid support was or not added. After 144 h of fermentation without solids (control), a PHB concentration of only 0.6 g/L was obtained. These results are in accordance with the observed loss in cell viability above commented (from 104 to 103 CFU/mL) as low PHB production may be associated with reduced cell viability. However, when solid supports were present in the fermentation broth, PHB was produced by the bacterium and concentrations between 6 and 7 g/L were achieved after 72 h. Comparing the production of PHB in fermentations carried out with BP and PSS, significant differences were not observed ($p > 0.05$), even though the cell growth was much higher in the case of the PSS. In both cases, the maximum yield was obtained after 120 h of fermentation with values around 36 mg PHB/g CBS (dry weight). The highest rate of PHB production occurred simultaneously to the exponential cell growth, during the 1st and 2nd day of fermentation, with an average biopolymer productivity of 0.11 g/L h. It has been reported that some bacteria of the *Bacillus* genus present a growth-associated PHB production and accumulate the polymer during the exponential phase (Yanti et al., 2021).

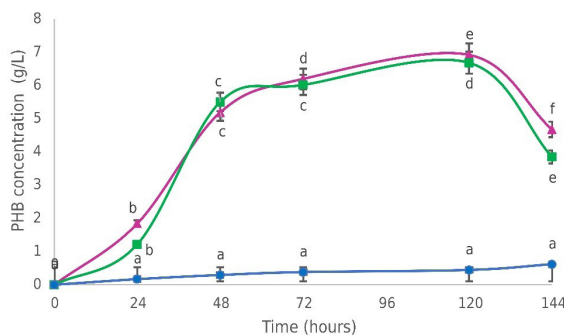


Figure 4: Concentration of PHB during fermentation process: control (●, ○), BP (■, □) and PSS (▲, △). For each fermentation, the means followed by different letters means statistic differences ($p < 0.05$) by Tukey's test.

Some other authors reported similar productions of PHB using as substrate other lignocellulosic residues. Amir et al. (2024) studied the addition of different dried and ground food wastes solids (i.e. potato peel, banana peel, corn peel and cassava bagasse) in the fermentation media for the production of PHB employing *Pseudomonas stutzeri* and reported a maximum concentration of PHB of 6.1 g/L after 5 days of fermentation. In addition, Soni et al. (2023) obtained approximately 0.9 g/L of PHB from banana peel waste hydrolyzed with 5% H_2SO_4 in a fermentation media without a solid support, values very similar to those obtained here with the medium without solids. Hassan et al. (2019) evaluated the use of different agrifood solid wastes (i.e., corn bran, corncob, wheat bran and rice bran) as a supplement to optimize the production of PHB by *Bacillus subtilis* and the maximum concentration achieved was only of 0.3 g/L of PHB when rice was employed as feedstock, almost the same concentration as that obtained here in the control broth.

This research has demonstrated that the availability of a solid support in the fermentation media, in which microorganisms can form a biofilm, improves not only bacterial growth and viability but also the production of PHB. However, the PHB concentrations obtained here with inert supports were still lower than those obtained in a previous work with CBS solids (20 g/L of PHB) (Sánchez et al., 2023b). Therefore, the presence of solids in the fermentation medium is indeed a key factor in the production of PHB, but also their composition and nature are fundamental since they can act as an additional source of micronutrients and/or growth factors for microorganisms.

4. Conclusions

CBS has been employed as feedstock for the production of PHB, a sustainable alternative to synthetic plastics, by fermentation with *B. firmus*. A very low production of PHB was observed when CBS hydrolysates without solids were employed, with a maximum concentration of PHB of 0.6 g/L. However, the use of inert supports (i.e., BP and PSS) notably increased the production and concentrations of PHB between 6 and 7 g/L were achieved after 72 h of fermentation, respectively. This production of PHB took place in parallel with an increase in *B. firmus* counts. In particular, in the PSS fermentation the concentration of viable cells increased notably from 6×10^3 to 1.2×10^5 CFU/mL. On the contrary, in absence of solids (control broth) microbial viability decreased from

the first day of fermentation. According to these results, it is clear the necessity of solid particles for bacterium growth and, therefore, to produce PHB from CBS. This is explained by the advantages derived from the formation of a biofilm on the surface of the solids, which protects bacteria from inhibitory compounds and adverse conditions.

The results obtained in this work highlight the potential of CBS to be employed as feedstock for fermentation with the objective of producing value-added products such as bioplastics. As future work, other fermentation conditions (i.e., inoculum, temperature, agitation, time, kind of solid particles, etc.) should be optimized in order to consider the possible scaling of the process.

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