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PRODUÇÃO DE CELULOSE BACTERIANA UTILIZANDO SUBSTRATOS ALTERNATIVOS NO MEIO DE CULTURA*

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RESUMO: O estudo tem como objetivo o desenvolvimento de celulose bacteriana (CB) produzidas a partir da bactéria *Gluconacetobacter xylinus*, em meios de cultura enriquecidos com vinhaça e *Aloe vera*. A análise de Espectroscopia na região do infravermelho, possibilitou concluir que ocorreram alterações químicas nas estruturas da CB incorporadas com *Aloe vera* e na incorporação com vinhaça. Na análise de Difração de Raio – X, foi possível identificar incorporações com a presença de celulose I e incorporações com a presença de celulose II. Mecanicamente, a incorporação CBB80 de *Aloe vera* demonstrou semelhança nos valores de módulo de elasticidade (MPa), tensão máxima (MPa) e alongamento (%), enquanto a incorporação com vinhaça, a CBV40 demonstrou semelhança nos valores de módulo de elasticidade (MPa), tensão máxima (MPa) e alongamento (%) quando comparadas às membranas CB. Os resultados mostraram o potencial uso de incorporações da vinhaça e *Aloe vera* como substratos alternativos, reduzindo os altos custos envolvidos na produção, a valorização do uso de resíduos agroindustriais e o potencial uso de plantas com compostos bioativos.

Palavras-chaves: celulose bacteriana, *Gluconacetobacter xylinus*, substratos alternativos, vinhaça, *Aloe vera.*

BACTERIAL CELLULOSE PRODUCTION USING ALTERNATIVE SUBSTRATES IN THE CULTURE MEDIUM

ABSTRACT: This study aimed to develop CB produced from the bacterium *Gluconacetobacter xylinus* in culture media enriched with vinasse and *Aloe vera*. The infrared spectroscopy analysis allowed us to conclude that there were chemical alterations in the structures of the CB incorporated with *Aloe vera* and in the CB incorporated with vinasse. In the X-ray diffraction analysis, it was possible to identify incorporations with cellulose I and incorporations with cellulose II. Mechanistically, when CBB80 was incorporated into Aloe vera, the modulus of elasticity (MPa), maximum stress (MPa) and elongation (%) were similar, while when CBB40 was incorporated with vinasse, the modulus of elasticity (MPa), maximum stress (MPa) and elongation (%) were similar to those of CB membranes. The results showed the potential for the incorporation of vinasse and *Aloe vera* as alternative substrates, reducing the high costs involved in production, the valorization of agroindustrial waste and the potential use of plants with bioactive compounds.

Keywords: bacterial cellulose, *Gluconacetobacter xylinus*, alternative substrates, vinasse, *Aloe vera.*

1. INTRODUCTION

Bacterial cellulose (BC) is produced from the biosynthetic pathways of microorganisms from the following genera:

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Komagataeibacter, Aerobacter, Achromobacter, Agrobacterium, Alacaligenes, Pseudomonas, Rhizobium, Sarcina and Salmonella. The bacterium produces cellulose membranes through fermentation in a medium rich in carbon (C) and nitrogen (N) sources. This CB membrane is considered chemically pure because it is free of lignin and hemicellulose and nontoxic, in addition to having high porosity and mechanical resistance (CHANG; CHEN, 2016; QIU *et al.*, 2016; HALIB *et al.*, 2019; WANG; TAVAKOLI; TANG, 2019).

The use and exploitation of agro-industrial, food and brewery waste products with potential therapeutic and/or medicinal properties as alternative substrates in culture media to obtain bacterial cellulose are increasing in the literature, including the use of glycerol (VAZQUEZ *et al.*, 2013), sisal liquid (LIMA *et al.*, 2017), cashew juice and soy molasses (SOUZA *et al.*, 2021b), orange peel residues (KUO *et al.*, 2019), banana peel waste (SIJABAT *et al.*, 2020), and a mixture of date syrup and cheese whey (RAISZADEH-JAHROMI *et al.*, 2020), among others. These byproducts are generally rich in sugars such as glucose, fructose, galactose and sucrose and may be promising for the industrial production of bacterial cellulose (WU; LIU, 2013).

Vinasse, a byproduct of ethanol manufacturing, is rich in liquid residual nitrate; has a high content of organic matter, macro- and micronutrients; has high amounts of cations such as potassium (K), iron (Fe), calcium (Ca) and magnesium (Mg); and has small amounts of nitrogen (N) and phosphorus (P) (FUESS, 2013). It is composed of 93% water and 7% organic and inorganic solids, and its composition may vary depending on the ethanol production process (ALBANEZ *et al.*, 2018). When deposited in the soil, vinasse acts as a fertility restorer in a process called fertigation (OLIVEIRA, 2015). However, the application of this process must be controlled to avoid impacts on the soil and groundwater, as when uncontrolled, it can lead to leaching (SULEIMAN *et al.*, 2018).

Popularly known as aloe vera, *Aloe vera is a plant belonging to the* Xanthorrhoeaceae family (LIM; CHEONG, 2015). The main characteristic is the high water content, approximately 99%, with the other 1% from 75 different active compounds, such as vitamins, minerals, enzymes, polysaccharides, phenolic compounds and organic acids, as well as anthracene derivatives, including aloins (e.g., barbaloin and isobarbaloin). It has great potential for application in medical areas such as tissue engineering, burn treatment and healing. (RAMOS; PIMENTEL, 2011; FREITAS; RODRIGUES; GASPI, 2014; RADHA; LAXMIPRIYA, 2015; GAO *et al.*, 2019).

Since the culture medium must contain, at a minimum, a carbon source (C), a nitrogen source (N), and other macro- and micronutrients necessary for the growth of the microorganism, such as phosphorus salts (P), sulfur (S), potassium (K) and magnesium (Mg), *Aloe vera* and vinasse are potential substrates in culture media for the production of bacterial cellulose, which could become an economically viable solution (JOZALA *et al.*, 2016; MOHAMMADKAZEMI;

Therefore, the objective of the present work was to evaluate the development of bacterial cellulose (BC) using culture media enriched with vinasse and *Aloe vera*.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Microorganism

The bacterium used to produce the bacterial cellulose membranes was *Gluconacetobacter xylinus* ATCC 23768, which was kindly donated by Prof. Dr. Sidney José Lima Ribeiro from the Universidade Estadual Paulista "Júlio de Mesquita Filho" – Institute of Chemistry of Araraquara – UNESP.

2.1.2 Culture medium: growth and maintenance

Alanine culture medium (replacing glucose with sucrose – crystal sugar) prepared in 1 L of distilled water was used, and the pH of the solution was adjusted to between 4.5 and 5,

as shown in Table 1. The growth and maintenance media were sterilized in a vertical autoclave at a temperature of 121°C and pressure of 1.2 kgf/cm2 for 15 min.

Table 1. Composition of the standard culture medium of albanane.

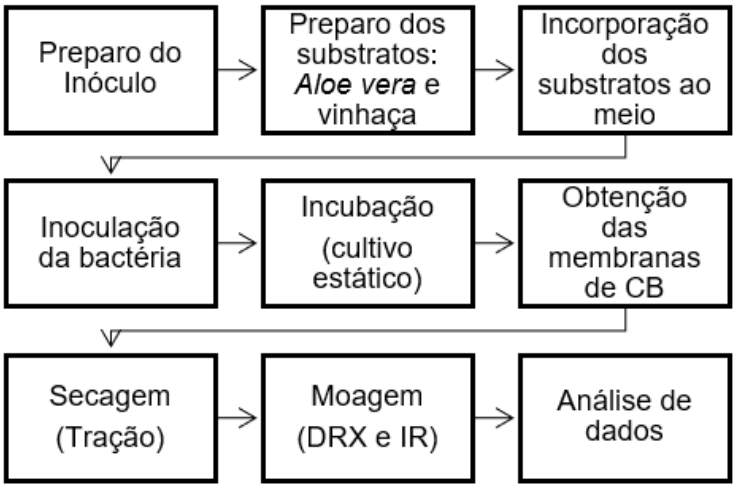
Components	Concentration (gL^{-1})
Crystal sugar ($C_{12}H_{22}O_{11}$)	150
Citric acid ($C_6H_8O_7$)	6
dibasic potassium phosphate (K_2HPO_4)	10
Yeast extract	5
Magnesium sulfate ($MgSO_4$)	0.12
Ammonium sulfate ($(NH_4)_2SO_4$)	0.4

Source: Prepared by the authors

2.2 Methods

The methodology used in this work is described in the flowchart shown in Figure 1.

Figure 1. Experimental flowchart of the steps



Source: Prepared by the authors

2.2.1 Preparation of the inoculum

For the formation of the inoculum, approximately 0.2 mL of the aliquot received was added to an Erlenmeyer flask with 5 mL of Alaban standard culture medium, sterilized at 121°C and pressure of 1.2 kgf/cm2 for 15 min, and incubated for a period of 72 hours in static culture at a temperature of 28 ± 2°C.

After 72 hours, 3 mL of the abovementioned solution was added to an Erlenmeyer flask supplemented with 50 mL of Alaban standard culture medium and incubated for a period of 72 hours without shaking at 28 ± 2°C.

2.2.2 Preparation of the *Aloe vera substrate*

Aloe vera leaves were collected from the external area of the Department of Bioprocesses and Biotechnology at the Faculty of Agricultural Sciences of the Universidade Estadual Paulista "Júlio de Mesquita Filho" - Campus Botucatu. The treatment of *Aloe vera* leaves was carried out in the RESIDUALL laboratory. First, the leaves were washed in running water to remove major impurities and then washed a second time with distilled water

to ensure the cleanliness of the material. To separate the gel, 2 cm pieces were cut from the basal portion of the leaves and 5 cm from the apical portion. To obtain the gel, the bark, which is morphologically known as the chlorophyll parenchyma, was removed from longitudinal sections and lateral spines. The gel, the reserve parenchyma, was scraped with a spoon and homogenized in a Walita brand blender at speed 3 for 2 minutes. The solution was transferred to a 2 L beaker and heated in an oven at 60 °C for 20 minutes to reduce the amount of foam that formed after mixing in the blender.

2.2.3 Incorporation of the *Aloe vera substrate into the* Albanan culture medium

Alcian standard culture medium and homogenized *Aloe vera* gel substrate were prepared weekly. A 250 mL Erlenmeyer flask was used to add 100 mL of culture medium, a volume represented by the Alaban culture medium + substrate. For the control CB samples, 100 mL of Alaban standard culture medium was added to 250 mL Erlenmeyer flasks. For the CBB20 samples, 80 mL of Alaban and 20 mL of *Aloe vera substrate*, CBB40 samples, 60 mL of Alaban and 40 mL of *Aloe vera substrate*, CBB60 samples, 40 mL of Alaban and 60 mL of *Aloe vera substrate* and CBB80 samples, 20 mL of Alaban and 80 mL of *Aloe vera substrate were used*. Certification of the homogeneous distribution of media and substrate fractions was carried out using graduated cylinders.

Table 2 shows how the Aloe vera standard culture medium was fractionated into percentages of 100%, 80%, 60%, 40% and 20%, and the *Aloe vera substrate was inserted*.

Table 2. *Aloe vera* substrate fractions in the standard culture media of albana vera.

Fractions		Bacterial cellulose membranes (CB)	
		Alabama	***Aloe vera***
CB	100%	100 ml	0 mL
CBB20	20%	80 mL	20 ml
CBB40	40%	60 mL	40 ml
CBB60	60%	40 ml	60 mL
CBB80	80%	20 ml	80 mL

Source: Prepared by the authors

2.2.4 Preparation of the vinasse substrate

The vinasse used in the production of bacterial cellulose membranes was kindly donated from a Still in the region of São Manuel, SP. The separation of the solid part and debris from the liquid part occurred using 122 BCP vacuum pumps. To preserve the material during the experiment, the vinasse was bottled and frozen in a CL580/86v ultrafreezer at -40 °C.

Albanan culture medium

Alanine standard culture medium and vinasse substrate were prepared weekly. For sample preparation, 250 mL Erlenmeyer flasks were used. Once the final volume of 100 mL (Alaban culture medium + substrate) was determined, the amount of 100 mL of Alaban standard culture medium was determined for the control CB samples: CBV20, 80 mL of Alaban and 20 mL of vinasse substrate; CBV40, 60 mL of Alaban and 40 mL of vinasse substrate; CBV60, 40 mL of Alaban and 60 mL of vinasse substrate; and CBV80, 20 mL of Alaban and 80 mL of vinasse substrate. Test tubes were used to verify the distribution of media and substrate fractions in a homogeneous manner. Table 3 shows how the Alaban standard culture medium was fractionated to 100%, 80%, 60%, 40% and 20%, and the vinasse substrate was added.

Table 3. *Aloe vera* substrate fractions in the standard culture media of albana vera.

Fractions		Bacterial cellulose membranes (CB)	
		Alabama	Vinasse
CB	100%	100 ml	0 mL
CBV20	20%	80 mL	20 ml
CBV40	40%	60 mL	40 ml
CBV60	60%	40 ml	60 mL
CBV80	80%	20 ml	80 mL

Source: Prepared by the authors

2.2.6 Sterilization and inoculation

The appropriate proportions of the alaban culture medium and alternative substrates (vinasse and *aloe vera*) were closed with cotton plugs and autoclaved in a vertical autoclave at a temperature of 121°C and pressure of 1.2 kgf/cm2 for 15 min. The same procedure was performed with *aloe vera*.

After being cooled at room temperature, they were placed in a vertical laminar flow microbiological hood that had already been sterilized with 70% alcohol. The UV light was turned on for 15 minutes to ensure that no contamination by other organisms occurred. Once the sterilization time in the dark was over, the inoculation procedure began.

The inoculum was made from a bacterial cellulose membrane grown in an Erlenmeyer flask with 100% Alaban for 7 days. The membranes were cut into 2 cm × 2 cm squares to inoculate the new bottles. The inoculated flasks were incubated in BOD (biochemical oxygen demand) at a temperature of 28 ± 2 °C by static cultivation for 7 days.

2.2.7 Purification and neutralization

To eliminate any traces of bacteria and ensure purification, the bacterial cellulose membranes were washed with 4% NaOH solution (m/v) at 60 °C for 1 hour in a water bath and subsequently washed in distilled water several times until neutralization.

2.2.8 Drying

To characterize the CB membranes, the drying process took place in an oven at a temperature of 50 ± 2 °C for 10 h. The membranes were placed in petri dishes previously lined with baking paper or absorbent paper, and another plate was placed on top to ensure that the dried CBs were stretched. Every 1 h, the sheets of baking paper and/or absorbent paper were changed, ensuring that the membranes did not stick. After drying, the membranes were transferred to designated petri dishes and placed in desiccators for future analysis.

2.2.9 Grinding

The bacterial cellulose membranes, which were already dry, were ground in a Willey knife mill. To standardize the particle size of the samples, they were first passed through a 10 mesh stainless steel sieve and then through a 20 mesh sieve. The CB powders were stored in Falcon tubes for future characterization analyses.

2.2. 10 Mechanical Properties: Tensile Test

To analyze the mechanical properties of the bacterial cellulose membranes, tensile tests were carried out on EMIC and DL3000 equipment from the Solid and Composite Waste Laboratory of FCA/Unesp/Botucatu – RESIDUALL.

These tensile tests were carried out following the ASTM D882 standard with adaptations. To this end, ten oven-dried specimens of each fraction, kept in a desiccator, were cut to 40 mm in length and 15 mm in width, and the average thicknesses were measured with a digital caliper at three different points. A 5000 N (500 kg) load cell was used.

A speed of 5 mm/min and an initial distance between claws of 20 mm were predetermined and standardized before the test

in the Tesc software, a measurement used as the initial length to calculate the percentage elongation.

Subsequently, in Excel spreadsheets, the averages of elongation (%), breaking stress (MPa) and modulus of elasticity (MPa) were calculated from the slope of the initial straight section of the strain *versus* stress curve obtained in the tensile test. .

2.2.11 Infrared (IR) spectroscopy

Analyses of the chemical structures of the CB samples were carried out using a PerkinElmer ® Spectrum Two spectrometer from the Center for Advanced Research in Matology (Nupam) at FCA/Unesp/Botucatu.

This technique determines the structural characteristics of the CB and the functional groups and bonds present. The samples were subsequently dried, ground and stored in Falcon tubes. For reading, the range from 400 to 4,000 cm^{-1} was determined. The generated data were processed using Origin® software.

2.2.12 X-ray Diffraction (XRD)

To analyze the crystallinity of the bacterial cellulose membranes, the samples were processed on a benchtop X-ray diffractometer (D2 Phaser) in the multiuser laboratory at the Londrina Campus of the Technological University of Paraná (UTFPR).

The samples were subsequently dried, ground and stored in Falcon tubes. The analysis was carried out on 9 CB powder samples using the following parameters: Cu-Kα (λ = 1.5406 Å), 0.2 mm slit + knife (Air Anti-Scattering Screen – AAS) 3 mm. The scanning of each sample was between 5° and 40° in the 2θ *range*, and the scanning speed was 0.02°/min. The generated data were exported to Excel spreadsheets, and the graphs were generated with Origin® software.

RESULTS AND DISCUSSION

The values of the mechanical properties are related to the amount of water present in the CB membranes. The tension can be affected by the thickness of the samples due to the inter- and intramolecular bonds in the membrane and the orientation of the fibrils. The different incorporations with the *Aloe vera substrate* presented variations in maximum tension from 0.91 to 2.32 MPa, elongation from 33.7 to 45.7% and elastic modulus from 3.41 to 13.05 MPa. These data are detailed in Table 4. Compared to the control CB, CBB80 presented higher values of elastic modulus, maximum tension and elongation.

The different enrichments with the vinasse substrate showed variations in maximum tension from 0.78 to 1.76 MPa, elongation from 28.9 to 47.9% and elastic modulus from 5.98 to 13.42 MPa; these results are presented in Table 5. Compared to the control CB, CBV40 had a greater modulus of elasticity, and CBV80 had greater elongation (%) and maximum tension (MPa).

The crystallinity index is also a factor that influences the mechanical properties of CB. The presence of type II cellulose may be related to its lower tensile strength (CLARO, 2017). The carbon source can also influence the mechanical properties of CB membranes, although it does not affect the chemical structure (NADZIR *et al.* , 2021) .

Table 4. Mechanical properties of the CB membranes obtained from the incorporation of the Aloe vera substrate into the standard culture media

CB	Modulus of Elasticity (MPa)	Maximum Stress (MPa)	Stretching (%)	Thickness (mm)
CB	13.05 ± 5.69	2.32 ± 1.23	33.7 ± 9.02	0.66 ± 0.13
CBB20	5.95 ± 2.10	0.91 ±0.24	35.1 ± 8.21	0.57 ± 0.14

CBB40	4.87 ± 1.02	0.91 ± 0.05	34.4 ± 6.55	0.45 ± 0.02
CBB60	3.41 ± 0.59	0.91 ± 0.26	45.7 ± 705	0.50 ± 0.06
CBB80	10.83 ± 7.27	1.91 ± 0.58	36.5 ± 12.39	0.20 ± 0.01

CB (100% Alaban)/CBB20 (80% Alaban and 20% *Aloe vera*)/CBB40 (60% Alaban and 40% *Aloe vera*)/CBB60 (40% Alaban and 60% *Aloe vera*)/CBB80 (20% Alaban and 80% *Aloe vera*).
Source: Prepared by the authors

Table 5. Mechanical properties of CB membranes obtained from the incorporation of vinasse substrate into the standard culture media of albanan

CB	Modulus of Elasticity (MPa)	Maximum Stress (MPa)	Stretching (%)	Thickness (mm)
CB	13.05 ± 5.69	2.32 ± 1.23	33.7 ± 9.02	0.66 ± 0.13
CBV20	5.98 ± 1.38	0.78 ± 0.15	36.9 ± 4.62	0.65 ± 0.06
CBV40	13.42 ± 2.26	1.61 ± 0.22	28.9 ± 6.23	0.37 ± 0.05
CBV60	10.15 ± 4.42	1.12 ± 0.42	33.4 ± 7.17	0.35 ± 0.05
CBV80	6.79 ± 1.96	1.76 ± 0.56	47.9 ± 9.05	0.19 ± 0.02

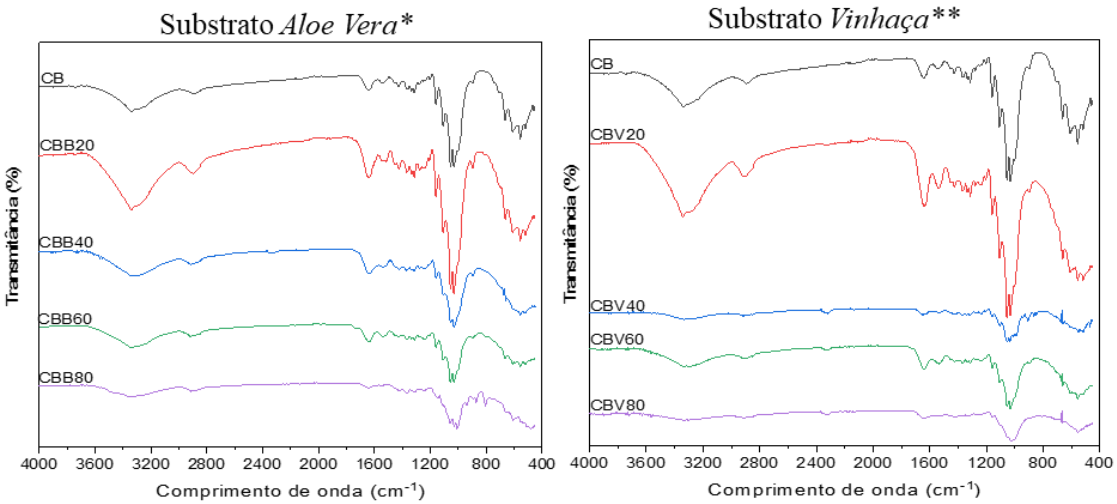
CB (100% Alaban)/CBV20 (80% Alaban and 20% vinasse)/CBV40 (60% Alaban and 40% vinasse)/CBV60 (40% Alaban and 60% vinasse)/CBV80 (20% Alaban and 80% vinasse).
Source: Prepared by the authors

In the spectra of CB, CBB20, CBB40, CBB60, CBB80 and CBV20, the band in the wavelength region between 3352 and 3246 cm^{-1} is related to the stretching vibration of hydroxyl groups (OH) and is characteristic of type I cellulose (REVIN *et al.* , 2020) . As the dilution of the components increases in relation to the medium, it is clear that some specific bands are still present, even if less pronounced, and other absorption bands appear. Second, (STANISŁAWSKA; STAROSZCZYK; SZKODO, 2020)CB IR spectra can also vary in relation to the applied strain, position and intensity of the bands.

The band at 1315 cm^{-1} present in CB, CBB20, CBB40, CBB60 and CBV20 suggests crystalline regions, that is, regions characteristic of bacterial formation membranes, since crystallinity is one of the essential factors in the characterization of CB samples, even if there are noncrystalline regions in the same CB (ZHONG, 2020) .

The IR spectra, presented in Figure 2, showed that the different incorporations, both of vinasse and *Aloe vera*, presented similar results, with changes in relation to the size and intensity of the spectra, mainly in the presence of bands related to cellulose of type I, CB characteristics (GÜZEL; AKPINAR, 2020) .

Figure 2. IR spectra of CB obtained after incorporation of the *Aloe vera* and vinasse substrates



* CB (100% Alaban)/CBB20 (80% Alaban and 20% *Aloe vera*)/CBB40 (60% Alaban and 40% *Aloe vera*)/CBB60 (40% Alaban and 60% *Aloe vera*)/CBB80 (20% Alaban and 80% *Aloe vera*). ** CB (100% Alaban)/CBV20 (80% Alaban and 20% vinasse)/CBV40 (60% Alaban and 40% vinasse)/CBV60 (40% Alaban and 60% vinasse)/CBV80 (20% Alaban and 80% stillage).
Source: Prepared by the authors

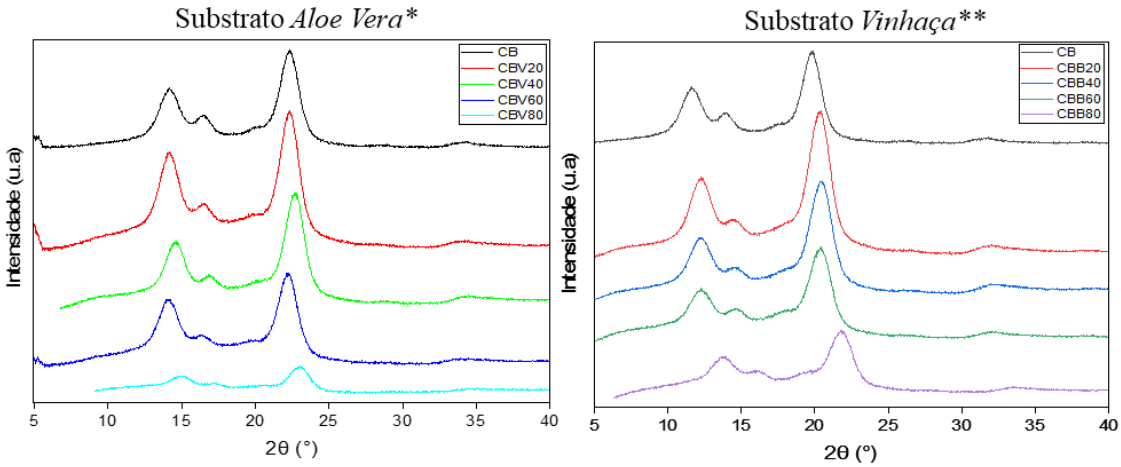
Cellulose I is a mixture of two crystalline forms, Iα and Iβ. Cellulose Iα (triclinic) is predominant in algae and bacteria, and cellulose Iβ (monoclinic), a characteristic of plant cellulose, is found in plants and tunicates. Cellulose I can be converted into cellulose II and III. Cellulose II, a polymorphic material in industry, is considered the most stable form. The difference between cellulose I and II is in the atomic orientation, and the parallel direction of cellulose I is opposite to that of cellulose II (SOUZA *et al.* , 2020a) .

Figure 3 shows the XRD patterns, and in all the samples, which were both enriched with vinasse and *Aloe vera*, cellulose I was identified in the second peak in the region with an angle of 2θ ≅ 14. Among the samples enriched with the *Aloe vera substrate*, CBB20, CBB40 and CBB60 presented peaks predominantly at 12.2° and 20.5°. The peaks at 2θ ≅ 12° and 20° are characteristic of cellulose II and present an antiparallel arrangement (THORAT; DASTAGER, 2018) .

CBV80 enrichment, with a proportion of 80% vinasse inserted into the culture medium, exhibited peaks close to 16°. The variation in intensities, mainly between angles of 2θ ≅ 16°, is related to changes in the crystallinity of cellulose (crystalline and amorphous cellulose) and is also associated with the different incorporations of vinasse into the culture medium (GHOZALI; MELIANA; CHALID, 2021) , possibly causing a change in the conformation of cellulose.

Some processes involved in the production of CB can influence the crystallinity of these membranes, such as the cultivation medium (static or dynamic), the carbon source, the pH of the medium, the temperature, the growth time, the drying method and the method of obtaining the CB powder (freeze dryer, ball mill, knife mill, among others) (KHAN *et al.* , 2021) .

Figure 3. CB XRD diffractograms obtained after the addition of the *Aloe vera* and vinasse substrates



* CB (100% Alaban)/CBB20 (80% Alaban and 20% *Aloe vera*)/CBB40 (60% Alaban and 40% *Aloe vera*)/CBB60 (40% Alaban and 60% *Aloe vera*)/CBB80 (20% Alaban and 80% *Aloe vera*). ** CB (100% Alaban)/CBV20 (80% Alaban and 20% vinasse)/CBV40 (60% Alaban and 40% vinasse)/CBV60 (40% Alaban and 60% vinasse)/CBV80 (20% Alaban and 80% stillage).
Source: Prepared by the authors

4 CONCLUSIONS

The results obtained for the different fractions of the substrates evaluated in the analyses point to promising data for the use of vinasse and *Aloe vera* as substrates in culture media for the production of bacterial cellulose.

In the XRD analysis, it was possible to identify characteristic peaks of cellulose I in the vinasse enrichments (CBV20, CBV40, CBV60 and CBV80) and of cellulose II in the *Aloe vera enrichments* (CBB20, CBB40 and CBB60).

Mechanically, the elastic modulus (MPa) and maximum tension (MPa) of the Aloe vera CBB80 membrane were similar to those of the CB membrane (control), while the elastic modulus (MPa) and elongation (%) of the CBV40 membrane were similar.

Morphologically, CB presented a dense microfibril network with no arrangement pattern, which was very similar to that of the control membranes. CBB80 demonstrated a network of microfibrils with *Aloe vera* gel adhered to the surface.

The use of vinasse promotes the reduction of the high costs involved in the production of CB and the valorization of agro-industrial waste in the CB production process. The use of *Aloe vera*, which has therapeutic and/or medicinal properties and is known and described in the literature, allows not only the reduction of costs in the production of CB but also its application in diverse areas, such as medicine, cosmetics and pharmaceuticals.

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