Universidad de Guayaquil
Facultad de Ingeniería Química

Ingeniería Química y Desarrollo
https://revistas.ug.edu.ec/index.php/iqd

Production and characterization of a biopolymer obtained from cocoa agroindustrial wastes (CCN-51)

Producción y caracterización de un biopolímero obtenido a partir de residuos agroindustriales del cacao (CCN-51)

Ing. Cecilia Katherine Uzca Sornoza, MSc * .[1]; Aguilar Siguenza Samantha Mishel[2]; Espín Mayorga Dennis Leandro[3]

Recibido: 22/09/2020 – Recibido en forma revisada: 19/11/2020 -- Aceptado: 09/03/2021
*Autor para la correspondencia.

Abstract
In the present work, the production of a biopolymer using the agroindustrial residues of the cocoa industry CCN-51 and a native isolate of Gluconobacter xylinus was carried out. The cocoa excess was evaluated as the only carbon source in the production medium (F1). In turn, the effect of two sugars in this medium was evaluated, F2 (sorbitol: cocoa excess = 1:1) and F3 (glycerol: cocoa excess = 1:1), for the production of the biopolymer. The biopolymer was characterized by several analyzes such as: scanning electron microscopy (SEM), differential scanning calorimetry (DSC), Fourier-transform infrared spectroscopy (FTIR), traction, humidity, color and sizing of the membranes. The variables were evaluated with an ANOVA statistical analysis. After 15 days of fermentation at a temperature of 35ºC, the characterization was made by SEM, showing a similar characteristic to bacterial cellulose fibers; DSC, obtaining degradation temperatures higher than 190ºC and at 195ºC for formulation 1 and 2, respectively. An FTIR also obtained peaks of the functional groups characteristic of a cellulose. The yield was evaluated based on the production medium (RVP) and carbon source (RFC), where the results were obtained 10.43 g/L and 21.29 g/kg, respectively, corresponding to F2. Through statistical analysis it was determined that the evaluated variables do not present significant differences, therefore the null hypothesis is approved.

Key words
Gluconobacter xylinus, biopolymer, bacterial cellulose, cocoa CCN-51.

Resumen
En el presente trabajo se realizó la producción de un biopolímero empleando los residuos agroindustriales de la industria del cacao CCN-51 y un aislado nativo de Gluconobacter xylinus. Se evaluó los excedentes de cacao como única fuente de carbono en el medio de producción (F1). A su vez se valoró el efecto de dos azúcares en este medio, F2 (sorbitol:excedentes=1:1) y F3 (glicerol:excedentes=1:1), para la producción del biopolímero. El biopolímero fue caracterizado mediante varios análisis como: microscopía electrónica de barrido (SEM), calorimetría diferencial de barrido (DSC), espectroscopia infrarroja (FTIR), tracción, humedad, color y dimensionamiento de las membranas. Posteriormente se evaluaron las variables con un análisis estadístico ANOVA. Pasados los 15 días de fermentación a una temperatura de 35 °C, se realizó la caracterización mediante SEM, presentando característica similar a las fibras de celulosa bacteriana; DSC, obteniendo temperaturas de degradación mayor a 190 °C y a 195 °C para la formulación 1 y 2, respectivamente. También se realizó un FTIR obteniendo picos de los grupos funcionales característicos de una celulosa. Se evaluó el rendimiento en base al, medio de producción (RVP) y fuente de carbono (RFC), donde se obtuvieron los resultados 10.43 g/lt y 21.29 g/kg, respectivamente, correspondientes a F2. Mediante análisis estadístico se determinó que las variables evaluadas no presentan diferencias significativas, por lo tanto, se aprueba la hipótesis nula.

Palabras claves
Gluconobacter xylinus, biopolímero, celulosa bacteriana, cacao CCN-51.

1. Introduction

The polymer industry has been developing for several decades. With advancements in other disciplines such as nanotechnology and biotechnology, and the global interest in reducing industrial pollution resulting from these processes, there has been growing interest in generating biopolymers using agro-industrial waste as raw material.

The uses of biopolymers are varied; in the medical field, they are used as drug coatings; in the food sector, they are utilized as containers for sausages. The medicine and food industries are the most favored areas highlighted by this technological development due to the applications of biopolymers in their functional structure. In developing countries, such as Ecuador, this topic is relatively unknown, as there is limited research related to potential

[1] Universidad de Guayaquil; https://orcid.org/0000-0002-0872-5167; cecilia.uzcas@ug.edu.ec .
[2] Investigador independiente; samantha.aguilars@hotmail.com.
[3] Investigador independiente; dennis_leandro23@hotmail.com

raw materials that could serve as sources for obtaining polymeric membranes.

In a larger percentage, the production of polymers is derived from synthetic origins, with a smaller percentage from plant sources. However, some polymers are being developed from natural sources or from combinations of natural and synthetic origins.

As new raw materials are discovered for the development of polymeric membranes, it is necessary to characterize the films to evaluate the effect of synthesis on the structural properties of the material, properties that directly influence the performance of the films.

At the Faculty of Chemical Engineering at the University of Guayaquil, research has been presented on polymers obtained from natural sources, specifically of animal origin, as well as combinations with plant-derived polymers [1].
Other research involves bacterial origins, using the species Gluconobacter xylinus, isolated from the tea fungus [2].

The production of bacterial cellulose has become of great interest in the last six years. Studies on bacterial cellulose biopolymers have focused on the synthesis of films, evaluating the sources or substrates present in the culture medium, the characteristics related to the structure and strength of the film, and its applications.

Most literature reports that the application of these biopolymers is oriented toward the food and health sectors.

Considering that Ecuador is the only country that produces and exports large quantities of Colección Castro Naranjal (CCN51) cacao, and that processing it generates waste, these residues are mostly used for generating biofuels, or for producing liquors. There is hope to utilize this waste by applying the synthesis of bacterial cellulose for the production of a biopolymer.

In this work, the use of industrial cacao surplus is proposed as a new raw material for the production of a polymeric membrane. It is expected that the generated product will represent a valuable source of material with structural properties that allow for subsequent application, and that the cacao CCN51 waste will signify an attractive source for generating the biopolymer.

Ecuador is one of the countries with the highest cacao production worldwide; for this reason, several studies have been developed focusing on the utilization of the surplus from this fruit to generate products. The surplus has been used as raw material for studies in various fields, such as food, pharmaceuticals, agriculture, among others, producing nectar, alcoholic beverages, cosmetics, herbicides, and more [3]. However, there is currently no research that generates a material using this type of raw material.

Cacao exudate and placenta contain a high percentage of sugars, which are utilized in various industries; for example, the food industry takes advantage of these sugars for liquor production, where fermentation occurs, allowing the liquor to have a characteristic aroma and flavor of cacao [4].

On another note, among the main biopolymers studied with the aim of creating biodegradable materials are: polyhydroxyalkanoate (PHA), cellulose (CAB), polylactic acid, and starch plasticization [5].

There are studies on the production of bacterial cellulose synthesized by bacteria belonging to the genera Acetobacter, utilizing sugars such as glucose, sucrose, and fructose, among others, as well as ethanol [6].

1.1. Colección Castro Naranjal (CCN-51)

The "Manual of Cacao Cultivation CCN-51" from the National Institute of Agricultural Research (INIAP) states that the cacao CCN-51 crop belongs to the species Theobroma cacao, one of the most well-known for its importance in the economic and social field of the country [7]. The CCN-51 clone is a medium-sized plant that can grow up to 20 meters tall, with simple, entire green leaves. The flowers of this plant grow in small clusters, and the cacao fruit is commonly referred to as a pod. This pod has a variable shape, ranging from spherical to elongated elliptical, and its coloration varies from yellow to orange and purple. The weight of the fruit ranges from 15g to 1kg, with an average of 50 beans connected to a column called the placenta inside the pod, as illustrated in Figure 1. The cacao bean or seed is covered by a sweet, acidic pulp known as aril or mucilage, as seen in Figure 2 [7].

The hydrolyzed cacao mucilage is also known in the industry as “exudate.” During fermentation, it provides the substrate for some microorganisms essential for

developing the precursors to chocolate flavor, which are expressed later in the roasting process. Additionally, fresh pulps can be frozen and used as flavoring in other fields, mainly in the food sector, to flavor ice creams and yogurts [8].

Fig. 1. Cocoa Pod
Source: [8]

Fig. 2. Cocoa Mucilage or Pulp
Source: [8]

In 2017, the Agricultural Public Information System (SIPA) highlighted in its cocoa production report that during that year, Ecuador had a total production of 289,102 tons of national and CCN-51 cocoa, representing 43% of the production in the first half of the year (January-June), and the remaining 57% in the second half (July-December). The report indicates that national cocoa production was 72% for the CCN-51 variety, while the national variety accounted for 28%. The weighted yield was 0.65 t/ha for CCN-51 cocoa, where it was noted that annually about 20% of the total production is waste, including husk, granza, crushed material, and maguey, and that the generated waste has no application in any activity [9].

1.2. Gluconobacter xylinus

Gluconobacter xylinus is found in fruits, naturally, in a state of decay. It is an obligate aerobic bacterium, rod-shaped, which can be found either alone or grouped in chains. The movement of some strains during cellulose production can be observed since some have flagella; it has a variable size greater than 2 µm, as shown in Figure 3 [10].

It has been used, so far, for the production of bacterial cellulose using fruit waste as substrates; it is mostly present at the air-medium interface where its presence can be verified if it produces a cellulose film. According to research conducted by S. Masaoka, 1993 [11], its production under optimal conditions is 36 g/d m^2, with a yield of 100%, using agro-industrial waste from tropical fruits as a substrate.

According to Chávez, L. et al., 2004 [12], the film of bacterial cellulose positions the bacteria at the air/liquid interface, facilitating the acquisition of the necessary oxygen for its growth. Bacterial cellulose not only serves as a flotation mechanism, but it also protects the bacteria from UV rays, functioning as a physical barrier. It allows the bacteria to better utilize substrates in the medium since it retains external moisture.

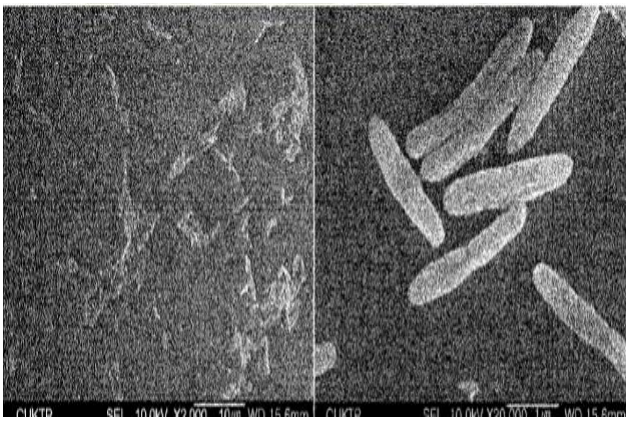

Fig. 3. Microphotograph of Gluconobacter xylinus

Source: [13]

1.3. Bacterial Cellulose

Bacterial cellulose (BC), also known as microbial cellulose, is a polymer primarily synthesized by bacteria of the genus Acetobacter. In nature, bacterial cellulose serves as protection for colonies of microorganisms, shielding them from varying environmental conditions. This membrane prevents the dehydration of natural nitrates present in decaying fruits, retaining moisture near the bacterial cells and, thanks to its low transparency to ultraviolet light, providing protection against the negative effects of solar radiation; it also prevents the colonization of other organisms such as molds and other bacteria in the medium [14].

BC was first discovered in 1886 by J. Brown when observing the formation of a film during acetic fermentations, when a microorganism was isolated that was cultivated under appropriate conditions, producing a translucent gelatinous layer over the liquid, where it was identified through various tests as cellulose since it was formed by the ability of the "vinegar plant" and was named bacterium xylinus, derived from the Greek Xýlinalína, meaning "wood tissue" [14].

1.4. Characteristics of Bacterial Cellulose

Despite the possibility of obtaining cellulose from plant sources, BC is preferred as it maintains the same structure, though it differs in its physical properties. It has been reported that BC, regardless of the raw material used, maintains greater purity, a higher degree of polymerization, crystallinity index, and has greater tensile strength and water retention capacity compared to plant cellulose (See Table 1). BC fibers are 10 times finer than plant cellulose, making the material very porous [15].

Table 1.
Comparison of Different Characteristics of Bacterial and Plant Cellulose.

Characteristic	Bacterial Cellulose	Plant Cellulose
Chain Dimensions (mm)	7.0-8.0 x 10^{-5}	Pine: 3.0-7.5 x 10^{-2} Birch: 1.4-4.0 x 10^{-2}
Degree of Polymerization	16,000-20,000	13,000-14,000
Young's Modulus (MPa)	4.9	Cotton: 0.085
Specific Density	0.99	Cotton: 0.19

Source: [2]

1.5. Effects of Medium Components and Growth Factors of Gluconobacter xylinus.

Cellulose production and growth depend on the optimal combination of various nutrient sources, including carbon, nitrogen, and mineral salts. High quantities of bacterial cellulose are produced by different strains on various substrates such as glucose, fructose, mannitol, sucrose, and glycerol [14]. The initial concentration of carbon sources in cellulose production has a significant effect since the formation of gluconic acid as a byproduct in the culture medium will lower the pH of the culture and ultimately decrease cellulose production. Studies have been conducted examining cellulose yield with different initial concentrations of sugars at 6, 12, 24, and 48 g/L, in which the consumption was 100, 100, 68, and 28% of the initial concentration, respectively [16].

1.6. Cultivation Media Used

In 1954, Hestrin and Schramm (HS) created a culture medium composed of: 0.5% peptone, 0.5% yeast extract (hydrogen source), 2% glucose (carbon source), 0.27% disodium phosphate, 0.115% citric acid, and an initial pH value of 6. This culture was carried out under the following conditions: at 30°C in static and agitated culture samples for 2 to 5 days. This type of culture has been used as a reference for determining other studies because efforts have been made to increase yields and reduce production costs (See Table 2). Higher yields have been observed in static media productions [17].

Table 2.
Yields, Methods, and Conditions of Static Production Media for Bacterial Cellulose.

Carbon Source	Yield (g/L)	Static Culture Production Technique	Microorganism	Reference
HS Medium	10.12	t: 7 days	*G. xylinus*	[18]

HS Medium with 1% lignosulfonate	16.32	t: 7 days	*G. xylinus*	[18]
Glucose	3.3	t: 15 days	*Komagataeibacter medellinensis*	[19]
Sucrose	3.3	t: 15 days	*Komagataeibacter medellinensis*	[19]
Fructose	0.38	t: 8 days	*Komagataeibacter medellinensis*	[19]
HS Medium nitrogen source with orange juice	6.90	t: 14 days	Acetobacter xylinus	[20]

1.7. Biopolymer

Sarango Y., 2017 [21], argues that natural polymers, also known as biopolymers, are macromolecules synthesized by living organisms that form during the life cycle of organisms.

According to Velde K. and Kiekens P., 2002 [22], biopolymers, like polymers, are macromolecules formed by the covalent union of small molecular units called mers, obtained through a polymerization process from smaller molecules known as monomers.

A biopolymer always interacts with a biological system. The biopolymer is a solid organic or inert type of substance. Some potential interactions between the biopolymer and the living organism have been investigated. Within the research of S. Stupp and P. Braun, several functions of biopolymers are described, including evaluating or correcting some tissue, organ, or function of the organism [23].

The European Committee for Standardization [24], defines biopolymers as bioplastics that have a biomass-based origin; therefore, they are considered renewable and can be biodegradable. They can be found in natural or synthetic forms, or a combination of both. It is important to note that biodegradable plastics do not always originate from biomass, and plastics derived from biomass are not always biodegradable. According to Tharanathan, 2003 [25], atural biopolymers come from four sources: animal origins such as collagen or gelatin, marine origins like chitin and chitosan, agricultural origins like lipids and fats and hydrocolloids: proteins and polysaccharides, and microbial origins (polylactic acid (PLA) and polyhydroxyalkanoates (PHA)). Due to their high biodegradability rates and excellent physicomechanical properties, PHAs and PLAs have proven to be the most widely used today.

The vast majority of biopolymers originating from renewable resources are becoming significant for the plastics industry [26] [27], They can undergo the same industrial processes as conventional plastics, such as blow molding, injection molding, or extrusión [28].

The National Technology Center for Food and Conservation, 2013 [29] , states that biopolymers resemble petroleum-derived polymers in their physicochemical and thermoplastic properties; with one significant difference being that once discarded, biopolymers biodegrade. From this comparison, the substantial advantages of substituting petroleum for polymer manufacturing are established, which would notably reduce environmental pollution.

According to Martínez T. et al., 2013 [30], biopolymers can be classified into two types: those that come from living organisms and those that must be synthesized, with a source derived from a renewable resource.

Biopolymers can be classified into three subgroups according to their source and market: polymers that come from a renewable resource (starch and cellulose), biodegradable polymers based on the derivation of monomers (oils and lactic acid), and biopolymers from microbial synthesis [31] [32].

Technology advances in parallel with the development of new biopolymers. Biopolymers play an important role in the composition of new therapeutic systems and also in tissue engineering [33]. This allows biopolymers to be situated in the medical and pharmaceutical fields.

Polymers with biodegradable characteristics derived from a renewable natural source can be used as coatings and edible films, as well as in the manufacture of nanofibers for the development of functional foods. This positions biopolymers within the food industry [34] [35].

1.8. Characteristics of Biopolymers

Biopolymers exhibit characteristics similar to synthetic polymers, but unlike synthetic ones, biopolymers come from renewable raw materials and their production is clean. Bacterial-origin biopolymers depend on the enzyme (synthase) and various substrates that determine whether they are short-chain or medium-chain polymers. Among their characteristics are: thermoplasticity, elasticity, and flexibility, depending on their composition. The chemical composition of the synthesis varies their properties. They have stability against UV radiation and low water permeability. Their mechanical properties, as well as their biocompatibility, will depend on the mixture or combination with other polymers and the use of other enzymes. All the above allows for a wide range of applications for this material [36].

1.9. Applications of Biopolymers

Biopolymers are replacing synthetic polymers in various uses due to their rapid degradation and zero pollution. The biodegradability of biopolymers has allowed them to be positioned in eco-friendly manufacturing, making them a competitive alternative to non-sustainable products [37]. Biopolymers are used as replacements for synthetic food packaging, but they represent a slightly higher cost [38]. In biomedical applications, biopolymers target drug release control as a solution to pH changes, enabling transport and diagnosis of cancer [39].

2. Materials and Methods

This research is of an experimental nature because it tests different formulations using cacao waste; and exploratory because it will characterize the material and detail its properties, thus facilitating the evaluation of the performance of the obtained material.

To obtain this biopolymer, the bacteria “Gluconobacter xylinus” was isolated from kombucha tea and activated in a modified H-S liquid medium (Carbon source; absence of citric acid) with cacao waste, resulting in the generation of the pre-inoculum.

The aforementioned H-S liquid medium was used to prepare the inoculum, which contains 10 ml of the pre-inoculum and the various modified and formulated H-S media with different carbon sources: cacao, sorbitol, and glycerol. These formulations were carried out in three replicates, and fermentation of each sample was performed, generating bacterial cellulose membranes. Once the different membranes were obtained, they were washed and bleached, and then the evaluation of their physical and mechanical characteristics was carried out, and their performance was determined through ANOVA analysis.

The research consists of producing a biopolymer using agro-industrial waste from the cacao CCN-51 and a native isolate of Gluconobacter xylinus as raw material. The carbon sources of the production medium were varied, employing cacao waste, sorbitol, and glycerol. The variations were made such that there was 100% sucrose from the agro-industrial cacao waste in the first formulation; 50% in formulations 2 and 3, in combination with other carbon sources, sorbitol and glycerol, respectively.

Table 3.
Carbon source formulations in the production medium represented as percentages.

Carbon Sources	Formulations		
	1	2	3
Cacao	100%	50%	50%
Sorbitol	0	50%	0
Glycerol	0	0	50%

Once fermentation was completed and the product obtained, the bacterial cellulose samples were evaluated through sensory analysis (color), moisture, dimensions (diameter and thickness), and their respective yield was calculated. Additionally, the samples underwent tensile mechanical testing and physical tests of SEM, DSC, and FTIR, which allowed for a deep characterization of the obtained material.

To evaluate the characteristics of this biopolymer, analyses of scanning electron microscopy (SEM) were conducted based on the ASTM E986 standard; differential scanning calorimetry (DSC) under ISO 11357-3 regulations; tensile testing according to ASTM D882; moisture content following GOST 16932-93 standards; and infrared spectrophotometry (FTIR) in reference to ASTM E1252 regulations; along with color and sizing of the films.

The results obtained were statistically evaluated using a simple analysis of variance (ANOVA).

2.1. Process Diagram

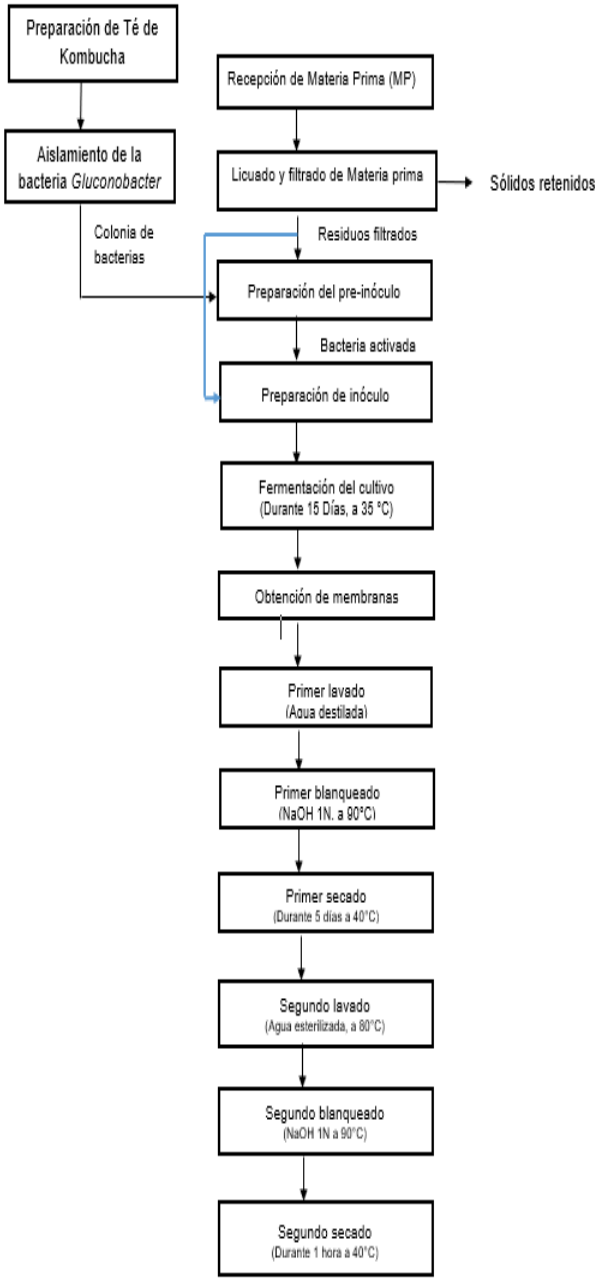

Fig. 4. Preparation of Kombucha Tea

2.2. Methodology for Yield Estimation

Yields were calculated based on the Volume of the Production Medium (RVP) and the weight of the Carbon Source (RFC), expressed in g/L and g/kg, respectively.

To calculate these yields, the masses of the cellulose membranes obtained in triplicate were used, and the yields were calculated by applying the formulas mentioned below:

$$Average\ Production\ Yield = \frac{Mass\ of\ Cellulose\ (g)}{Volume\ of\ Production\ Medium\ (lt)} \quad (1)$$

$$Carbon\ Source\ Yield = \frac{Mass\ of\ Cellulose\ (g)}{Weight\ of\ Carbon\ Source\ (kg)} \quad (2)$$

The yield of cellulose or biopolymer from the culture media was analyzed using a bar chart, which displays the average yields along with their respective standard deviations.

2.3. Methodology for Moisture Analysis

To determine the moisture content of these membranes, a sample of 2 g of the product in its wet state is taken and dried for 3 hours at a temperature of 105 ± 3 ºC. Subsequently, it is allowed to cool in a desiccator until reaching room temperature. This procedure was carried out according to GOST 16932 - 93. The following formula was applied:

$$w = \frac{m - m1}{m} x100 \quad (3)$$

Where:
m: $mass\ of\ the\ sample\ before\ drying.$
$m1$: $mass\ of\ the\ sample\ after\ drying.$

2.4. Methodology for Tensile Analysis

Tensile strength was evaluated for the different membranes according to ASTM D882. This standard assesses tensile properties with the fundamental principle of Young's Modulus, but it was carried out with modifications. The dimensions of the membranes were altered while still adhering to the same principle. The membrane was

supported at both ends, and a weight holder was placed at the bottom, to which weight was added until the membrane broke.

2.5. Methodology for SEM Analysis

The scanning electron microscopy test was conducted at the LEMAT-ESPOL Metrology and Materials Testing Laboratory, following the methodology based on ASTM E986 "Standard Practice for Scanning Electron Microscope Beam Size Characterization.".

2.6. Methodology for DSC Analysis

This analysis was performed at the LEMAT-ESPOL Metrology and Materials Testing Laboratory, in accordance with ISO 11357-3, using the SDT Q600 Thermal Analyzer, EM-004. It was conducted on two samples of different formulations.

2.7. Methodology for FTIR Analysis

This analysis was carried out at the LEMAT-ESPOL Metrology and Materials Testing Laboratory, evaluated based on ASTM E1252. The analysis of the samples was performed using the PerkinElmer Spectrum Version 10.4.2.

For the subsequent results of the FTIR graph, the functional groups that are intended to be found in cellulose should be considered. These functional groups maintain a range of values according to the spectrum, as shown in the following table.

Table 4.
Interpretation of FTIR Spectra.

Links	Type of Vibration	Wavelength Range (cm-1)
C-H	Alkanes (stretch)	3000 - 2850
	-CH3 (bend)	1450 and 1375
	-CH2- (bend)	1465
	Alkanes (stretch)	3100 – 3000
	(out of plane)	1000 - 650
	Aromatics (stretch)	3150 – 3050
	(out of plane)	900 - 600
	Alkyne (stretch)	3300
	Aldehyde	2900 – 2700
O-H	Alcohols, free of phenols	3650 – 3600
	Carboxylic acid	3400 – 2400
C≡C	Alkyne	2250 – 2100
C-O	Alcohols, esters, ethers, carboxylic acid, anhydrides	1300 – 1000

Source: [40]

2.8. Methodology for Mass Balance

A mass balance was conducted to determine the total amount of raw material obtained, applying the filtration operation, using average weights of the parts of interest (mucilage, placenta, and leachate) from 5 cacao pods of the CCN-51 variety. The following equation was used to perform the mass balance:

Waste Balance

$$Study\ Waste = Solid\ Waste\ (Retained) + Liquid\ Waste\ (Filtrate) \quad (4)$$

Cultivation Medium Balance

$$\text{Mass of Medium H} - \text{S} = \text{Mass of Cultivation Medium} * \text{Concentration of Medium H} - \text{S} \quad (5)$$

$$\text{Masa de } residuos\ filtrados = \text{Masa del } medio\ de\ cultivo * \text{Concentracion de residuos filtrados} \quad (6)$$

$$\text{Mass of Filtered Residues} = \text{Mass of Cultivation Medium} * \text{Concentration of Filtered Residues} \quad (7)$$

$$\text{Mass of Sorbitol} = \text{Mass of Cultivation Medium} * \text{Concentration of Sorbitol} \quad (8)$$

3. Results

Table 5 presents a statistical summary, evaluating the variables for each of the formulations, where we observe the obtained values of the mean, standard deviation, and coefficient of variation.

The statistical summaries indicate that the standard deviation of the data obtained concerning the mean for each of the formulations is high for the RVP and RFC variables. Meanwhile, this deviation is low for moisture and traction. Similarly, the relationship between the mean size and the variability of the variable (coefficients of variation) shows high values for RVP and RFC and low values for moisture and traction, allowing us to conclude that production, and therefore RVP and RFC, may have been affected by external factors, such as variations in fermentation temperatures (due to electrical instability in the facilities); a factor that can inhibit the growth of Gluconobacter xylinus and, in turn, its ability to produce bacterial cellulose. This is due to the difference in the masses obtained in production for each formulation, as reported by Jaramillo et al., 2012 [41]. On the other hand, for moisture and traction, the coefficients of variation report low dispersion; therefore, we assume that the methodologies used to obtain the data for the variables are reliable, as explained by Castro, 2015 [42].

Table 5.
Statistical Data of the Evaluated Variables

RVP Statistics				
Formulation	**Count**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
F1	3	5,90262	3,19657	54,1551%
F2	3	10,4436	7,52525	72,0558%
F3	3	0,277452	0,247674	89,2673%
Total	9	5,54124	6,0149	108,548%
RFC Statistics				
Formulation	**Count**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
F1	3	12,8443	6,95586	54,1551%
F2	3	21,2958	15,3449	72,0558%
F3	3	0,578647	0,516543	89,2673%
Total	9	11,5729	12,3456	106,676%
Moisture Statistics				
Formulation	**Count**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
F1	3	68,7833	2,58666	3,7606%
F2	3	67,4667	3,72201	5,51681%
F3	3	---	----	-----
Total	9	45,4167	34,1426	3,7606%
Traction Statistics				
Formulation	**Count**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
F1	3	1200,0	264,575	22,0479%
F2	3	1300,0	50,0	3,84615%
F3	3	----	----	----
Total	9	833,333	640,8	76,896%

The obtained data were evaluated in a box-and-whisker plot, which is useful for making graphical comparisons between the studied variables. The analysis of the tabulated data and the graphs allows us to accept the null hypothesis, indicating that there is no significant difference between the means of the variables of each formulation and their respective yields [42].

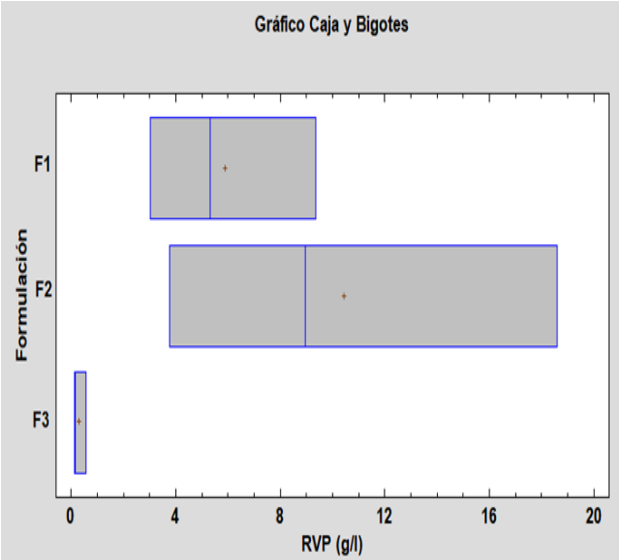

Fig. 5. Box and Whisker Plot for the RVP Variable

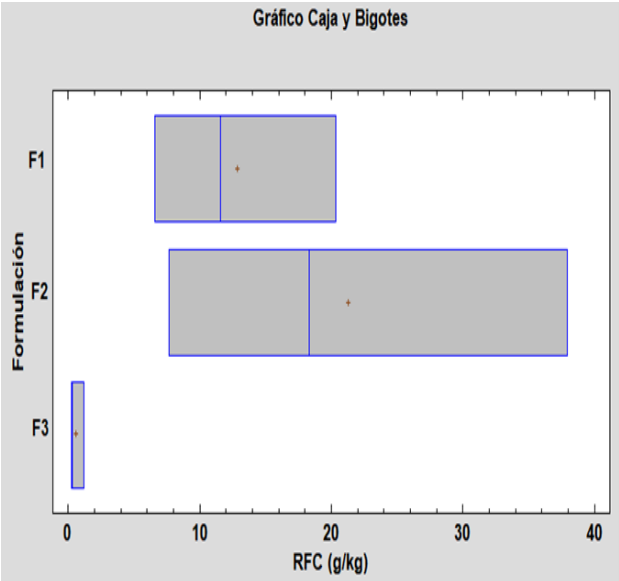

Fig. 6. Box and Whisker Plot for the RFC Variable

There are no statistically significant differences between means, with a 95% confidence level; we can say that the moisture and traction variables are not significantly influenced by the formulations, but F2 shows a better result..

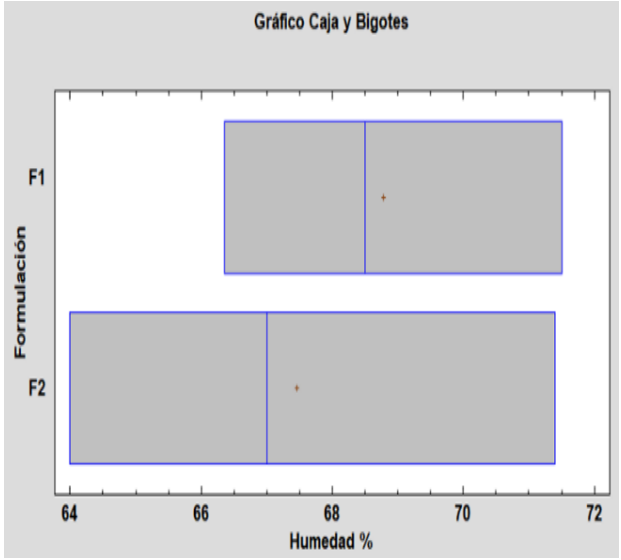

Fig. 7. Box and Whisker Plot for the Moisture Variable

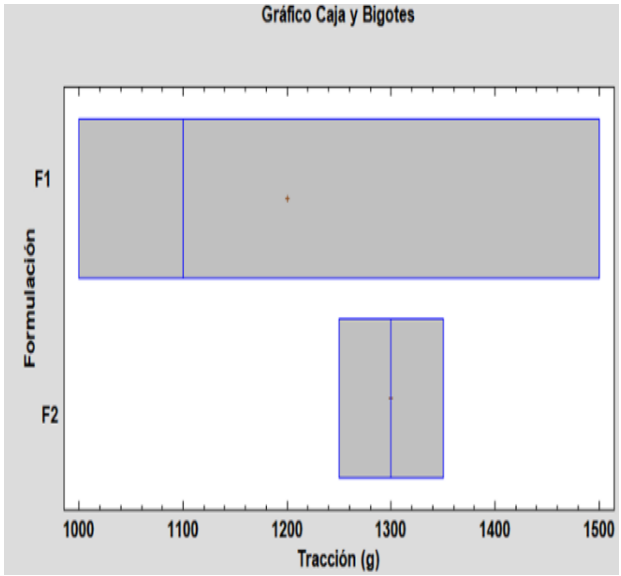

Fig. 8. Box and Whisker Plot for the Traction Variable

3.1. Balance of Raw Material

The balance of raw material was performed to determine the total amount of liquid waste required, resulting in a need for 2,457 g of liquid waste.

In the culture medium balance, the amount of liquid waste necessary for each of the formulations is observed,

resulting in the following: for F1, 409.5 g of filtered waste is required; for F2, 204.75 g of filtered waste and 204.75 g of sorbitol; for F3, 204.75 g of filtered waste and 204.75 g of glycerol.

4. Conclusions

- The bacterium Gluconobacter xylinus was isolated from kombucha tea, and its presence was demonstrated by performing a Gram staining procedure, which confirmed that they had the same morphology.
- Production was carried out, and the physicomechanical characteristics of the obtained biopolymers were determined based on the different formulations presented.
- The methodology applied in the process was optimal for obtaining a biopolymer from agro-industrial waste of cocoa CCN-51, specifically through static fermentation at 35°C for 15 days, followed by washing, bleaching, and drying.
- Using the proposed methodology, a biopolymer was obtained by applying three different formulations concerning carbon sources, which include F1 (cocoa waste); F2 (cocoa waste: sorbitol); and F3 (cocoa waste: glycerol), where F2 achieved the best yield by production volume (10.43 g/L) and by carbon source (21.29 g/kg), when compared to its alternative sources.
- As a result, F2, which is the combination of cocoa waste (55%) and sorbitol (45%), demonstrated greater strength, higher yield, greater production, and degradation temperature compared to the other studied formulations. The averages of these variables were higher than those of the other formulations, though they did not maintain significant differences, as confirmed by statistical analysis.
- FTIR analysis verified that cellulose was obtained, as it presents the characteristic functional groups of cellulose.
- SEM analysis confirmed that the structure of the membranes exhibited characteristics similar to those of cellulose when compared to its alternative source.
- It is concluded that cocoa type CCN-51 is an organic waste that can be utilized for the production of bacterial cellulose (biopolymer)

References.

[1] Bustamante R ; Peralta M, «Caracterización De Biopolímero Obtenido A Partir,» *Universidad de Guayaquil*, pp. 51- 62, 2018.

[2] Carreño L.D. , «Efecto de las Condiciones de Cultivo y Purificación sobre las Propiedades Fisicoquímicas y de Transporte en Membranas de Celulosa Bacteriana,» Bogotá, Colombia , 2011.

[3] Quimbita, F., Rodriguez, P., Vera, E., «Uso del exudado y placenta del cacao para la obtención de subproductos,» *Departamento de Ciencia de Alimentos y Biotecnología*, vol. 26, pp. 8-15, 2013.

[4] Rodriguez P. Quimbita F., «Aprovechamiento del exudado y la placenta del cacao (Theobroma cacao) para la producción de una bebida alcohólica de baja concentración y elaboración de nectár.,» Quito, 2008.

[5] Pillai, K., «Nanocompuestos de celulosa bioinspirados,» Virginia Polytechnic Institute and State University, Blacksbrug, Virginia, 2011.

[6] Santos, S; Carbajo, J; & Villar, Juan., «Celulosa Bacteriana,» *ResearchGate*, 2015.

[7] C. Suárez Capello, M. Moreira Duque y J. Vera Barahona, "Manual del Cultivo de Cacao N°25", Estación Expeimental Tropical Pichilingue: INIAP, 1994.

[8] Arteaga Y., «Estudio del desperdicio del mucílago de cacao en el cantón Naranjal,» *ECA. Sinergia*, vol. 4, pp. 54-55, 2013.

[9] SIPA, Ministerio de Agricultura y ganadería, 2015.

[10] Gama M.G, Bacterial NanoCelullose: A Sophisticated Multifunctional Material, 2012.

[11] S. Masaoka, T. Ohe y N. Sakota, «Production of cellulose from glucose by Acetobacter xylinum,» *Journal of Fermentation and Bioengineering*, vol. 75, pp. 18-22, 1993.

[12] L. Chávez, M. S, M. Contreras y E. E., Celulosa bacteriana en Gluconacetobacter Xylinu; biosíntesis y aplicaciones, Mexico, 2004.

[13] Jeonju, «Jeonju- Korea Patente nº WO2013073722». Korea Patente WO2013073722, 2013.

[14] Dufresne, A. T, Biopolymer Nanocomposites processing, properties and applications, New Yersey: John Wiley & Sons, 2013.

[15] Shoda, M. S., «Recent advances in bacterial cellulose production.,» *Biotechnol, Bioprocess Eng*, pp. 1-8, 2005.

[16] Chawla P.B., «Microbial Cellulose: Fermentative Production and Applications,» *Food Technol. Biotechnology*, pp. 107-124, 2013.

[17] Hestrin & Schramm, «Factors affecting production of cellulose at the air/liquid interface of a culture of Acetobacte xylinum,» *J. gen. Microbiol*, pp. 123-129, 1954.

[18] Keshk, S & Sameshima, K., «Influence of lignosulfonate on crystal structure and productivity of bacterial cellulose in a static culture,» *Enzyme and Microbial Technology* , 2006.

[19] Ramirez C, Castro M, Osorio M, Taborda M, Gomez B , «Effect of different carbon sources on bacterial nanocellulose production and structure using the low pH resistant strain Komagataibacter Medellinensis,» *Materials (Basel)* , 2017 .

[20] Kurosumi A, Sasaki, Chizuru, Yamashita, Yuya & Nakaruma, Y., «Utilization of various fruit juices as carbon source for production of bacterial cellulose by Acetobacter xylinum,» *Carbohudrate Polymers* , pp. 333-335, 2009.

[21] Y. Sarango, «Extracción y acracterización de un bipolímero natural a partir del cáliz de Physalis Peruviana L.,» Loja, 2017.

[22] K. Velde y P. Kiekens, Polymer Testing, vol. 21, 2002, pp. 433-442.

[23] S, Stupp; P, Braun, Role of Proteins in Microstructural Control:Biomaterials, Ceramics & Semiconductors,, vol. 277, Science, 1997, p. 1242.

[24] I. J. Quiñones, «Vigilancia Tecnológica Aplicada para Identificar las Tendencias Tecnológicas en los Biopolímeros y Plásticos Biodegradables,» *Revista Informador Técnico*, p. 54, 2009.

[25] Tharanathan, Biodegradable films and composite coatings: past, presente and future, vol. 14, 2003, pp. 71-78.

[26] J. Philp, R. Ritchie y K. and Guy, «Biobased plastics in a bioeconomy,» *Trends Biotechnol*, vol. 31, pp. 65-67, 2013.

[27] B. DiGregorio, «Biobased performance bioplastic:Mirel,» *Chem Biol*, pp. 1-2, 2009.

[28] M. Valero, Y. Ortegon y Y. Uscategui, «Biopolimeros: Avances y Perspectiva,» pp. 172-173, 2013.

[29] CTC-CSIC;Centro tecnologico nacional de la conserva y alimentacion, «Biopolimeros,» *Agro Waste*, p. 1, 2013.

[30] C. R. Morin, Martinez-Tenorio y L. A.-M. Vigil, «Biopolimero y su integracion con polimeros convencionales como alternativa de empaque de alimentos,» *Temas selectos de ingenieria de Alimentos*, pp. 42-45, 2013.

[31] L. Yu, K. Dean y L. li, «Polymer blends and composites from renewable resources, Progress in Polymer Science,» vol. 6, nº 31, pp. 576-602, 2006.

[32] M. V. Uscategui y Y. O. Yomaira, «Biopolímeros: Avances Y Perspectivas,» *Dyna*, vol. 80, nº 181, pp. 171-180, 2013.

[33] S. s. Mata y J. D. Y, «Expanding Fron-tiers in Biomaterials,» *MRS Bulletin*, vol. 30, pp. 864-873, 2005.

[34] H. V. Velasco y H. A. Reinado, «Biopolímeros Naturales Usados En Empaques Biodegradables,» *Temas Agrarios*, vol. 12, nº 2, pp. 5-13, 2007.

[35] Grupo de Investigación de Biopolímeros en Alimentos (GIBA) , «seminario internacional sobre Innovaciones y tendencias en la aplicación de biopolímeros,» de *Innovaciones y tendencias en la aplicación de biopolímeros en la industria de alimentos en seminario*, Chile, 2015.

[36] García, L. A., Betancur, J. A. N., Ramírez, A. M. F., & Arboleda, L. M. H, « Estudio de la síntesis de biopolímeros de origen microbiano,» *Revista QUID*, nº 25, 2015.

[37] Ospina, S, «Biopolímeros y su aplicación en medio ambiente,» *Revista Colombiana de Biotecnología*, vol. 17, nº 2, pp. 5-6, 2015.

[38] Cruz-Morfin, R., Martínez-Tenorio, Y., & López-Malo, A., «Biopolímeros y su integración con polímeros convencionales como alternativa de empaque de alimentos,» *Temas de Selección de Ingeniería de Alimentos*, vol. 7, nº 2, pp. 42-52, 2013.

[39] Neira-Carrillo, A., Muñoz, D. Y., Zazzali, P. A., Marini, Y. A., Vilches, S. V., & Palma, R. E, «Encapsulación de biomoléculas usando polímeros naturales:“un nuevo enfoque en la entrega de

fármacos en medicina",» *Avances en Ciencias Veterinarias,* vol. 28, nº 2, p. 31, 2013.

[40] Villar A., «Caracterización de polímeros materiales.,» *Agilent technologies,* vol. 1, 2012.

[41] P. Jaramillo Ruben L, I. Tobio Wladimir y P. Escamilla José, «Efecto de la sacarosa en la producción de celulosa por Gluconacetobacter xylinus en cultivo estático,» *MVZ Córdoba,* vol. 2, nº 17, pp. 3004-3013, 2012.

[42] M. C. Riascos., «Determinación de las condiciones de cultivo para la producción de celulosa por la bacteria nativa Gluconacetobacter medellinensis,» *Título de Magister en Biotecnología,* pp. 40-45, 2015.