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Obtaining bacterial cellulose from kombucha by replacing black tea with coffee husk tea.

Obtención de celulosa bacteriana a base de kombucha por sustitución de té negro por té de cáscara de café.

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Received: 29/05/2021 – Revised received: 14/07/2021 -- Accepted: 21/10/2021
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Abstract
The present investigation sought to obtain bacterial cellulose by inoculation of the Gluconacetobacter xylinus fungus present in the Kombucha drink by means of a static culture and with the replacement of black tea with an infusion of coffee husk. Two concentrations of 20 and 10 % sugar and 5 treatments of 0, 25, 50, 75 and 100 % substitution of coffee husk tea were evaluated. The rectangular reactors used were 1.5 L capacity. These were kept at room temperature for 15 days until the cellulose harvest. Both the fermented liquid and the membrane were evaluated for physical, chemical, mechanical and functional properties. The analysis of variance was carried out and the results indicate that the highest production of cellulose was with 10 % sugar and with a 75 % replacement of the coffee husk infusion. This treatment showed a biweekly yield of 25 % cellulose production, with better hardness properties and firm structure compared to the other treatments.

Keywords
Kombucha, bacterial cellulose, culture media, black tea, coffee husk.

Resumen
La presente investigación buscó obtener celulosa bacteriana por inoculación del hongo Gluconacetobacter xylinus presente en la bebida Kombucha mediante un cultivo estático y con la sustitución del té negro por una infusión de cáscara de café. Se evaluaron dos concentraciones de 20 y 10 % de azúcar y 5 tratamientos de 0, 25, 50, 75 y 100 % de sustitución del té de cáscaras de café. Los reactores rectangulares usados fueron de 1.5 L de capacidad. Estos se mantuvieron a temperatura ambiente durante 15 días hasta la cosecha de la celulosa. Tanto el líquido fermentado como la membrana fueron evaluados en las propiedades físicas, químicas, mecánicas y funcionales. Se realizó el análisis de varianza y los resultados indican que la mayor producción de celulosa fue con 10 % de azúcar y con una sustitución del 75 % de la infusión de cáscaras de café. Este tratamiento mostró un rendimiento quincenal de 25 % de producción de celulosa, con mejores propiedades de dureza y estructura firme respecto a los demás tratamientos.

Palabras claves
Kombucha, celulosa bacteriana, medios de cultivo, té negro, cáscara de café.

1. Introduction

Polymers were once ideal materials, but over time they caused damage to the environment [1]. This led to the search for similar ecological alternatives in terms of functionality [2]. Recently, bacterial cellulose has been studied in various sciences [3]. In medicine it helps in the treatment of burns and in food engineering it replaces synthetic coatings [4]. Although many bacteria produce biopolymers [5], Acetobacter Xylinum is considered the best for cellulose production due to its symbiotic behavior with other microbes, thriving in any culture medium [6].

This research aims to replace black tea with tea made from coffee husks in the production of Kombucha beverages. The objective is to achieve adequate cellulose production and reduce the costs of the bacterial culture medium, add value to coffee waste and benefit the environment. In recent decades, cellulose production has been an empirical process focused on generating structural polymers from

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plants to meet an exponentially growing demand [2]. The consequent greenhouse effect, pollution, oxygen reduction and scarcity of resources led to the search for an alternative polymer. Although the applications of cellulose polymers cannot be reduced, research began to focus on finding other naturally available sources. The reuse of coffee by-products in cellulose production can be a sustainable and environmentally friendly solution [7].

The manufacture of plastics, the high costs and limited availability of synthetic cellulose were also problematic for industries due to the use of chemicals and structural degradation. To address these problems, the search for a new and more effective material began, free of chemicals and pollutants that could damage the environment [8]. In 1953, Doctors Hestrin and Schramm investigated the production of cellulose from the Acetobacter Xylinum bacterium. Their work was based on previous studies demonstrating that these bacteria could synthesize cellulose from non-proliferative cells in oxygenated environments using a carbon substrate. Hestrin and Schramm developed an ideal culture medium, defining glucose as the basic carbon source for microbial growth. They also recommended nutrients such as yeast extract and low pH compounds, nicknaming the medium "HS" [9].

Subsequent research increased cellulose yield by optimizing substrate and nutrient ratios [10]. Doctors Hassid and Barker proposed using sucrose for being chemically more stable than glucose, while maintaining the other nutrients. Zhou [11] varied the proportions of glucose and sucrose along with corn liquor and sulfated compounds as nutrients. Zhou [12] also proposed using low concentrations of ethanol while maintaining their medium. The studies of Cakar, [13] Gomes [14] and Pacheco [15] replaced the basic carbon source with agricultural waste to enhance cellulose without external nutrients [16]. Coffee husk can replace black tea, providing functional alkaloids such as caffeine and theobromine as an inexpensive residue. Illana [17] found that Kombucha black tea was an ideal culture medium, since tea provides optimal substrates and alkaloids such as theine that aid in the development of the cellulosic membrane during incubation.

1.1. Bacterial cellulose

Bacterial cellulose is an extracellular polymer synthesized by fermentation of microorganisms, mainly the Acetobacter bacteria. This microbe was first discovered in 1886 by Adrian J. Brown as "a translucent gelatinous membrane that grows on the surface of the culture medium". However, other bacteria such as Rhizobium, Agrobacterium and Sarcina can also produce celluloses [18]. Theories on why microbes produce biopolymers include: aerobes generate a membrane to remain at the air-medium interface, microbes produce cellulose to avoid UV rays and bacteria form a "framework" to protect themselves from external threats such as heavy metals while better assimilating nutrients by diffusion [19]. Bacterial cellulose is chemically like plant cellulose but has important structural differences [20]. A key distinction is the greater purity of microbial cellulose, which lacks associated hemicellulose and lignin [14]. Bacterial cellulose forms cellulose Iα and Iβ crystals [21].

1.2. Coffee

The coffee plant is a lush shrub of the genus Coffea, from the Rubiaceae family. It thrives in tropical and subtropical equatorial climates. Its composition is a fleshy fruit in the shape of a cherry that contains high percentages of alkaloids [22]. The husks surrounding the seed, called parchment, and mucilage have a high sugar content and low in methylxanthines compared to the cherry. The pulp is the largest portion and determines the varieties by its chemical structure [23].

Fig. 1. *Part of a coffee bean*
Source: [24]

1.3. Nutrients in coffee husk.

Among the benefits and properties of coffee husk tea, they have a low caffeine content, antioxidant capacity related to flavonoids, a total phenolic compound concentration like other teas. It has high prebiotic and antioxidant activity, even greater than that of vitamin C [25].

Coffee husk represents approximately 12% of the dry coffee bean, and is constituted as an excellent source of cellulose, lignin, pentosans, silica, ash, and other compounds in smaller proportions [26].

The physical and chemical composition of coffee husk according to Fonseca [27] is:

Table 1. Physical and chemical composition of coffee husk

Biomass	Moisture (%)	Volatiles (%)	Ash (%)	C (%)	H (%)	O (%)	N (%)
Coffee husk	10,1	82	1,2	50,3	5,3	43,8	0,39

Source: Fonseca [27]

1.4. Composition of Coffea Arábica coffee

As mentioned above, the Caturra variety from the Bourbon genus retains most of the chemical characteristics of its predecessor, Coffea Arábica [28]. On the other hand, more than a hundred volatile compounds are formed during coffee roasting that contribute to the aroma and flavor during infusions, such as: acetic acid, caffeic acid, acetaldehydes, ketones, compounds from furfural, furans and esters that mostly disappear due to the high temperatures to which the coffee beans are usually subjected [29]. Furthermore, coffee drinks reach an optimal pH that ranges between 4.9 and 5.2 making them slightly acidic.

The following table shows the nutritional information of Arabica coffee as an infusion:

Nutritional value per 100g of coffee infusion.

Table 2. Chemical composition of Coffea Arábica

Nutrient	Value	Units
Carbohydrates	0	
Fats	0,02	g
Proteins	0,12	g
Water	99,40	g
Caffeine	40	mg
Thiamine (vit. B1)	0,014	mg
Riboflavin (vit. B2)	0,076	mg
Niacin (vit. B3)	0,191	mg
Pantothenic acid (vit.. B5)	0,254	mg
Vitamin B6	0,001	mg
Vitamin E	0,01	mg
Calcium	2	mg
Magnesium	3	mg
Manganese	0.023	mg
Phosphorus	3	mg
Potassium	49	mg
Zinc	0,02	mg

Source: Taken from USDA, 2018

Although most of these components are concentrated in the pulp, the husk contributes 14.76% furfural, and the rest is complemented by 41.86% lignin in addition to fats and pentoses. In addition, nitrogen, and phosphorus concentrations of 0.39% and 28 mg can be found respectively [30].

For every 100 g of fresh coffee cherry there is the following composition [23], where 6% corresponds to the husk.

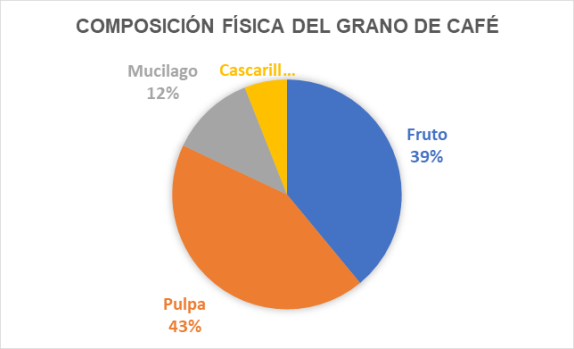

Fig. 2. *Physical composition of the coffee bean*
Source: Merchán & Tigre

1.5. National production

The following are the coffee growing areas in Ecuador distributed by the Coffea Arábica and Coffea Robusta genres. In addition, it should be noted that the Caturra genus comprises more than 90% of the existing varieties in the country [31].

Table 3. Production of Coffea Arábica and Coffea Robusta in Ecuador

Provinces	Coffea arábica (ha)	Coffea robusta (ha)
Manabí	70.050	0
Santa Elena	1.800	0
Guayas	11.195	425
Los Ríos	4.770	6.610
El Oro	9.730	0
Carchi	195	0
Imbabura	300	0
Pichincha	1.300	1.300
Santo Domingo	0	5.300
Cotopaxi	2.000	1.600
Tungurahua	0	0
Bolívar	3.410	3.780
Chimborazo	880	0
Cañar	370	0
Azuay	420	0
Loja	29.345	0
Sucumbíos	0	17.320
Orellana	0	20.000
Napo	120	4.800
Pastaza	150	0
Morona Santiago	290	120
Zamora Chinchipe	6.350	0
Galápagos	1.100	0

Source: Aguilar [32]

In 2016, crops of the Coffea Arábica genus decreased compared to 2015, having 3,905 tons. However, coffee production in 2017 increased by 12% due to the entry of new coffee plantations with the "Reactivation Project of Ecuadorian Coffee Growing", where the variety was introduced to provinces whose climatic conditions were optimal for its development, Loja and Manabí standing out. In historical data, Ecuador was the fifth largest producer and exporter of coffee worldwide until 2015; however, it currently ranks 28th [33].

2. Materials and methods

The tea substitution was evaluated through experiments. First, black tea was replaced with coffee husk tea at 25%, 50%, 75% and 100%, using 200g of sucrose according to Aguilar and Espín (2019). In the second experiment the sucrose concentration was modified, and the control HB medium was added (0.25% yeast extract, 0.25% calcium sulfate, 0.5% potassium phosphate, 0.83% ammonium sulfate, 0.2% magnesium sulfate) for optimum bacterial growth without altering the cell structure. The constants for both experiments were 15 days of incubation, 25±3°C temperature, initial pH of 3.5 and amount of inoculum. The containers were identical in shape, volume, and material for each treatment. The first experiment had 5 treatments with 3 replicates each in 5 cultures. The second had 5 treatments with 3 replicates in 2 cultures.

Table 4. Experiment 1 conditions. 20% sugar.

Identifications E1	**Tea infusion substitution (%)**				
Treatment designation	T1	T2	T3	T4	T5
Black tea concentration (%)	100	75	50	25	0
Concentration of coffee husk infusion (%)	0	25	50	75	100

Source: Merchán & Tigre

The reagents used for the second experiment control were reagent grade and their formulation was made according to the HB medium content.

Table 5. Experiment 2 conditions. 10% sugar

Identifications E2	**Tea infusion substitution (%)**					
Treatment designation	Control (HB)	T1	T2	T3	T4	T5
Black tea concentration (%)	0	100	75	50	25	0
Concentration of coffee husk infusion (%)	0	0	25	50	75	100
HB medium concentration	100	0	0	0	0	0

Source: Merchán & Tigre

2.1. Raw material analysis

The coffee and tea raw materials were evaluated for their moisture content according to the NTE INEN 1114 standard and ashes through gravimetry using the TAPPI T 211 ash test standard for cellulose. It was performed in 3 replicates.

2.2. Finished product analysis

The fermented liquid and the cellulose membrane are considered as the finished product. The liquid was evaluated for: pH and soluble solids content. While the cellulose was evaluated for: wet basis weight, dry basis weight, daily and biweekly yields, moisture, ashes, thickness, tension, permeability, absorption, determination of cellulose percentage, FTIR spectrum characterization, scanning electron microscopy (SEM).

2.3. Daily and biweekly yields.

For the biweekly yield calculation, the grams of dry cellulose membrane were taken and divided by the amount of substrate added per liter. Said substrate varied in weight according to experiment 1 and experiment 2.
The following formula was used.

$$Biweekly\ performance = \frac{Cellulose\ mass\ in\ dry\ membrane\ (g)}{Sucrose\ mass\ (g)} * 100\% \quad (1)$$

The daily yield of the culture medium was calculated by dividing the result of Eq. 1 by the number of planting days (15 days).

$$Daily\ performance = \frac{Biweekly\ performance}{sowing\ days} * 100\% \quad (2)$$

2.4. Moisture determination

It was carried out through the gravimetric method by weight difference, according to the NTE INEN 1114 standard [34]. It consisted of drying a container with its lid for 1 hour in the oven at the product drying temperature. Using tweezers, the capped capsule was transferred to the desiccator and allowed to cool for 5 to 10 minutes. The container was weighed with the lid (m1). The sample was placed in the aluminum container and recorded (m2). The sample with the uncovered container and lid were placed in the oven at 105°C for 4 hours. After this time, the capped sample was removed from the oven and cooled in a desiccator for 5 to 10 minutes. The drying procedure was

repeated until the variations between two successive weighings were constant. It is recorded (m3). The determination was performed in triplicate.

The product moisture was expressed as a percentage with Eq 3:

$$\% H = \frac{[m2] - [m3]}{[m2] - [m1]} \times 100 \quad (3)$$

Where:
H = Moisture
$[m1]$= Empty container weight with lid.
$[m2]$= Mass of container with lid and sample before drying.
$[m3]$ = Mass of container with lid plus sample after drying.

2.5. Ash determination

The procedure of the TAPPI T 211 standard for cellulose in the ash test was used [35]. Therefore, 1 gram of dry sample films was taken in a previously weighed crucible, its initial weight was measured, and it was calcined in a muffle furnace at 525 °C. The crucible with the ash sample was allowed to cool in a desiccator and then its final weight was recorded.

The ash percentage was calculated using Eq 4:

$$\%Ash = \frac{[(C_r + C_e) - C_r]}{(C_r + M_u) - C_r} \times 100 \quad (4)$$

Where:
$[C_r]$= Crucible mass
$[C_e]$= Ash mass (final)
$[M_u]$= Sample mass (initial)

2.6. Water absorption capacity

The test was performed according to the method described by Joaquín [36]. Cellulose sheets of 2 cm x 2cm were evaluated for 30 min, to determine how much water, they absorb. To do this, the weight of each cellulose sheet (M1) was recorded using an analytical balance. In a 50 ml beaker with distilled water, the sheet was placed and kept under constant stirring for 3 hours. The sheet was removed with tweezers to a watch glass. The new weight (M2) was recorded. They were analyzed in triplicate.

$$Water\ absorption\ capacity = \frac{\mathrm{M2} - \mathrm{M1}}{\mathrm{M1}} \mathrm{x}\ 100\% \quad (5)$$

Where:
M2 = Wet sample weight.
M1 = Initial dry sample weight.

3. Results

3.1. Morphological identification of the Acetobacter xylinum bacterium.

According to illustrations 5 and 6, several colonies of elongated bacilli with various structures are observed. In both images a violet - fuchsia coloration can be seen, which allows us to infer that these bacteria are Gram negative belonging to the genus "Acetobacter", whose groupings are given by means of long chains and ovoid in shape.

In figure 4, a 10X magnification was made using the optical microscope with low light intensity. Figure 3 shows a 40x magnification, where the structured morphology of the bacterial genus can be better appreciated.

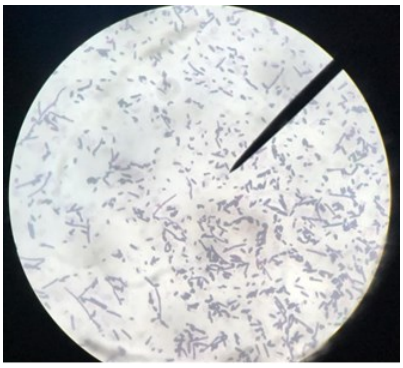

Fig. 3. *Shows a 40x magnification, where the structured morphology of the bacterial genus can be better appreciated.*
Source: Merchán & Tigre.

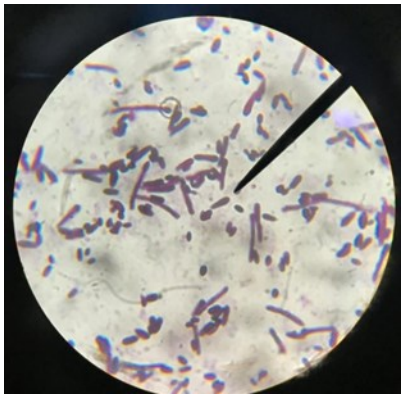

Fig. 4. *Optical microscopy of the Acetobacter xylinum bacterium at 10x magnification.*
Source: Merchán & Tigre

3.2. Raw material results

The moisture and ash results are presented in Table 6, it is observed that the ash values of the coffee husk are like those obtained by Manals [24]. The moisture value presents a dry raw material free of possibility of contamination by fungal growth.

Table 61. Result of moisture and ashes of black tea and coffee husk

	Moisture (%) and ashes (%) of the raw material							
	*C C1	CC 2	CC 3	X±S	**T N1	TN 2	TN 3	Average
% Moisture	10.08a± 0.13	10,07a± 0.06	10.39a± 0.61	10,075± 0,007	6,14a± 0.05	6,13a± 0.04	6,12a± 0.24	6,13 ± 0,009
% Ashes	1,37a± 0.467	1,71b± 0.418	2,04c± 0.1621	1,706± 0,335	4,56a± 0.15	4,67a± 0.03	4.70a± 0.04	4,685± 0,067

*CC represents coffee husk, ** TN represents black tea
*Equal letters indicate that there is no significant difference between them at a significance level of 0.05.
Source: Merchán & Tigre

3.3. Finished product analysis

Two products were obtained because of Kombucha fermentation, one is the fermented liquid and the other is the cellulose membrane.

Figure 5 shows the variation in results with reference to pH and soluble solids of the fermented Kombucha syrup. The pH value between the E1 treatments is constant, as are the pH values of E2. The lowest pH value is equal to 2.20 ± 0.50, which corresponds to the E2 control, while the highest pH value is 3.25 ± 0.27 and corresponds to treatment T3 of E1. According to Lestari [10] the values of an optimal pH for the growth of cellulose producing bacteria are between 2.0-3.0, indicating that, under these conditions, microorganisms develop better.

According to figure 5, the T1 soluble solids value is greater than the values of T2, T3, T4 and T5 of experiment 1; while the values of the treatments of Experiment 2 are not significantly different. The soluble solids (SS) value of T1E1 is greater than that of the other treatments of E1, which does not contain the coffee husk infusion. Therefore, the higher the dissolved solids value means, the greater the presence of sucrose and the lower consumption by the microorganisms and thus the lower cellulose production. That is, the carbon source or sucrose is better assimilated in the treatments where the coffee husk infusion is present.

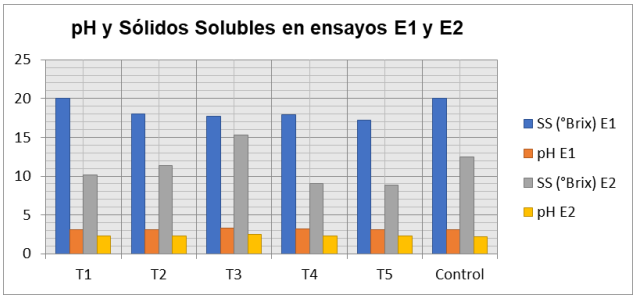

Fig. 5. *pH and soluble solids (SS) of E1 and E2*
Source: Merchán & Tigre

This shows that the T5 of both experiments, which contains an infusion only of coffee husk, exhibits the highest ash content of 7.56% while the lower ash value of 3.23% corresponds to T1 tea infusion only, with significant difference between these treatments. This result allows us to infer that a higher concentration of coffee produces cellulose membranes with higher mineral and inorganic components.

Table 7. Composition of cellulose membranes: moisture, ashes, and thickness of dry membranes

		Treatments				
Experiments (E)	Parameters	T1	T2	T3	T4	T5
1	Moisture (%)	17,21a ± 0,87	10,38 b ± 0,21	10,41 b ± 0,76	18,11 a ± 2,33	10,14 b ± 0,68
	Ashes (%)	3,23a ± 1,49	5,72a b ± 2,31	6,34a b ±1,95	4,81a b ±0,84	7,56b ± 0,57

2	Thickness (mm)	0,16 a ± 0,02	0,26b ± 0,01	0,26b ± 0,001	0,73c ± 0,04	0,38d ± 0,02
	Moisture (%)	12,614a± 0,47	17,65b ± 0,189	14,04 4b ± 0,306	17,02a ± 0,284	18,648b ± 0,289
	Ashes (%)	3,098a ± 0,75	5,992 ab ± 0,75	4,616 ab ± 1,30	4,214 ab ± 0,635	7,56b ± 0,883
	Thickness (mm)	0,694a ± 0,15	0,766 b ± 0,29	0,533 b ± 0,076	0,733 c ± 0,12	0,493 d ± 0,15

* Equal letters determine that there is no significant difference between them.
Source: Merchán & Tigre

The thickness of each membrane allows it to behave in a specific way, being less flexible as the thickness is greater. T4 presents a firmer and more solid structure where it can be inferred that there was a higher degree of polymerization by the cellulose.

Figure 6 shows the moisture, ash, and thickness networks of the finished membranes of both E1 and E2, in each treatment. The moisture content in E1 of treatments T1 and T4 does not vary significantly between them, however, it is significantly different from the values of treatments T2, T3 and T5. The membranes were subjected to the same drying treatment, however; the highest moisture value of the T4 membrane with 18.11% indicates that it should be dried additionally since otherwise it would be vulnerable to yeast and fungal growth.

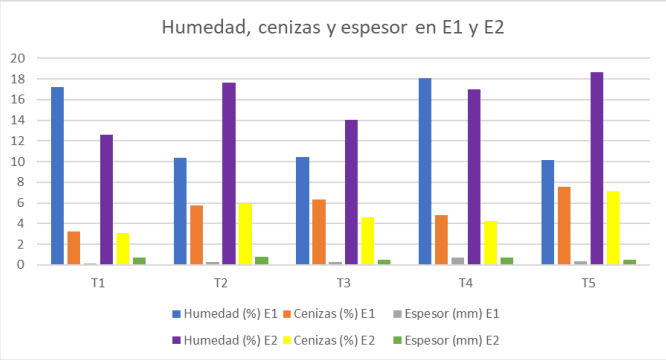

Fig. 6. *Moisture, ashes and thickness of membranes*
Source: Merchán & Tigre

Table 8. Cellulose production result of experiment 1

Cellulose production 200 gram crop					
	T1	**T2**	**T3**	**T4**	**T5**
RDMS (%)	0,19a ± 0,01	0,20a ±0,01	0,25ca ±0,01	0,53b ±0,003	0,29c ±0,003
RDMH (%)	4,78a ±0,13	5,01a ±0,13	7,12ac ±0,10	9,93b ±0,17	7,87c ±0,55
RQMS (%)	2,88a ±0,61	2,98a ± 1,43	3,67ac ±0,73	8b ±1,22	4,31c ±0,38
RQMH (%)	71,67a ±1,89	75,17a ±1,89	106,83ac ±1,44	149,00b ±2,60	118,00c ±8,26
PMS (g)	5,75a ±0,41	5,95a ±0,40	7,33ac ±0,34	15,99b ±0,11	8,62c ±0,09
PMH (g)	143,33a ±3,79	150,33a ±3,79	213,67ac ±2,89	298,00b ±5,20	236,00c± 16,52

* Equal letters indicate that there is no significant difference between them at the 0.05 significance level.
Source: Merchán & Tigre

Table 8 shows the cellulose production values, whether as daily performance, biweekly, wet, or dry weight of E1. This table indicates that the cellulose production of T1, T2 and T3 does not differ between them, while with respect to treatments T4 and T5 if there is a significant difference between these treatments.

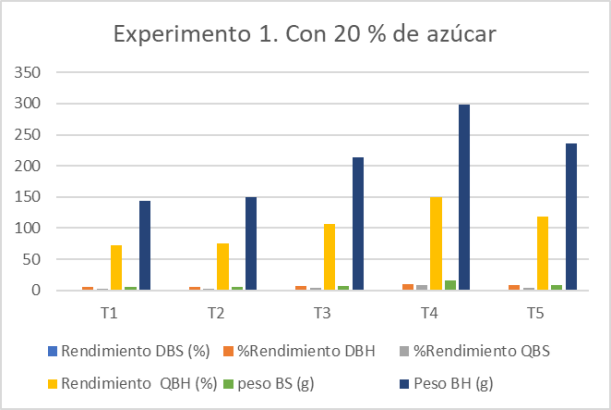

Fig. 7. *Experiment 1 using 200 grams of sugar*
Source: Merchán & Tigre

Treatment 4 exhibits the highest cellulose production results, while the result of treatment 3 is similar to that of treatment 5.

The production of T1 and T2 are similar and although T2 and T3 do not show significant differences, the increase in biweekly and daily production is observed due to the inclusion of higher coffee husk infusion content. The positive effect of the coffee husk infusion in kombucha begins to be analyzed from the 50% substitution, however, it is observed that when 100% is substituted, the resulting cellulose value decreases, so it is convenient to maintain a percentage between 50 to 75% black tea infusion to obtain better results. The average performance value of T4 is

higher than the 6% performance value presented according to Zhou [11], while the other tests exceed the 3% performance obtained by Joseph [37].

Although the general statistical analysis was performed by a two-way ANOVA, the cellulose percentage analysis comprises a one-way ANOVA, which is the cellulose percentage for each treatment performed. Through the Tukey Test it is known that in the third culture of experiment 1, the cellulose percentage of treatments T1 (62.83 ±0.31), T3 and T5 express that T1 is significantly different from T3 and T5 (Figure 7). However, between T3 and T5 there is no significant difference, which allows us to infer that with respect to T4 there would be no significant difference in this parameter, since its value will be between both treatments. The high values of cellulose purity in treatments with the inclusion of coffee husk tea indicate the favorable effect of the addition with respect to the one that does not have it.

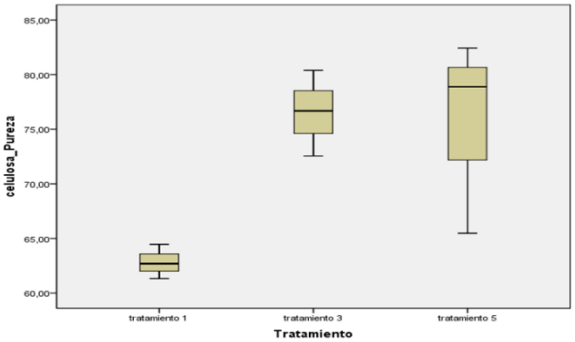

Fig. 8. *Cellulose percentage*
Source: Merchán & Tigre

It could also be indicated that as a greater proportion of coffee husk is included in the culture medium as an infusion, the cellulose membranes have a better purity.

The water absorption capacity was independent of the tea or coffee husk infusion content, however; the value of the T4 and T2 films are similar, so it is stated that these membranes have a greater capacity to receive and contain external fluids such as water. This value is reciprocal to the wet membrane weight of treatment 4, indicated in the table. According to Costa [38] a biopolymer has a water absorption capacity of 80 to 85%, similar values to those obtained in this research.

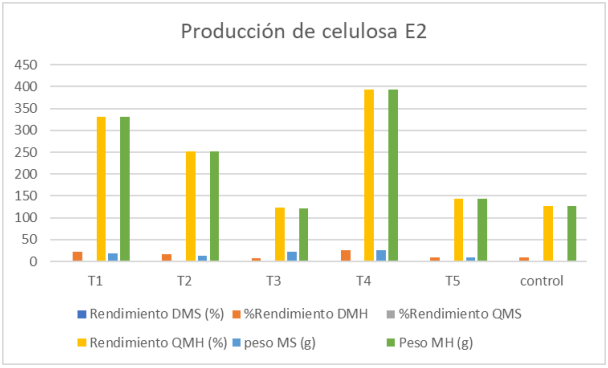

Fig. 9. *Cellulose production. Experiment 2*
Source: Merchán & Tigre

Cellulose production in E2 or with 10% sugar varies with respect to E1. According to the two-factor analysis of variance shown in Table 9, there is a significant difference between E1 and E2 in performance at a confidence level of 95%.

Table 2. Two-factor Anova. Biweekly performance

Dependent variable: Biweekly performance					
Source	Type III sum of squares	gl	Quadratic mean	F	Sig.
Corrected model	1998,452a	10	199,845	675,090	,000
Intercept	2594,795	1	2594,795	8765,390	,000
Experiment	1210,847	1	1210,847	4090,323	,000
Treatment	943,764	5	188,753	637,619	,000
Experiment * Treatment	198,718	4	49,679	167,821	,000
Error	6,513	22	,296		
Total	5271,993	33			
Total corrected	2004,964	32			
a. R-squared = ,997 (Adjusted R-squared = ,995)					

Source: Merchán & Tigre

By performing an ANOVA in the experiment (E2), in relation to cellulose production, it is observed that there is a significant difference between the different treatments.

Through the two-factor ANOVA it was observed that in cellulose production there is a significant difference between experiments E1 and E2 at a confidence level of 95%.

Table 3. Comparative evaluation of weight and performance of E1 and E2

Comparative evaluation of weight and performance of E1 and E2					
	E1 and E2	N	Media	Standard deviation	Mean standard error
Weight	With 200 sugar E2	3	15,9939	,06355	,03669

	With 100 sugar E1	3	25,0330	,87413	,50468
RQMH	With 200 sugar E2	3	7,9970	,03176	,01834
	With 100 sugar E1	3	25,0330	,87413	,50468

Source: Merchán & Tigre

According to Table 10, the maximum weight and performance values correspond to treatment T4 experiment 2 and through the T test it is verified that there is a significant difference compared to treatment T4 of experiment 1. Through this, the null hypothesis raised is rejected and the alternative is accepted, in the sense that if there is a significant difference in the cellulose production performance by including the coffee husk infusion.

According to Table 11, the F value of the Levene test indicates that the p value (probability) is less than the significance value 0.05, so the equal variance hypothesis is rejected, and it is analyzed according to unequal variances. Since the p value obtained is less than 0.05, the equality of the hypothesis or equality of treatments in experiments E1 and E2 is rejected.

Table 11. T test of means

Test of independent samples										
		Levene's Test for Quality of Variances		t-test for equality of means						
		F	Sig.	T	Gl	Sig. (bilateral)	Difference in averages	Standard error difference	95% confidence interval of the difference Inferior	Upper
Weight	Equal variances are assumed	13,397	,022	-17,863	4	,000	-9,03913	,50601	-10,44405	-7,63422
	Equal variances are not assumed			-17,863	2,021	,003	-9,03913	,50601	-11,19465	-6,88362
RQMH	Equal variances are assumed	14,522	,019	-33,734	4	,000	-17,03603	,50501	-18,43817	-15,63389
	Equal variances are not assumed			-33,734	2,005	,001	-17,03603	,50501	-19,20345	-14,86861

Source: Merchán & Tigre

The FTIR characterization of the cellulose samples showed typical spectra of plant cellulose.

If a perpendicular is drawn at the wave number corresponding to 1500 cm^{-1}, two regions are obtained in the infrared spectrum plane corresponding to the diagnostic zone on the left side, and the fingerprint zone on the right side.

Within FTIR spectroscopy, the wave band that characterizes cellulose is between 3335 and 3290 cm^{-1} [39]. In all the treatments analyzed, peaks are observed within these values, which shows that all the membranes have a structure equal to plant cellulose but free of lignin. In addition, there are peaks between 1000 - 1100 cm^{-1}, which demonstrates the presence of C-O-C bonds, and whose compounds form the beta glucose molecules that give rise to cellulose [40].
In addition, all cellulose films have spectra at a band length of 1630 cm^{-1}, which is very characteristic in celluloses with a high moisture content, contained in the pulp of the paper [39].

According to Rosma [41], the structure of bacterial cellulose based on Kombucha comprises bands from 891.59 - 1424.18 cm^{-1} comparing it with a commercial microcellulose and corroborating the peaks obtained in all the samples.

The fingerprint zone allows differentiating the structure of each membrane, where treatments 4 and 5 are the only results that show spectra between 1227, 1159 and 1028 cm^{-1}, and whose vibrations belong to the C-O-C, C-OH and β-glucopyranose bonds of cellulose. However, this is not the case in the other treatments, which allows us to affirm that the high concentrations of the coffee husk infusion in the cellulose composition increase the crystallinity and hardness of the membranes [42].

Analyzing the diagnostic zone again, there is no peak between the absorption bands of 1740 - 1850 cm^{-1}, ruling out the presence of acid anhydrides and acyl chlorides whose vibrations are within said wavelength bands [40].

On the other hand, the average wave number corresponding to ketones and carboxylic acids is 1715 cm^{-1}, therefore their presence is equally ruled out within the samples since there is no peak to corroborate their composition [41].

La ausencia de estos compuestos demuestra que se llevó a cabo un buen tratamiento de las membranas al realizar el lavado con hidróxido de sodio al 0,1 N y agua destilada a 80 °C, principio por el cual fue eliminar la presencia de dichos componentes orgánicos [43].

4. Conclusions

According to the analysis of results, the replacement of black tea with coffee husk infusion positively influences the production of bacterial cellulose. T4 (75% coffee husk and 25% black tea) showed the best production yields, with a polymer with better mechanical characteristics than the other treatments, where the tensile strength of said treatment was 85 N `per 45 mm elongation, presented a thickness of 0.788 mm, and the infrared spectra varied from 3400 to 1750 cm^{-1} which is typical of a cellulose membrane. However, despite the 4% ash analysis and its 17% moisture, the substitution with the coffee husk infusion was even more effective with experiment 2 that allowed obtaining the highest yield (25%) and highest purity (87%) of all treatments.

On the other hand, its total absence affects cellulose production performance. This was demonstrated in treatment 5 (100% coffee husk), whose average value was 11%.

The variation in substrate consumption expressed in Brix degrees and the acidity level given in pH for each experiment shows significant difference, however, their values remain constant between treatments. pH values fluctuate between 2 - 2.5, returning the culture medium optimal for the development of the Acetobacter genus.

The production costs to develop the cellulose membrane are below $9 per film including the current VAT. According to Nava [44], the cost of a membrane fluctuates at $11 considering the high cost of the Kombucha inoculum and the substrates of the culture medium. The low price of coffee husk provides a proportional decrease in the added value in obtaining cellulose.

The use of coffee husk in the production of bacterial cellulose will make it possible to add value to this by-product and additionally reduce the production costs of the polymer.

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