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Assessment of Bacterial Cellulose from *Komagataeibacter medellinensis* Derived from Agricultural Waste and its Influence on *Artemia* Larval Culture

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ABSTRACT

The production of bacterial cellulose is an innovative and sustainable biotechnological process that harnesses the extracellular metabolism of bacteria to convert diverse organic substrates into cellulose. These substrates may include sources of renewable raw materials such as agricultural residues. Their feasibility of acquisition provides them with applications in multiple industrial sectors, including the aquaculture industry. Thus, this research aimed to assess the potential of two agricultural residues to produce bacterial cellulose and identify their impact on the development of *Artemia* larvae. In this study, five treatments were evaluated: treatment T1 (control) based on standard culture medium with glucose as the carbon source, treatments T2 and T3 with banana peel extracts at different concentrations (10 and 25%, respectively) as alternative carbon sources, and treatments T4 and T5 with pineapple peel extracts at different concentrations (10 and 25%, respectively) as a second alternative carbon source. These were incubated for 7 days, during which productivity parameters were calculated. It was found that treatment T2 exhibited the highest values in productivity parameters, yield, and substrate conversion rate. This can be attributed to the fibrous composition of the material, which also proved to be an efficient substrate for the growth of *Artemia* larvae, which will be beneficial in the feeding of aquatic organisms.

INTRODUCTION

Bacterial cellulose (BC) is a natural extracellular biopolymer secreted by certain types of bacteria as part of their metabolism. It appears in the form of fibrils of glucan chains linked by hydrogen bonds, forming a fibrous network **(Esa *et al.*, 2014; Reshmy *et al.*, 2021)**. In contrast to plant cellulose, BC lacks lignin, pectin, and hemicellulose, which facilitates its purification and avoids the use of aggressive chemicals, resulting in lower energy investment **(Azeredo *et al.*, 2019)**.

In nature, there are several genera of bacteria capable of producing this type of cellulose, such as *Alcaligenes, Achromobacter, Agrobacterium, Sarcina, Pseudomonas, Aerobacter, Gluconacetobacter, Rhizobium*, and *Azotobacter*; however, the commercially used strain belongs to the *Gluconacetobacter* genus (*Komagataeibacter*) **(Lin *et al.*, 2013)**.

The production of bacterial cellulose is achieved through fermentation processes, which can be carried out under various conditions, whether in bioreactors or in stirred or static vessels, with the latter being the most common, where a membrane is formed at an air-liquid interface **(Wang *et al.*, 2019)**.

The composition of the culture medium is crucial for achieving an efficient production of bacterial cellulose. Often, a commercial medium known as Hestrin-Schramm (HS) is employed, which contains glucose as a carbon source and yeast extract with peptone as a nitrogen source **(Corujo *et al.*, 2016)**. However, positive growth of microorganisms has also been observed in other alternative carbon sources such as agricultural waste. In the case of Ecuador, banana and pineapple peels are identified as potential substrates due to their high content of carbohydrates and fermentable sugars **(Tibolla *et al.*, 2018; Mishra *et al.*, 2023)**.

The bacterial cellulose obtained from this process possesses unique properties that allow its use as a renewable polymer in various fields, such as the food, pharmaceutical, and medical industries, among others **(Klemm *et al.*, 2005)**. Its applications range from food packaging, the production of food hydrocolloids, the development of cosmetics and medical instruments, ethanol production, and even as a key component in electrical and magnetic materials **(Cacicedo *et al.*, 2016; De Oliveira Barnd *et al.*, 2016)**.

In the aquaculture industry, bacterial cellulose presents numerous potential applications such as nutrient or medication encapsulation, contaminant removal, or the construction of aquatic structures. However, its outstanding feature lies in its suitability as a substrate for the optimal cultivation of fish, shrimp, or mollusk larvae due to its high retention capacity and porosity **(Masoomi *et al.*, 2018)**. *Artemia* larvae are small crustaceans used as a food source for shrimp larvae during their early developmental stages. They provide essential nutrients such as proteins, lipids, and minerals crucial for their growth. The inclusion of this food source in their diet enhances the performance in the shrimp farming industry (**Maldonado-Montiel & Rodríguez-Canché, 2005)**.

The aim of this research was to quantitatively compare the potential of two agricultural residues (banana and pineapple peels) as substrates in bacterial cellulose production and to identify the impact of bacterial cellulose obtained from each substrate on the development of *Artemia* larvae. This study seeked to provide a comprehensive assessment of the potential of agricultural residues as substrates for bacterial cellulose production, as well as their impact on aquaculture.

MATERIALS AND METHODS

Bacterial cellulose-producing strain

Bacterial cellulose producers were obtained from homemade vinegar in an HS medium consisting of glucose (20g/ L), peptone (5g/ L), yeast extract (5g/ L), dibasic sodium phosphate (2.7g/ L), and citric acid (1.15g/ L). The pH was adjusted to 6 by adding 1N acetic acid. To prepare petri dishes in Hestrin-Schramm (HS) medium, 15g/ L of agar was added **(Hestrin & Schramm, 1954)**. Selected samples were inoculated onto

plates at a dilution of 10^{-4}-10^{-5} and incubated at 30°C for 72- 96h. Individual colonies on the plates were isolated and inoculated into HS broth (pH 6). Subsequently, the cultures were incubated at 30°C under static conditions for 5 days, and those with a thick gelatinous film were selected. The culture was then purified to obtain pure isolates of bacterial cellulose producers. The strain was identified as *Komagataeibacter medellinensis* by comparing the 16S rRNA database using the BLAST algorithm in GenBank.

Collection of agricultural waste and nutritional analysis

Samples of pineapple and banana peels were collected from the market and farms near the city of Milagro, Guayas province, Ecuador. The nutritional composition of these peels was evaluated in samples previously dehydrated at a temperature of 70°C for 24 hours, following standardized analytical procedures. The ash, fat, protein, and carbohydrate content were determined using the methodology established by **Horwitz and Latimer (2005)**. Crude protein content was analyzed using the Kjeldahl methodology, employing a conversion factor of 5.9. The quantity of carbohydrates was calculated using the formula: 100 - (% of protein + % of fat + % of ash). Moisture content was determined by applying the formula: ((weight of wet substrate - weight of dry substrate) / weight of dry substrate) x 100.

Obtaining aqueous extracts from agricultural residues

Aqueous extracts were obtained from previously collected pineapple and banana peels. For the pineapple peel extract, 400g of peels were squeezed, and 80ml of water were added to the extracted juice, which was heated in a container. The mixture was stirred until fully cooked. The pH was adjusted to 5 with acetic acid, and the medium was allowed to cool to a room temperature before storing it for later use **(Sardjono *et al.*, 2019)**.

The banana peel extracts were obtained using the methodology described by **Sijabat *et al.* (2019)**. Banana peels were cleaned, cut, weighed, and boiled in water. After cooling, they underwent an extraction and sieving process to remove solid residues and obtain the extract. This extract was heated to 100°C, and its pH was adjusted to 4 with acetic acid. After cooling, it was stored in an airtight container. Both extracts were sterilized in an autoclave and used as mother solutions for banana and pineapple wastes.

Activation of bacterial inoculum

The previously purified strain was introduced into 10ml aliquots of liquid HS medium using an inoculation loop, and incubated at 30°C for 48 hours under static conditions before being inoculated into the production medium.

Production of bacterial cellulose

In this study, five different treatments were tested, where T1 was considered as the control group using the standard HS medium and treatments T2 to T5 included an alternative carbon source. The treatments consisted of the selected carbon source + complementary nutrients. The complementary nutrients included peptone (5g/ L), yeast extract (5g/ L), sodium dibasic phosphate (2.7g/ L), and citric acid (1.15g/ L).

T1: Glucose (20g/ L) + Complementary nutrients (standard HS medium).
T2: Banana peel extract (10% v/ v) + Complementary nutrients.
T3: Banana peel extract (25% v/ v) + Complementary nutrients.
T4: Pineapple peel extract (10% v/ v) + Complementary nutrients.
T5: Pineapple peel extract (25% v/ v) + Complementary nutrients.

Subsequently, the pH of the medium was adjusted to 6 by adding an acetic acid. A specific amount of activated bacteria was inoculated into 250ml Erlenmeyer flasks containing 50ml of production medium, and they were incubated for 7 days in the dark.

Purification and quantification of bacterial cellulose

Following the incubation period, the gelatinous film formed at the air-liquid interface of the medium was purified **(Costa *et al.*, 2017)**. This process involved washing the film with 0.4M potassium hydroxide (KOH) at 80°C for 25 minutes on two consecutive occasions to remove cells adhered to the film, followed by rinsing with distilled water until reaching a neutral pH (7) (off-white color). Finally, the purified films were dried at 100°C until reaching a constant weight to quantify their concentration: bacterial cellulose mass (g)/ volume of culture medium (L).

Productivity parameters of bacterial cellulose

After 168 hours of incubation, productivity parameters were calculated using standardized formulations **(Carreira *et al.*, 2011)**. The cellulose production obtained (g/ L) was calculated by relating the total cellulose produced to the volume of the culture medium (mBC/ V). To calculate the yield (%), the weight of the cellulose was related to the difference between the initial concentration and the residual concentration of cellulose obtained ((mBC/ V)/ (Si-Sf))*100. The substrate conversion ratio α (%) related the difference in cellulose concentrations to the initial concentration ((Si-Sf)/ Si) *100.

Cultivation of *Artemia* larvae

Artemia franciscana cysts were provided by Ecuahidrolizados S.A. and stored at -10°C until use. Subsequently, 0.5g of cysts were hatched under the following parameters: 35g/ L salinity, water temperature at 25± 1°C, pH of 8, constant illumination, and aeration. Upon obtaining newly hatched nauplii, they were transferred to 160L plastic tanks with the same proposed salinity. Simultaneously, the density was adjusted to one organism per 100mL **(Castro-Mejía *et al.*, 2011)**. Larvae were fed with 25g/ L of bacterial cellulose obtained for each treatment. Organisms were cultivated until the twentieth day of growth. After this period, fifty sexually mature individuals were isolated to obtain length data. Preservation method involved the application of acetic acid drops, and length was measured under a dissecting microscope with camera.

Statistical analysis

The data obtained were statistically analyzed using a one-way analysis of variance (ANOVA) to determine significant differences at $P< 0.05$ using the SPSS program (Version 26). The productivity parameters of the bacterial celluloses obtained from each treatment and the measurement of *Artemia* larvae lenght were expressed as mean ±

standard deviation (SD) of five replicates for each treatment. Once the different statistical methods were determined, the Duncan test was established using $\alpha = 0.05$.

RESULTS AND DISCUSSION

Chemical composition of agricultural residues

The chemical composition of banana and pineapple peels showed the presence of various organic compounds, such as carbohydrates, lipids, proteins, and ash, as well as their respective percentage of moisture (Table 1). Banana peel exhibited the highest percentage of carbohydrates compared to pineapple peel due to its fibrous content of lignocellulosic nature. The main carbohydrates present in banana peels are oligosaccharides, starch, dietary fiber, and simple sugars. In turn, they showed the highest percentage of ash content, proteins, and fats **(Hassan *et al.*, 2018)**. The fat content in fruit peels is low due to their high fibrous content, and its slight variations depend on other factors such as ripeness, growing conditions, and fruit varieties. Various studies have found that the protein content of this material is mainly determined by the ripeness of the fruit **(Nisha & Radhamany, 2020)**. The moisture content varies according to the composition of the materials: in the case of banana peel, it is thicker and denser compared to pineapple, giving it hygroscopic properties that retain water and thus increase moisture **(Happi *et al.*, 2007)**.

Table 1. Nutritional composition of the agricultural wastes

Component (%)	Banana peel	Pineapple peel
Carbohydrate	74.14 ± 0.43	54.2 ± 1.19
Ash	6.11 ± 0.07	4.18 ± 0.31
Fat	4.11 ± 0.07	1.77 ± 0.12
Protein	5.57 ± 0.13	3.3 ± 0.15
Moisture	9.6 ± 0.08	6.03 ± 0.06

*All values are means ± standard deviation of the measurement.

Productivity parameters

Table (2) displays the results of the productivity parameter calculations (weight, yield, and substrate conversion ratio) exhibited by the cellulose obtained in the five treatment groups under study.

Table 2. Productivity parameters of the bacterial cellulose obtained in the five treatment groups under study

Treatment	Production (g/L)	Yield (%)	Substrate conversion ratio α (%)
T1	2.31 ± 0.01 ^{A}	12.5 ± 0.02 ^{A}	81.81 ± 0.10 ^{A}
T2	2.52 ± 0.02 ^{B}	7.46 ± 0.01 ^{B}	84.42 ± 0.08 ^{A}
T3	2.38 ± 0.01 ^{A}	6.97 ± 0.01 ^{B}	77.75 ± 0.02 ^{B}
T4	0.73 ± 0.02 ^{C}	3.96 ± 0.01 ^{C}	75.12 ± 0.02 ^{B}
T5	0.54 ± 0.01 ^{C}	2.84 ± 0.01 ^{D}	75.90 ± 0.01 ^{B}

* In each column, the different letters (A, B, C, D) indicate significant differences between the values ($P < 0.05$, Duncan, n= 5).

Based on the results, within the production parameter, it was observed that treatment T2 showed significant differences compared to the other treatments studied. This demonstrates that the treatment based on 10% banana peel extract had the highest production, surpassing the control treatment. Regarding the yield parameter, significant differences were observed in treatment T1, which had the highest value compared to the others, followed by treatments T2 and T3 based on banana peels, which approached this value. In terms of substrate conversion ratio, treatments T1 and T2 showed significant differences compared to the other treatments owing to the proximity of the data between the control and the treatment based on 10% banana peel extract.

Aqueous extracts of agricultural residues have been used in various studies to replace glucose in the conventional HS medium, such as sugarcane bagasse extracts, which typically weigh around 0.3g/ L **(Costa *et al.*, 2017)**. Additionally, several studies have shown that supplementation with complementary nutrients such as nitrogen or citric acid to the carbon source is important for increasing cellulose production, with an increase of up to 1g/ L in cellulose production observed in supplemented media compared to unsupplemented media **(Kurosumi *et al.*, 2009)**. Whereas, components like peptone and yeast extract present in the conventional culture medium serve as nitrogen sources; however, alternative sources such as ammonium sulfate are also being employed **(Lima *et al.*, 2017)**.

The concentration of the carbon source used in the medium also constitutes an important factor for the efficiency of cellulose production, as demonstrated in various studies. A lower substrate concentration has been shown to result in a higher production rate due to nutrient saturation **(Molina-Ramírez *et al.*, 2017)**. This phenomenon occurs since cellulose polymerization generates byproducts such as glycolic acid, which increases the pH in the medium, thereby limiting bacterial cellulose production **(Gullo *et al.*, 2019)**.

Table (3) displays the results of shrimp length supplemented with different bacterial celluloses obtained for each study treatment.

Table 3. Measurement of *Artemia* larvae length in the different bacterial celluloses obtained in the five treatment groups under study over 20 days

Treatment	Day 8	Day 11	Day 14	Day 17	Day 20
T1	4134 ± 0.01 ^{A}	4225 ± 0.03 ^{A}	4967 ± 0.01 ^{A}	5000 ± 2.23 ^{A}	6558 ± 1.12 ^{A}
T2	3050 ± 0.02 ^{B}	3997 ± 0.01 ^{A}	5113 ± 0.02 ^{B}	6080 ± 1.70 ^{B}	7020 ± 2.65 ^{B}
T3	1102 ± 0.03 ^{C}	2045 ± 0.02 ^{B}	3077 ± 0.01 ^{C}	3101 ± 0.01 ^{C}	6899 ± 3.04 ^{A}
T4	2356 ± 0.12 ^{D}	2450 ± 1.04 ^{B}	4185 ± 0.02 ^{A}	5832 ± 0.02 ^{A}	5956 ± 0.01 ^{C}
T5	1124 ± 0.21 ^{C}	3225 ± 1.12 ^{C}	3124 ± 1.34 ^{C}	4878 ± 0.03 ^{D}	5330 ± 0.03 ^{C}

*Length is expressed in µm. Additionally, in each column, different letters (A, B, C, D) indicate significant differences between values ($P < 0.05$, Duncan, n= 5).

Based on the results, it was observed that, on the eighth day of *Artemia* larvae growth, all treatments exhibited significant differences among them, with the highest value noted for the control treatment T1, followed by treatment T2, which utilized 10% of the banana peel extract cellulose. On the eleventh day, treatment T5, based on 25% of the pineapple peel extract cellulose, showed significant differences with a value below

the control treatment. On days 14 and 17, significant differences were observed among all treatments, with the highest values reported for treatment T2. On the final day of analysis, the significant difference was detected for treatment T2, demonstrating superior results in this treatment.

CONCLUSION

In conclusion, banana peel extract has proven to be a potential residue serving as an alternative carbon source for the efficient production of bacterial cellulose. This, in turn, provided a material with superior chemical and biological characteristics that acted as a growth-promoting substrate for *Artemia* larvae, a key feed in the aquaculture industry.

Bacterial cellulose obtained from banana peel extract at the lowest concentration (10%) exhibited improved productivity parameters, with values close to the control treatment (weight of 2.52g/ L and yield of 7.46%). The low concentration of carbon source allowed microorganisms to efficiently utilize nutrients from the medium and grow while carrying out their metabolism. This treatment also resulted in obtaining *Artemia* larvae with larger sizes of up to 7020µm in length.

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