Bacterial Cellulose Production from Acerola Industrial Waste Using Isolated Kombucha Strain

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Abstract

Bacterial Cellulose (BC) production is still considered expensive and challenging for industries. Herein, BC was produced through an acetic acid bacteria isolated from the kombucha consortium and an extract from acerola juice-industrial waste. The isolated bacterium was characterized through different assays (biochemical characterization and 16S rRNA gene) being identified as *Komagataeibacter rhaeticus*. BC production with static cultivation mode by the isolated strain was compared using traditional Hestrin-Schramm (HS) medium and acerola waste (AC) (5% w/v). The kinetic behavior of BC production was slightly higher in the HS medium reaching 2.9 g/L after 12 days of fermentation, while 2.3 g/L in the AC medium. Minor differences were observed between crystallite size and d-spacing, highlighting BC produced by the AC medium with higher crystallinity of 93.9% and two-fold breaking stress resistance in comparison with the conventional medium, with high-temperature stability and economically feasible, promissory results for further application of this synthetized cellulose obtained from industrial residues.

1. Introduction

Bacterial cellulose (BC) is a practical alternative to plant cellulose replacement enabling to use waste as synthesis precursors, resulting in high purity and highly crystalline nanostructured BC (Ruka et al. 2013; Zhang et al. 2018; Abol-Fotouh et al. 2020; Gao et al. 2021). BC is synthesized by gram-negative bacteria in a liquid sugar matrix and is made up of a high molecular weight linear polymer composed of glucose units linked by 1,4-β-glycosidic linkages, resulting in high-order crystalline arrangements (Park et al. 2010; Petersen and Gatenholm 2011; Ruka et al. 2013; Zhang et al. 2018; Kumar et al. 2019; Ye et al. 2019).

BC is one of the new era of Green Chemistry products and can be used in nanotechnological and biomedical applications such as wound dressing (Sajjad et al. 2020; Mao et al. 2021; Wang et al. 2021), sensing/biosensing (Farooq et al. 2020), food ingredients (Aydinol and Ozcan 2018; Guo et al. 2018; Santosa et al. 2020), and packaging material (Salari et al. 2018; Atta et al. 2021). Also, physical, chemical, or enzymatic treatments and functionalization can lead to different crystallinity, purity, surface reactivity, crystalline structure, morphology and therefore broadening the applications landscape, facilitating its use due to its great versatility (Liu et al., 2020; Machado et al., 2018).

The primary cellulose-producing bacterium is *Komagataeibacter xylinus* (Laavanya et al. 2021). However, several other microorganisms have been reported in the literature: *K. pasteurianus* and *K. lovaniensis* (Çoban and Biyik 2011), *K. sacchari* (Gomes et al. 2013), *K. medellinensis* and *K. saccharivorans* (Gayathri and Srinikethan 2019), *K. hansenii* (Güzel and Akpınar 2019), *K. rhaeticus* (He et al. 2020), and *Gluconacetobacter oboediens* (Jahan et al. 2018). Many of the mentioned are isolated using a kombucha consortium, which is a promising source of BC-producing strains.

BC production is still considered expensive, which can be a challenge for industries. Thus, an alternative reported by some authors is to replace the synthetic medium with an inexpensive carbon/nitrogen source (Azeredo et al., 2019; Güzel and Akpınar, 2019). Agro-industrial wastes have been shown an excellent

alternative for BC, because their availability and low-cost, while add value to agri-food residues, in addition BC present superior suitability for fermentation processes (Hussain et al. 2019; Ul-Islam et al. 2020). Agro wastes have been successfully used in the production medium obtaining high production BC yields, such as 3.2 g/L using sugar cane juice and pineapple residue as carbon source (Algar et al. 2014), 2.7 g/L using durian shell as carbon source (Luo et al. 2017), 2.8 g/L using pineapple peel and sugar cane juice as a nitrogen source (Castro et al. 2011).

In our previous study (Leonarski et al. 2021), it was disclosed that acerola waste (5% w/v) fermented by a kombucha consortium showed high BC production (4.0 g/L). A recent study by Devanthi et al. (2021) reported that the symbiotic consortium of bacteria and yeasts (SCOBY) of kombucha could negatively affect BC production, obtaining lower production compared to an isolated strain. Besides showing improvement in the production of BC, the strain isolated from kombucha also presented physicochemical, mechanical, and morphological properties similar to those of BC synthesized by *G. xylinus* and could be a candidate for commercial strain for BC production (Machado et al. 2018; Zhang et al. 2018).

Herein we aimed to isolate the cellulose-producing bacteria from the kombucha consortium and produce BC without interference from other microorganisms, comparing the outcome of both approaches. To the best of our knowledge, this report is the first study that details the BC production in acerola industrial waste with isolated bacteria. For the production of BC, in addition to the medium with acerola waste (AC), experiments were also carried out using the traditional medium Hestrin-Schramm (HS). The morphological and physicochemical properties of BC obtained in both media were evaluated and lastly compared in terms of relative cost of production.

2. Material And Methods

2.1 Materials

Glucose, fructose, cycloheximide, ampicillin, Coomassie brilliant blue, bromocresol green and TEMED (N,N,N',N'-tetramethylethane-1,2-diamine) from Sigma-Aldrich (St. Louis, USA); citric acid, mannitol from Vetec (Rio de Janeiro, Brazil); Agar Potato Dextrose (PDA) and peptone bacteriological from Himedia (Mumbai, Índia); yeast extract, peptone, agar medium and tryptone from KASVI (São José dos Pinhais, Brazil); Congo red from Metaquímica (Jaraguá do Sul, Brazil); sodium acetate, bromothymol blue, calcium carbonate, disodium phosphate from Dinâmica (Indaiatuba, Brazil); sodium lactate and ethanol PA from Neon (Suzano, Brazil).

Acerola waste was obtained from the juice clarification step (without seeds and peels), supplied by a juice-producing industry (Ceará, Brazil). The waste, consisting of residual pulp of immature fruits, was dried in a vacuum oven at 40°C for 48 h and then ground in a knife mill (1.0 mm).

2.2 Methods

2.2.1 Kombucha culture

The kombucha consortium was obtained from a local source in Florianópolis (Brazil) and maintained in sweetened green tea. The tea was filtered under sterile conditions, then 35 g/L of glucose and 35 g/L of fructose, 10% (v/v) liquid broth, and 4% (w/v) of biofilm were added. Fermentation was performed with static cultivation mode at 30°C for 10 days.

2.2.2 Acetic acid bacteria isolation

The BC-producing strain was isolated from the kombucha consortium, using a serial dilution with 0.1 mL of kombucha tea and 0.9 mL of peptone (0.1%). The strains were grown on Luria Bertani (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, 16 g/L agar medium, without the salt component) at 30°C for 5 days. Congo red (0.04 g/L) and Coomassie brilliant blue (0.02 g/L) were added to the medium to visualize cellulose (Römling and Lünsdorf 2004).

Isolation was carried out in HS medium (Hestrin and Schramm (1954): 20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L disodium phosphate, 1.15 g/L citric acid, 15 g/L agar medium containing 500 mg/L cycloheximide to inhibit yeast growth. This procedure was repeated consecutively four times (30°C for 7 days each).

After isolation, yeast extract was used as a selective medium for the growth of acetic acid bacteria at 30°C for 5-7 days; calcium carbonate glucose agar (GYC): 50 g/L glucose, 10 g/L yeast extract, 5 g/L $CaCO_3$ and 2 g/L agar medium (El-Salam 2012).

2.3 Biochemical characterization of acetic acid bacteria

The gram staining technique was conducted, and the evaluation was performed under an optical microscope (Olympus CX21, Zhejiang, China) with a 100x objective lens. The oxidase test was conducted with 50 µL of bacterial suspension deposited on a filter paper strip (previously sterilized). Then, a drop of 1% aqueous solution of TEMED was deposited on the culture. If there is no color change, the result is negative (expected for the *K. rhaeticus* strain); it is positive if the color turns purple. A colony was deposited on a slide for catalase testing. Then, a drop of 3% (v/v) hydrogen peroxide was deposited on the strain. If bubbles appear, the result is positive (expected for the *K. rhaeticus* strain); if there are no changes, it is negative (Videira et al. 2007).

Oxidation of ethanol was performed using Carr medium (30 g/L yeast extract, 20 g/L agar, 0.02 g/L bromocresol green, and 2% (v/v) ethanol), incubated at 30°C for 4-6 days (Maal et al., 2010; Mukadam et al., 2016). Oxidation of acetate and lactate followed the methodology of Leifson (1953), with some modifications. The medium was composed of 0.3% peptone, 0.2% yeast extract, 0.2% sodium acetate, 0.002% bromothymol blue, and pH 6.5. Incubation was performed at 30°C for 20 days. Gram-negative strain must present an alkaline reaction, which turns the medium dark-blue.

2.4 Bacteria identification

The identification of bacteria was performed through high-performance sequencing of the V3/V4 regions of the 16S rRNA gene using primers 341F (CCTACGGGRSGCAGCAG) (Wang and Qian 2009) and 806R

(GGACTACHVGGGTWTCTAAT) (Caporaso et al. 2012).

The preparation of the libraries followed a proprietary protocol (Neoprospecta Microbiome Technologies, Brazil), sequenced using the MiSeq Sequencing System (Illumina Inc., USA), V2 kit, 300 cycles and single-end sequencing. Sequences were analyzed using a proprietary pipeline (Neoprospecta Microbiome Technologies, Brazil). In short, all the DNA sequences resulting from the sequencing passed, individually, through a quality filter, based on the sum of the error probabilities of their bases, allowing a maximum of 1% of accumulated error. Subsequently, the sequences were removed from the DNA corresponding to the Illumina technology adapters. The sequences that passed through the initial procedures and showed 100% identity were grouped into phylotypes/clusters and used for taxonomic identification by comparing a database of accurate sequences of 16S rRNA or ITS1 sequences.

2.5 BC membrane production and purification

The pre-inoculum was prepared using a colony of the isolated strain in 10 mL of HS medium, maintained at 30°C for 7 days. Afterward, 10% (v/v) pre-inoculum was added to the HS medium at 30°C for 7 days. This solution was used in the subsequent steps.

The production of BC was performed in an alternative medium using extract obtained from acerola waste (5% w/v) and in HS medium as a control. Acerola extract (AC) was produced by hydrothermal extraction conducted at 121°C for 15 min. The medium was filtered under sterile conditions. Previous analyses verified that the extract contained 0.6 ± 0.01 g/L of glucose, supplemented with 20 g/L of glucose (previously sterilized). HS medium was sterilized at 121°C for 15 min. In both media, 10% (v/v) inoculum was added at room temperature and then distributed in a 6-well cell culture plate containing 10 mL in each well. BC growth kinetics (30°C) was evaluated by measuring the pH and dry weight up to the 12th day at 2-day intervals. The membranes were purified by immersion in 0.1 M NaOH at 90°C for about 1-2 h. Afterward, washing was performed with distilled water at 50°C for 24 h and then every hour until the neutral pH was achieved.

2.6 pH measurement

The pH value was measured in triplicate during fermentation with a pHmeter K39-2014B (Kasvi, São José dos Pinhais, Brazil).

2.7 BC concentration

After purification, BC membranes were frozen for 24 h, and lyophilized (Liotop 101, Liobras, São Carlos, Brazil) for 48 h. The cellulose concentration was expressed as grams of dry weight per liter of medium (g/L).

2.8 Morphological and physicochemical analysis

The morphological characteristics of cellulose were evaluated using Scanning Electron Microscopy JSM 6390LV (JEOL, Tokyo, Japan), coupled to a tungsten electron source, a secondary electron detector, and an accelerated voltage of 10 kV. Fourier transform infrared spectra (FTIR) of lyophilized cellulose were

recorded in a Cary 600 Series (Agilent Technologies, St. Clara, United States), in attenuated total reflectance (ATR) mode using a wavelength range of 4000 to 500 cm^{-1}, with a 4 cm^{-1} resolution and accumulation of 16 scans. The crystallinity was determined by X-ray diffractometry (XRD) MiniFlex600 (Rigaku, Tokyo, Japan), using reflection mode with $Cu_{K\alpha}$ radiation, a voltage of 40 kV, filament emission of 1.5 mA. Each sample was scanned from 5 to 50° 2θ range with a scan speed of 0.05°/step. The interplanar distances (d-pacing), and crystallite size were calculated according to Bragg's law Eq. (1), and Scherrer's formula Eq. (2), respectively:

$$\text{d} - \text{spacing (nm)} = \left(\frac{\lambda}{2 \cdot \sin(\theta)}\right) \quad (1)$$

where θ is the angle between the plane and the diffracted, and λ is the wavelength of the X-rays

$$\text{Crystallite size (nm)} = \left(\frac{0.9 \cdot \lambda}{FWHM \cdot \cos(\theta)}\right) \quad (2)$$

where FWHM is the width of the peak at half the maximum height, θ is Bragg's angle, and λ is the wavelength of the X-rays.

The crystallinity (%) was determined using peak-fitting of Gaussian functions, and calculated according to Eq. (3), described by Mohammadkazemi et al. (2015):

$$\text{Crystallinity (\%)} = \left(\frac{S_c}{S_t}\right) \times 100 \quad (3)$$

where S_c is the sum of the net area, and S_t is the sum of the total area.

Crystal allomorphs (cellulose Iα and Iβ) were analyzed by the Eq. (4) on the basis of Z discriminant function (Wada et al. 2001):

$$Z = 1693 \cdot d_1 - 902 \cdot d_2 - 549 \quad (4)$$

where, d_1 is the d-spacing peak (100), and d_2 is the d-spacing peak (010). $Z < 0$ connotes that cellulose is rich in Iβ form while $Z > 0$ signifies that Iα is the predominant form.

Thermogravimetric (TG) curves of the dried samples were recorded (TA SDT 2960, TA Instruments). Samples were heated in open α-alumina pans from 40°C to 720°C under a nitrogen atmosphere (flow rate: 70 mL/min) at a heating rate of 10°C/min.

Differential Scanning Calorimetry (DSC) analysis was performed in a Jade-DSC (Perkin Elmer) equipped with intercooler system 2P. The samples were equilibrated for 1 min at 25°C after the temperature

increased from 25°C to 400°C at 10°C/min. The nitrogen flow rate was 50 mL/min.

Mechanical properties of wet BC were determined in a texturometer (TA-HDplus, Stable Micro Systems,) using a 500 N load cell. Specimens were 35 mm wide and 35 mm long. The two ends of the test specimens were placed between the upper and lower instrument jaws, leaving a 10 mm sample gap between the two claws. The thickness for each sample was determined using a caliper with an average of three repeated measures randomly along the length of materials, and the instrument split rate was 1 mm/s. Young's modulus, yield, and rupture strength were calculated from each corresponding stress-strain curve. The tensile stress (MPa) and strain at the breaking point of the strip were recorded, and the apparent Young's modulus (MPa) was assessed by the slope of the linear region of the strain-stress curve.

3. Results And Discussion

3.1 Biochemical tests for identification of acetic acid bacteria

The results of the biochemical characterization of the isolated acetic acid bacteria are shown in Table 1. The isolation was confirmed by the halo formed by acidification of the GYC medium (Fig. 1A). Acetic acid bacteria produce halo in the GYC medium due to acid hydrolysis of $CaCO_3$ (Vashisht et al. 2019).

Table 1
Biochemical characterization of acetic acid bacteria isolated from kombucha.

Biochemical assay	Result
Gram staining	Gram-negative
Catalase	+
Oxidase	-
Oxidation of acetate	+
Oxidation of lactate	+
Carr Medium	from green to yellow than green again

The strain was identified as gram-negative (Fig. 1B), catalase-positive (due to the bubble formation according to the methodology), and oxidase negative (no color change was observed in the assay), in agreement with previous reports isolating cellulose-producing bacteria from kombucha obtained by Semjonovs et al. (2017). In addition, gram-negative bacteria produce an alkaline reaction in a medium containing lactate or acetate due to oxidation. This was observed due to the increase in pH and color

change (dark blue) of the medium as the bacteria grew. This set of analyzes and results allows classifying the strain as belonging to the genus *Acetobacter* (Mukadam et al., 2016; Thanh, 2019).

The classification was also confirmed by growth in Carr medium, generally used to differentiate strains of the genus *Acetobacter* from *Gluconobacter*. *Acetobacter* strains are able to oxidize ethanol to acetic acid and subsequently to CO_2 and H_2O through the tricarboxylic cycle under acidic (pH 4.5) and neutral (pH 7.0) conditions. *Gluconobacter* strains have a non-functional tricarboxylic cycle, being unable to oxidize most organic acids (Kadere et al. 2008). Therefore, strains of the genus A*cetobacter* change the medium from green to yellow and back to green (Fig. 1C). Those of the genus *Gluconobacter* change the medium from green to yellow but do not return to green. Therefore, the biochemical assays confirmed that the isolated bacterium belongs to the genus *Acetobacter*. The method described in Section 2.4 confirmed that the isolated bacterium was pure and was identified as *K. rhaeticus.*

3.2 BC production

3.2.1 Kinetic evaluation of pH

The pH variation was evaluated during BC production. After inoculation, the pH of the HS and AC medium was 5.6 and 3.6, respectively (Fig. 2). The lower pH in the AC medium is due to the acidic character of the fruit. The most significant drop in pH occurred for both media in just 2 days of fermentation, decreasing to 4.0 in HS medium and 3.1 in AC medium. The pH remained practically constant after the 8th day of fermentation in HS medium and after 10 days in AC medium, reaching 3.4 and 2.7, respectively. He et al. (2020) also verified a higher drop in the pH value in just two days of fermentation for *K. rhaeticus* in the HS medium. The author also demonstrated that after 7 days, the pH remained practically constant until the end of the fermentation, reaching around 3.6. Semjonovs et al. (2017) found a final pH of 4.5 and 4.2 using apple juice and cheese whey as substrates, respectively, for BC production by *K. rhaeticus*.

3.2.2 Kinetic evaluation of BC production

BC production in both media was the same until the sixth day of fermentation. It is important to highlight that the production rate was the same in both media even with different initial pH. After that day, the HS medium stood out, reaching a final concentration of 2.9 g/L, while 2.3 g/L was produced in the AC medium. In fact, despite the difference in the BC concentration, the kinetic behavior was similar in both media, described by a linear increase until 6-8 days showing productivity of approximately 0.28 g/L/d, and subsequently a sharp increase in BC concentration between 10 and 12 days with a productivity of 0.24 and 0.19 g/L/d for HS and AC medium, respectively.

According to these results, the final pH does not appear to have affected BC production in both media (Fig. 2). A previous study also observed this behavior using the kombucha consortium on the same acerola waste extract (Leonarski et al., 2021). BC concentration showed a marked increase at the end of the fermentation between 9 and 15 days and pH 2.6, which can be described as a pattern behavior of this strain. In the study of Gupte et al. (2021), the authors also showed an increasing trend in the concentration of BC produced by *K. rhaeticus* (isolated from kombucha) in HS medium after 14 days of

fermentation and pH 3.5. However, BC production by *K. intermedius*, isolated from organic waste (pineapple and chikoo), did not increase after 7 days, correlated with the medium's low pH.

The AC medium supplemented with glucose resulted in slightly less BC production compared to the HS medium, only 20% lower. No other components were added despite the glucose supplementation, while the HS medium is formulated with yeast extract and peptone. These results contribute to discussing the enormous potential of using agro-industry byproducts in BC production (Hussain et al. 2019). In the case of acerola waste, a simple thermal pre-treatment was used to prepare the extract to achieve satisfactory BC concentration. For example, Algar et al. (2014) evaluated the production of BC by *G. medellinensis* using sugarcane juice and pineapple residues as sources of carbon and other nutrients and obtained 3.24 g/L of BC in 13 days. Urbina et al. (2017) reached 2.5 g/L by *G. medellinensis ID13488* in 14 days of fermentation using apple and sugarcane byproducts. Machado et al. (2018) partially replaced glucose with sugarcane molasse in the HS medium, obtaining 4.0 g/L BC by *K. rhaeticus* in 5 days. Using same bacteria, Pacheco et al. (2017) produced 6.0 g/L BC in 7 days using HS medium supplemented with cashew residues. The above-mentioned approaches led to higher BC production in this study. However, both authors used the HS as medium in their formulations.

3.3 Morphological and physicochemical properties of cellulose

BC morphology was verified by scanning electron microscopy (SEM). From Fig. 3, we can observe that both samples revealed a dense structure and uniform fibrils with almost non-existent interfibrillar space. There was no difference in BC produced by HS or AC medium. A similar morphological structure was observed in BC produced by *G. xylinus* (Ruka et al., 2013; Bandyopadhyay et al., 2018) and *K. rhaeticus* (He et al. 2020).

FTIR spectra similar to that shown in Fig. 4A were also shown by other authors, even using different media, indicating that the chemical structure is compatible with BC (Algar et al., 2014; Pacheco et al., 2017; Machado et al., 2018). Characteristic bands of bacterial cellulose were observed in 3346 cm^{-1} (-OH stretching), 2887 cm^{-1} (-CH stretching), and 1632 cm^{-1} corresponding to adsorbed water molecules. The 1433 cm^{-1} band is one of the main peaks associated with symmetric (CH_2) bending vibration of cellulose I-α type. Bands at 1370, 1320, and 1060 cm^{-1} correspond to -CH bending vibration, OH in-plane bending, and C–O stretching, respectively (Thorat and Dastager, 2018; Ashjaran and Sheybani, 2019; Illa et al., 2019).

Cellulose is described as a two-phase combination: crystalline (ordered) and amorphous (less ordered) regions (Illa et al. 2019). Changes in BC morphology due to chemical and mechanical treatments can be observed by two parameters provided by the XRD analysis: crystalline peak angle and interplanar distance variation (Dima et al. 2017). In Fig. 4B, it was observed that both BC reveal crystalline peaks around 14.5°, 16.8°, and 22.5°, which is similar to the pattern of cellulose type-I. The values of Full Width at Half Maximum (FWHM), interplanar distances (d-spacing), crystallite size, Z value, and degree of

crystallinity are shown in Table 2. The values of FWHM and d-spacing were close for the HS and AC medium when calculated at the same peak. Grande et al. (2009) found a value of 1.93° for the FWMW at the peak close to 15°, which is lower than those reported in this study. At 22.5°, Lee et al. (2011) found 1.71° for pure BC, close to that found in this study for HS sample. Ruan et al. (2016) and Dubey et al. (2017) presented similar values to those reported in their studies for the interplanar space (d-pacing) between planes in crystallites, 0.61, 0,53, and 0.39 nm at 2θ about 14.5°, 16, 8°, and 22.6°, respectively.

Table 2
Full width at half maximum (FWHM), interplanar distances (d-spacing), crystallites size, and crystallinity degree of bacterial cellulose produced in HS and AC medium.

Sample	2θ	FWMH (°)	d-spacing (nm)	Crystallite size (nm)	Z value	Crystallinity (%)
HS	14.34	2.18	0.62	3.74	+11.2	88.7
	16.49	1.82	0.54	4.42		
	22.37	1.77	0.40	4.58		
AC	14.71	2.14	0.60	3.74	+1.08	93.9
	17.06	1.48	0.52	5.44		
	22.88	2.07	0.39	3.91		

HS media indicated higher crystallite for the peak 22.37° (4.58 nm), although this value is not very different from the one reported for the other peaks. For AC medium the largest crystallite size was reported at peak 17.06° (5.44 nm). However, the average crystallite size was 4.25 nm for HS and 4.36 nm for AC medium, indicating a similar crystallite size.

BC can be composed of two polymorphs: monoclinic structure Iα (the cell contains one chain) or triclinic structure Iβ (contains two parallel chains) (Soemphol et al. 2018; Illa et al. 2019). The Z value is a parameter used to discriminate whether BC is enriched in Iα or Iβ type. The results in Table 2 showed that both samples (HS and AC) are Iα-rich type. Dubey et al. (2017) and Khan et al. (2021) reported celluloses produced by strains *K. europaeus* SGP37 and *K. xylinus* IITR DKH20, respectively, also enriched with Iα type. The crystalline peaks around 14.5°, 16.8°, and 22.5° correspond to triclinic Iα crystallographic planes: (100), (010), (110), and monoclinic Iβ crystallographic planes: (110), (110) and (200) (Illa et al. 2019; Anwar et al. 2021). The value of Z shows that the Iα structure was dominant in both samples. DRX of BC showed that it is enriched with the Iα form of cellulose I.

The crystallinity of both samples was high, reaching 88.7 and 93.9% for the HS and AC medium, respectively. He et al. (2020) found 85% crystallinity in BC produced by *K. rhaeticus*, whereas Güzel and Akpınar (2019) found 87.5% using *K. hansenii*, both in HS medium. Güzel and Akpınar (2019) also verified the crystallinity of BC using lemon peel, mandarin peel, orange peel, and grapefruit peel as a medium source, obtaining values between 79-92%. In our previous work (Leonarski et al., 2021),

crystallinity between 81.4 to 96.7% was found for BC produced using the same acerola waste medium in the kombucha-like beverage fermentation.

Alkaline treatment applied to remove bacteria, proteins, and other fermentation residues are used to obtain cellulose purification (Dima et al. 2017). However, if this treatment is intense, it can cause mercerization of the cellulose, changing it from type I to type II (Moharram and Mahmoud, 2008; Vazquez et al., 2013). According to Bandyopadhyay et al. (2018), cellulose type-I has better mechanical properties than type II. In this study, the alkaline treatment did not change cellulose structure to transform it into type II.

The thermal stability and degradation profile were assessed by thermogravimetric analysis (TGA). Both samples presented a typical two-step degradation profile (Fig. 5A-B), the first event correspond to a slight loss of mass among room temperature and 130°C was observed, associated to the loss of moisture in cellulose, also reported by other authors (Abidin and Graha 2015; Kumar et al. 2020; Liu et al. 2020b). The second event start at about 310°C for BC produced by HS (Fig. 5A) and at about 300°C for BC produced by the AC medium (Fig. 5B). The degradation range of both samples was similar, reaching the end at about 375°C and 360°C for BC produced by HS and AC, respectively. The end of this process is usually BC pyrolysis (Tomé et al. 2010; Figueiredo et al. 2015). DTG (Fig. 5A-B) illustrates that the derivative of mass loss and the maximum decomposition rate is similar for BC from both media (HS and AC). Overall, the samples produced in this study have a higher thermal resistance stability since this step conventionally can start from 200 ºC and even below for cellulose obtained using sulfuric acid hydrolysis (Huang et al. 2014; Pa'e et al. 2018).

DSC measures the heat released or absorbed by a material due to temperature or time. The results obtained are depicted in Fig. 5C. In our study, it was not possible to identify the glass transition for both samples. Some authors have found values between 40-54°C (George et al. 2005; Mohite and Patil 2014; Kumar et al. 2020). Two endothermic peaks can be observed in Fig. 5C. BC produced in HS medium showed the first peak around 70.5°C, while in AC medium, around 79.6°C. The first endothermic peak usually occurs between 60-100°C and refers to the loss of water molecules from the samples, which is in agreement with the results of other authors (Abidin and Graha 2015; Liu et al. 2020b). The nature of the substance and its degree of purity can be identified by a physical parameter: the melting point (Guirguis and Moselhey 2012). Another endothermic peak was observed at 355.8°C ($\triangle$H 116.1 J/g) for HS and 352.6°C ($\triangle$H 70.8 J/g) for AC, indicating the melting point (T_m). Abidin and Graha (2015) verified a melting temperature of 350.3°C for native BC. Until the evaluated temperature (400°C) no degradation peak was verified. The results suggest that both BC produced by HS and AC have strong thermostability.

The stress-to-strain analysis is shown in Fig. 5D. The breaking stress values for HS and AC were 0.102 ± 0.001 MPa and 0.191 ± 0.002 MPa, respectively. BC produced by AC medium showed breaking stress and strain higher than BC produced by HS medium. Breaking stress is directly linked to crystallinity (Liu et al. 2019), the sample that showed greater crystallinity (AC) also presented greater breaking stress. Both samples showed similar behavior, initially showing a non-linear behavior (up to 2.5% strain for HS and

4.5% for AC, and later showed almost linear behavior until breaking stress was reached. Similar curves were reported by Chen et al. (2018) for BC produced by different *Komagataeibacter* strains. Chen et al. (2018) also found breaking stress values between 0.12-0.68 MPa, varying according to the strain used for BC production. The Young's modulus was calculated using the slope curve of the stress-to-strain analysis. The values obtained for HS were 0.059 ± 0.0005 MPa and for AC 0.033 ± 0.0001 MPa. Godinho et al. (2016) presented Young's modulus for pure BC equal to 0.027 MPa, a lower value compared to BC produced in this study. According to Chen et al. (2018), cellulose concentration and fiber orientation are the factors that most affect tensile properties. In this case, the higher concentration of cellulose obtained in the HS medium led to a higher Young's modulus.

3.4 Comparison of the cost of HS and AC media for BC production

In Fig. 6A, the raw materials unitary cost for the production of HS and AC media are shown. The value for production of the HS medium is 3.14 US$/L, while for the AC medium, it is 1.14 US$/L (Fig. 6B), considering that the acerola medium is composed of residue and supplemented with glucose, its value is lower compared to the synthetic medium (HS). Still in Fig. 6B, in terms of BC production (US$/kg of BC), the AC medium presented a 52.4% of relative cost reduction, and as seen previously, comparable properties. We report a higher cost reduction in comparison with Pacheco et al. (2017), ranging from 16.5 to 33.0% when using cashew residues for BC production. Furthermore, Avcioglu et al. (2021) reported a cost reduction of approximately 30% when using kombucha for the production of BC. Therefore, using AC medium and kombucha strain, as presented in this work, can be considered a more viable and promising alternative for BC production, adding value to agro-industrial residues.

4. Conclusion

The possibility of using agro-industrial residues is an alternative to lower the production cost of BC. The main difference between the media was the absence of yeast extract and peptone in the AC medium. Furthermore, the polymer properties were alike, with the advantage of the higher crystallinity obtained in AC medium. Considering all properties of BC, applications in the food industry are an interesting alternative mainly due to the thermal characteristics associated with the processing and the economic feasibility of the presented approach, which can be still optimized in terms of its cultivation parameters in the AC medium, to improve productivity presenting a higher cost-effective yield.

Declarations

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Author's contributions

Eduardo Leonarski: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft. Karina Cesca: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Resources, Writing - review & editing**.** Sergio Y. G. González: Writing - review & editing.Débora de Oliveira: Writing - review & editing. Patrícia Poletto: Investigation, Resources, Writing - review & editing, Visualization, Supervision, Project administration.

Conflict of interest

The authors declare that they have no conflict of interest.

Human and animal participants

This paper does not contain any studies with human participants or animals performed by any of the authors.

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Figures

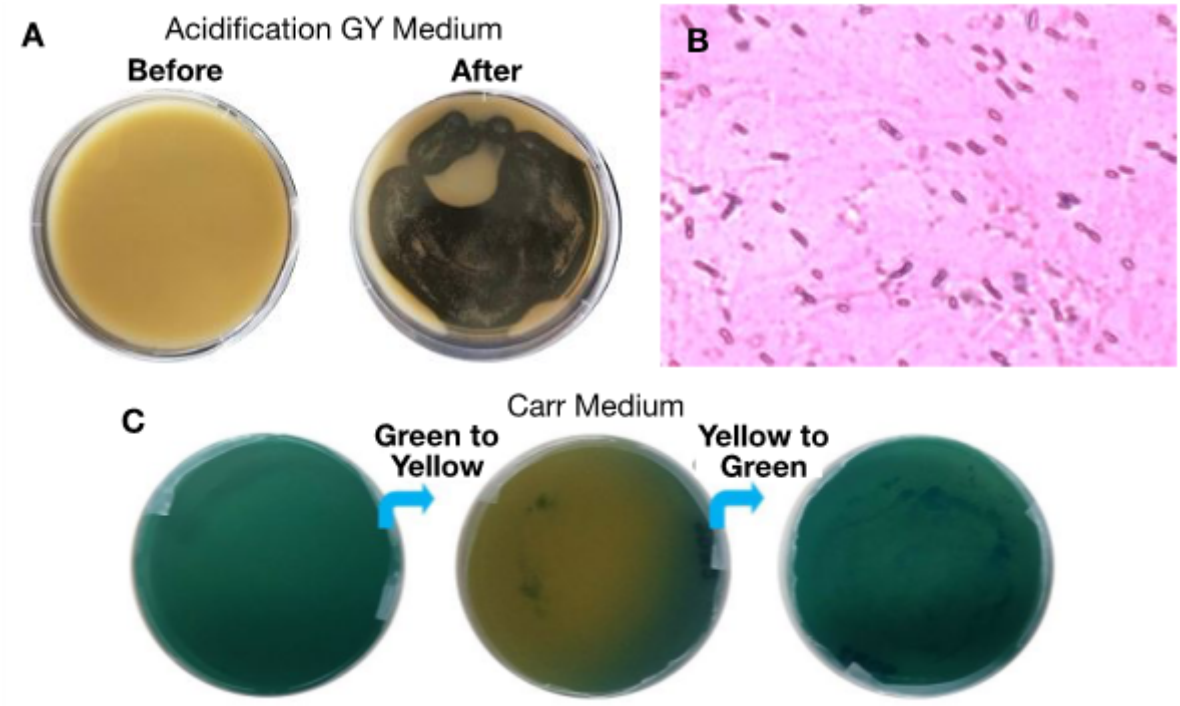

Figure 1

(A) Growth of isolated strain in GYC medium. (B) Gram staining assay observed under the optical microscope (100x). (C) Growth of isolated Acetobacter strain in Carr Medium.

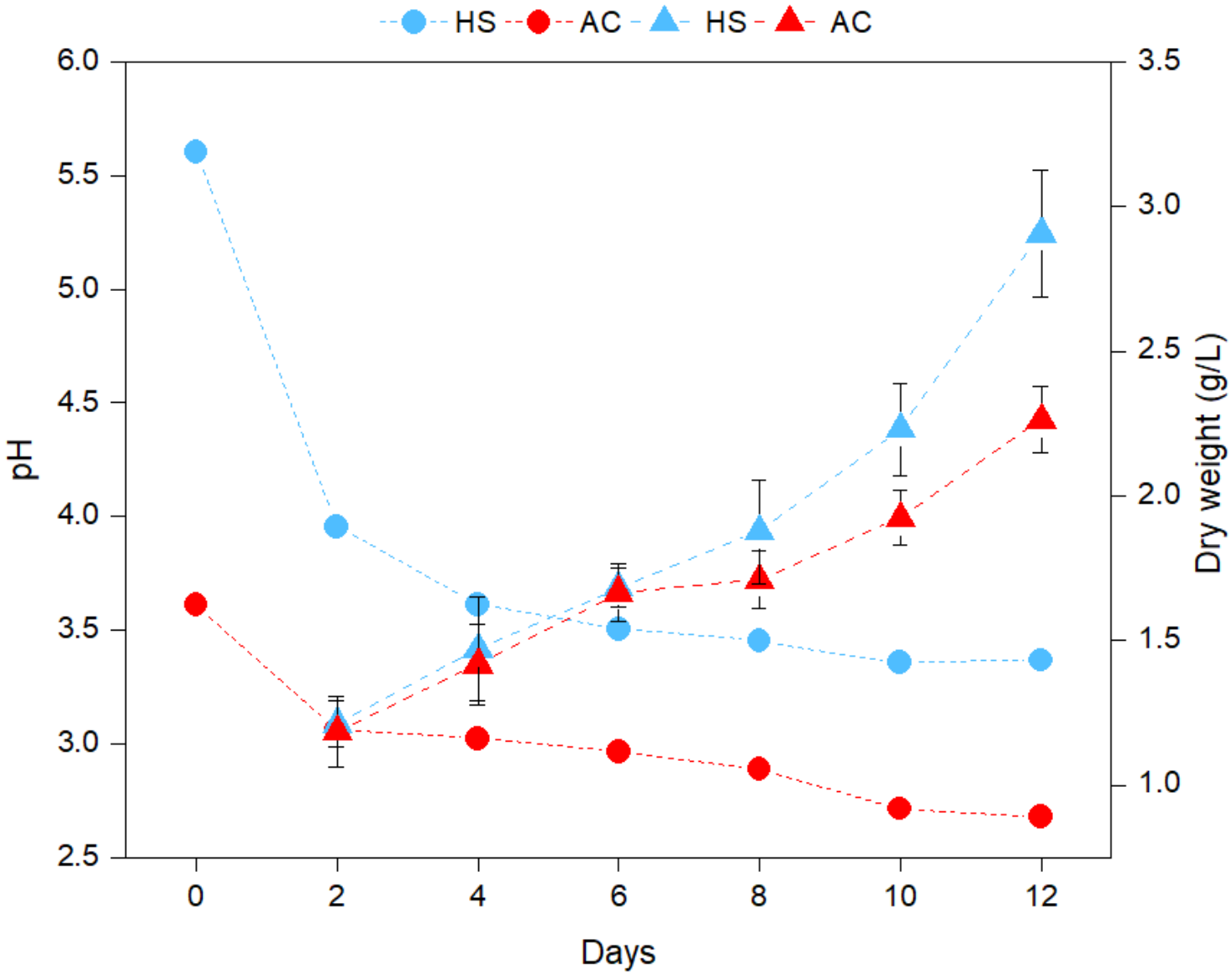

Figure 2

Kinetic profile of pH and bacterial cellulose concentration in HS and AC medium for 12 days at 30 °C.

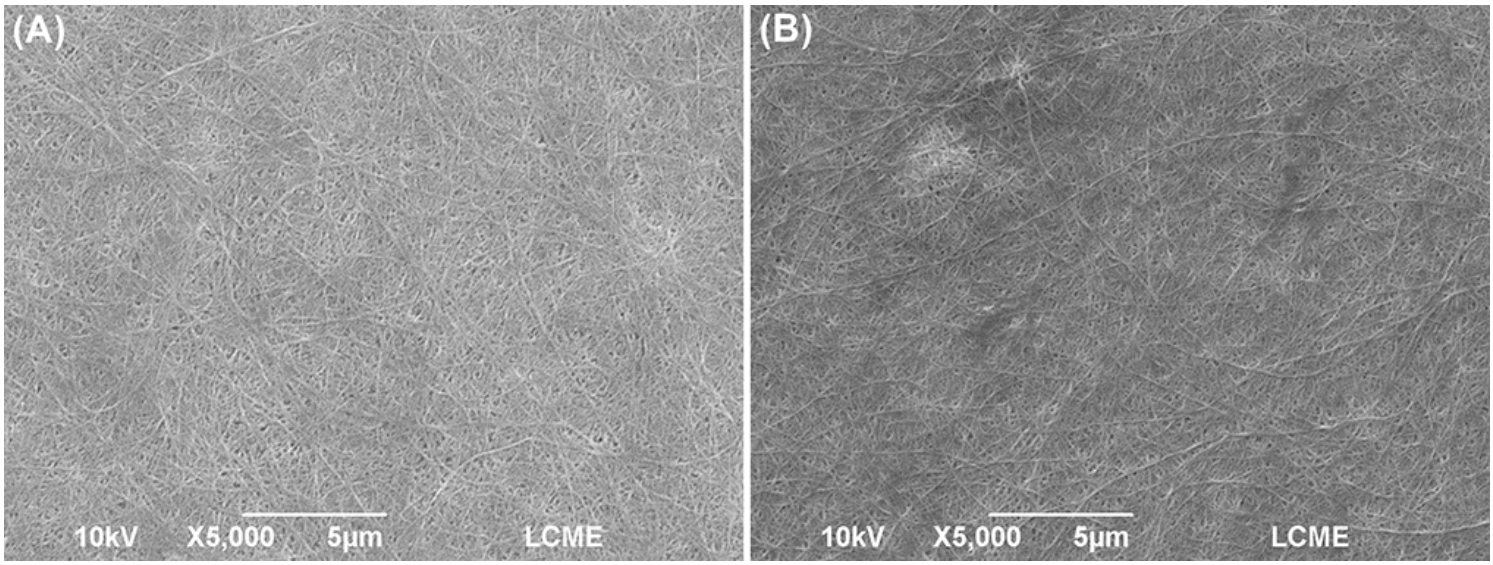

Figure 3

Scanning electron microscopy (5000x). (A) bacterial cellulose produced in HS. (B) bacterial cellulose produced in AC medium.

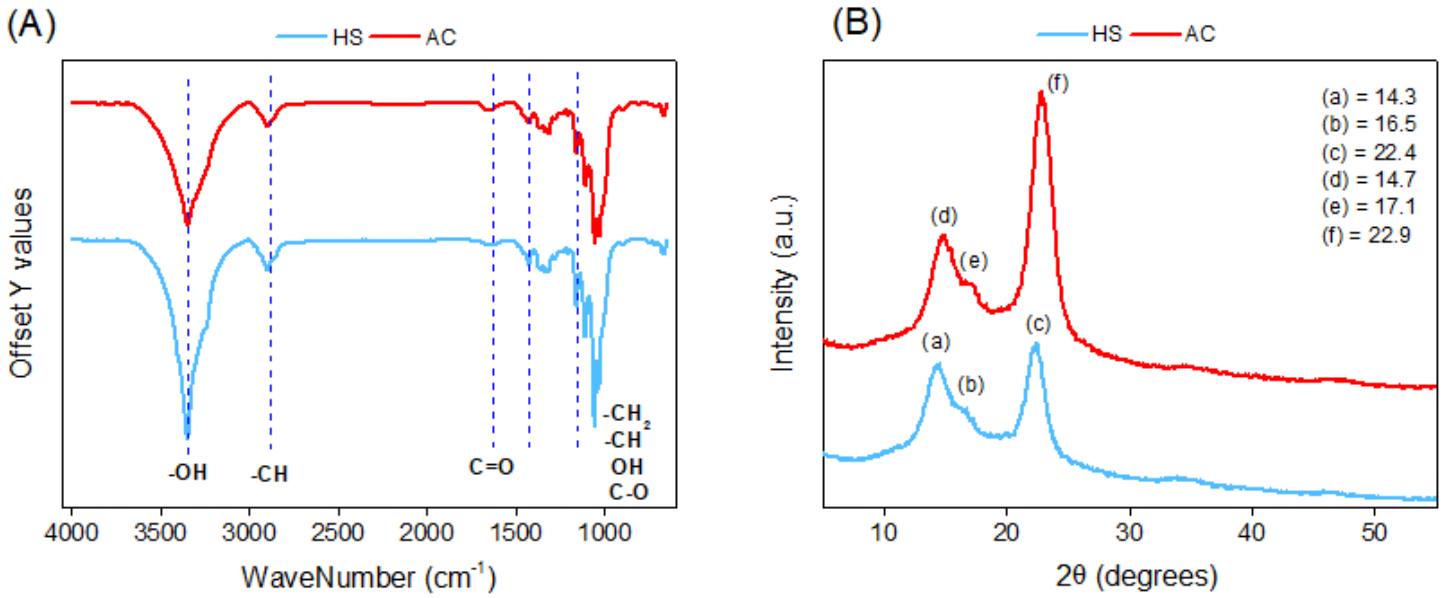

Figure 4

(A) FTIR, and (B) XRD of bacterial cellulose produced in HS and AC medium. (arbitrary units: a.u.)

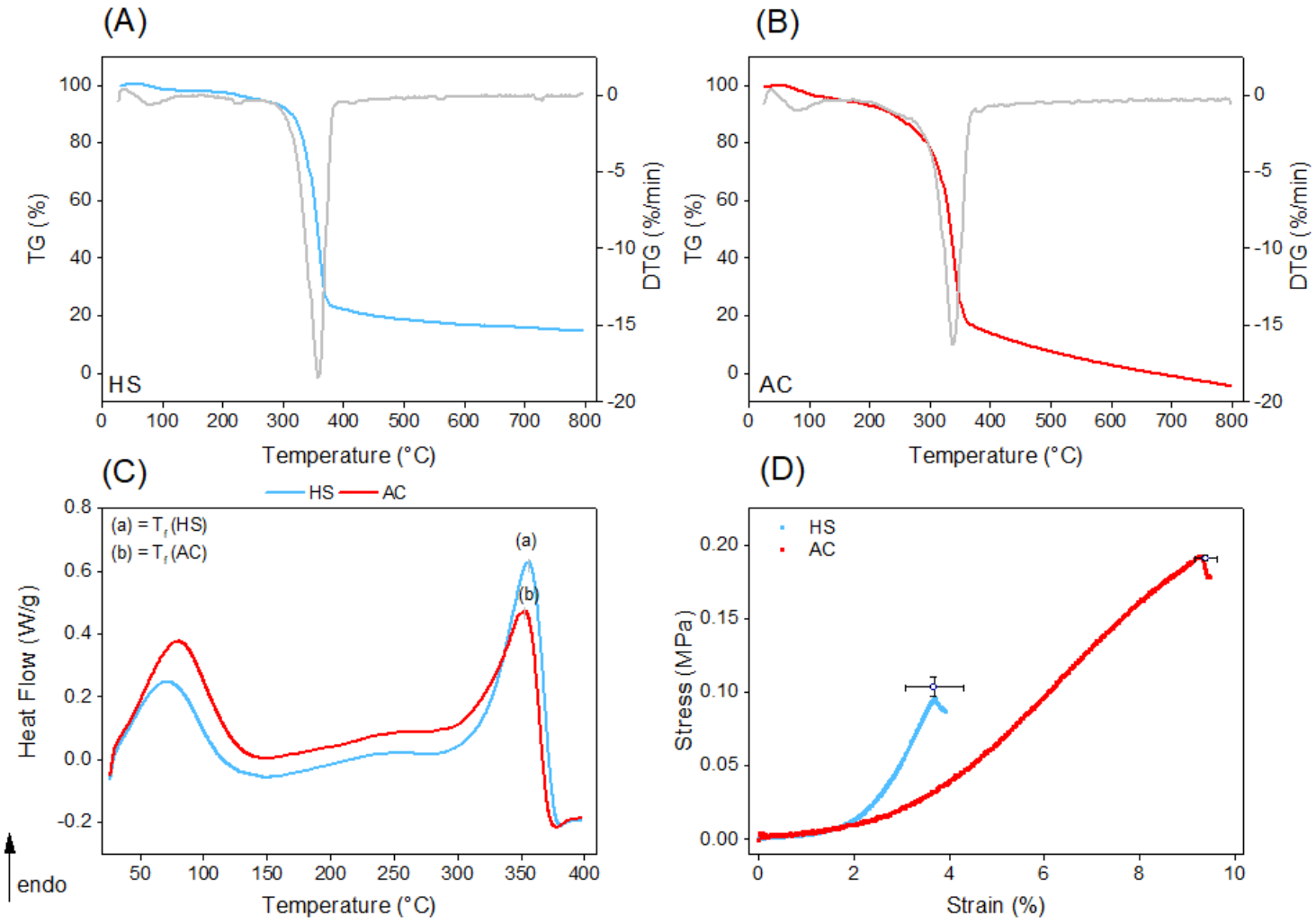

Figure 5

(A) TGA of BC from HS, (B) TGA of BC from AC, (C) DSC, and (B) Mechanical properties from BC of both media (HS and AC).

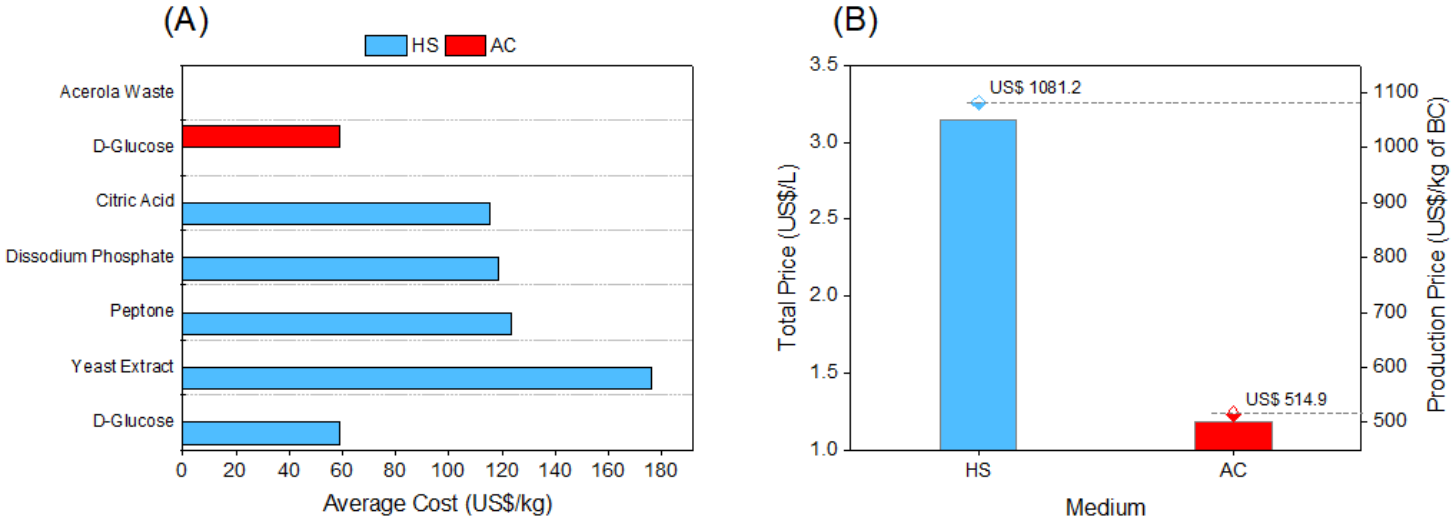

Figure 6

BC Production average cost for HS and AC medium (A) (prices taken from sigmaaldrich.com website in October 2021), and total price (bars) and (B) production price (symbols) for BC production of both media (HS and AC).