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Residue From the Production of Sugar Cane: an Alternative Nutrient Used in Biocellulose Production by *Gluconacetobacter Hansenii*

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The bacterial cellulose (BC) is a biomaterial produced by many microorganisms that use carbon and nitrogen sources available in the culture medium. The standard medium HS, represents a high cost for the BC production on an industrial scale, because it is formulated with synthetic compounds. Industrial wastes are being explored for use in many biotechnological processes. Sugar cane molasses can be used as an alternative substrate for BC production. Molasses is a viscous liquid obtained as a residue from the manufacture of sugar or refining of raw sugar. In Brazil, molasses is the main by-product of the sugar industry, being produced in the proportion of 40 to 60 kilos per ton of processed cane. Thus, alternative culture media containing three samples (designated as A, B and C) of rawhide sugar cane molasses (R), molasses inverted by high temperature (IHT) and molasses inverted by acid and high temperature (IA/HT) as substrates, combined with different nutrients (glucose, yeast extract, peptone, citric acid and Na_2HPO_4) were used, resulting in 36 alternative media. The results obtained showed that the best cost and yield was reached by the alternative medium formulated with 15 g/L of rawhide molasses (sample B), 5 g/L glucose, 1.5 g/L acid citric and 2.7 g/L Na_2HPO_4, without any nitrogen source addition (medium 3), which demonstrated 52, 59 and 65% in dry, hydrated mass and yield, respectively, when compared to the standard HS medium. The use of sugar cane molasses can be an attractive and alternative option to reduce the costs for producing BC for industrial use. The results obtained favor researches that aim not only at industrial applications of BC at a reduced cost, but also the lower environmental impact in terms of pollution load and energy consumption caused by the use of vegetable cellulose and by the disposal of industrial waste.

1. Introduction

Bacterial cellulose (BC), or biocellulose, was first reported by Brown (1986), which identified the growth of an unbranched film with the structure chemically equivalent to vegetable cellulose (VC) (Esa et al. 2014). BC is made of fibrils of 1.5 nm assembled into nanofibrils of 2–4 nm (but up 25 nm) width composed of 10–250 single polymeric chains and 1–9 nm length equivalent from 2000 up 18,000–20,000 glucose units organized into nanoribbons of 40–60 nm width (Ruka et al., 2014). The most common cellulose producing bacteria are members of the family *Acetobactereaceae* and particularly belongs to the genera *Komagataeibacter* (former Acetobacter first and later as *Gluconacetobacter* genus), *Agrobacterium*, *Rhizobium* and *Sarcina* recently revisited (Costa et al., 2017). BC is very versatile and can be obtained in the forms of nanofibrils, micro and nanoparticles (by chemical or enzymatic modification), and as biofilm matrix with different degrees of crystallinity just by choosing appropriate microbial strain and/or combined within different fermentation

strategies. The novel trends in Chemistry using soft and environmental benign techniques are making green methods for BC modification more attractive (Arjmandi et al, 2017).
Bacterial cellulose has high purity since it is not associated with other components, such as lignin and hemicellulose from the VC. Its nanofibrillar network in 3D shows water absorption capacity and high tensile strength property. Bacterial cellulose has also been suggested in the production of healthy foods, cosmetics, pharmaceuticals and biomedical products, packaging, high quality paper reinforcement, diaphragms for electroacoustic transducers, additives for paints, coatings, reinforcement for optically transparent films, among others (Li et al., 2015; Arjmandi et al, 2017). Bacterial cellulose production has traditionally been performed using defined culture medium, commonly named HS honoring Hestrin and Shramm who develop the media in 1954. The HS medium is composed of glucose, peptone, yeast extract, disodium phosphate, citric acid and adjusted to pH 6. However, changes in carbon source, nitrogen source, pH and inductors starting from this culture medium allowed to modify the BC productivity (Costa et al., 2017; Castro et al., 2011).
Since 1990s, researchers have focused on studying factors that influence the overall yield, rate of production, structural features, properties and application aspects of bacterial cellulose. The most crucial parameters are cultivation conditions such as degree of fluid mixing and oxygenation, sources of carbon and nitrogen (Park et al., 2004). The components of the fermentation medium of biotechnological processes represent additional costs, considering that glucose is essential for nutritional enrichment in addition to the addition of yeast extract and peptone, which raise the BC production costs. Thus, one of the major challenges of fermentation processes is to find a new, low-cost, high yield medium. New sources of carbon that meet the needs of high yield cellulose with a low cost must be found for the production of BC (Costa et al., 2017).
In this context, molasses has been researched as an attractive substrate in biotechnological processes due to their composition and reduced cost (Ahmad Sanadi et al., 2017).
Depending on how it is produced, molasses has different levels of sucrose, glucose and fructose in addition to significant amounts of calcium, iron, magnesium, potassium and vitamin B6. In Brazil, molasses is the main by-product of the sugar industry, being produced in the proportion of 40 to 60 kilos per ton of processed cane. Due to the high content of total reducing sugars and other components, molasses is mainly used in the manufacture of ethyl alcohol, but are also applied in biotechnological processes as raw material for the production of protein and animal feed (Albuquerque, 2009). Thus, the present work evaluated the influence of rawhide sugar cane molasses and the levels of sucrose, glucose and fructose present in sugar cane molasses after chemical and heat treatment in the production of BC.

2. Materials and Methods

2.1 Microorganism

For BC production, a strain of *Gluconacetobacter hansenii* UCP1619, obtained from the culture collection of Nucleus of Resource in Environmental Sciences, Catholic University of Pernambuco, Brazil, was used. The strain was maintained in the HS medium described by Hestrin and Schramm (1954) and modified by Hungund and Gupta (2010).

2.2 Characterization of sugarcane molasses

Three samples of sugarcane molasses, designated as A, B and C, were used in the experiments. Molasses were supplied by mills in the states of Pernambuco State (sample A) and in Paraíba State (samples B and C), Brazil. The physical-chemical characterization of molasses was carried out in triplicate and using specific methodologies of the sugar-alcohol area (Caldas, 2012). The following determinations were performed: soluble solids content (°Brix), polarization (Pol), purity in sucrose, starch, reducing sugars, total reducing sugars, moisture, colour ICUMSA, ashes and acidity.

2.3 Inversion of sucrose

The sugarcane molasses inversion was carried out using thermal and acid treatments to become glucose and fructose monomers be more easily consumed as available nutrients by the microorganisms (Tyagi and Suresh, 2016). The method proposed by Tyagi et al. (2016) for the pretreatment of molasses suggests a dilution of 1:5 (v/v) with distilled water and centrifugation at 6,484 rpm for 20 min. After this step, the solids of the supernatant that were subjected to the thermal and acid/thermal treatments were separated to remove the growth inhibitors. In the heat treatment, the supernatant was heated at 120°C for 20 min, and kept standing for 12 hours at room temperature and then centrifuged. In the acid / thermal treatment the molasses solution was adjusted to pH 3.0 with 2 M solution of H_2SO_4 and then the supernatant was heated at 120 ° C for 20 min and kept standing for 12 hours at room temperature and then centrifuged.

2.4 Formulation of alternative media for BC production

The experiments were carried out with alternative media formulated with three samples (A, B and C) of rawhide sugar cane molasses (R), of molasses inverted by high temperature (IHT) and of molasses inverted by acidic and high temperature (IA/HT), replacing 75 and 100% the carbon source. The presence of nitrogen sources was also varied. The combinations resulted in 36 alternative media formulations, as shown in Table 1. The culture medium number 1 constitutes the HS standard medium.

Table 1 – Composition of the alternative culture media for BC production

Culture Medium	Nutrients (g/L)					Molasse samples and types according to treatment* (g/L)								
						A	B	C	A	B	C	A	B	C
	Glucose	Peptone	Yeast extract	Na_2HPO_4	$C_6H_8O_7.H_2O$	R	R	R	IA/HT	IA/HT	IA/HT	IHT	IHT	IHT
1	20	5	5	2.7	1.5	-	-	-	-	-	-	-	-	-
2	5	-	-	2.7	1.5	15	-	-	-	-	-	-	-	-
3	5	-	-	2.7	1.5	-	15	-	-	-	-	-	-	-
4	5	-	-	2.7	1.5	-	-	15	-	-	-	-	-	-
5	5	-	-	2.7	1.5	-	-	-	15	-	-	-	-	-
6	5	-	-	2.7	1.5	-	-	-	-	15	-	-	-	-
7	5	-	-	2.7	1.5	-	-	-	-	-	15	-	-	-
8	5	-	-	2.7	1.5	-	-	-	-	-	-	15	-	-
9	5	-	-	2.7	1.5	-	-	-	-	-	-	-	15	-
10	5	-	-	2.7	1.5	-	-	-	-	-	-	-	-	15
11	-	5	5	2.7	1.5	20	-	-	-	-	-	-	-	-
12	-	5	5	2.7	1.5	-	20	-	-	-	-	-	-	-
13	-	5	5	2.7	1.5	-	-	20	-	-	-	-	-	-
14	-	5	5	2.7	1.5	-	-	-	20	-	-	-	-	-
15	-	5	5	2.7	1.5	-	-	-	-	20	-	-	-	-
16	-	5	5	2.7	1.5	-	-	-	-	--	20	-	-	-
17	-	5	5	2.7	1.5	-	-	-	-	-	-	20	-	-
18	-	5	5	2.7	1.5	-	-	-	-	-	-	-	20	-
19	-	5	5	2.7	1.5	-	-	-	-	-	-	-	-	20
20	5	5	5	2.7	1.5	15	-	-	-	-	-	-	-	-
21	5	5	5	2.7	1.5	-	15	-	-	-	-	-	-	-
22	5	5	5	2.7	1.5	-	-	15	-	-	-	-	-	-
23	5	5	5	2.7	1.5	-	-	-	15	-	-	-	-	-
24	5	5	5	2.7	1.5	-	-	-	-	15	-	-	-	-
25	5	5	5	2.7	1.5	-	-	-	-	-	15	-	-	-
26	5	5	5	2.7	1.5	-	-	-	-	-	-	15	-	-
27	5	5	5	2.7	1.5	-	-	-	-	-	-	-	15	-
28	5	5	5	2.7	1.5	-	-	-	-	-	-	-	-	15
29	-	-	-	2.7	1.5	20	-	-	-	-	-	-	-	-
30	-	-	-	2.7	1.5	-	20	-	-	-	-	-	-	-
31	-	-	-	2.7	1.5	-	-	20	-	-	-	-	-	-
32	-	-	-	2.7	1.5	-	-	-	20	-	-	-	-	-
33	-	-	-	2.7	1.5	-	-	-	-	20	-	-	-	-
34	-	-	-	2.7	1.5	-	-	-	-	--	20	-	-	-
35	-	-	-	2.7	1.5	-	-	-	-	-	-	20	-	-
36	-			2.7	1.5	-	-	-	-	-	-	-	20	-
37	-			2.7	1.5	-	-	-	-	-	-	-	-	20

* rawhide sugar cane molasses (R), inverted by high temperature (IHT); inverted by acidic and high temperature (IA/HT)

2.5 Inoculum and cultivation conditions

The inoculum culture was prepared by transferring the *G. hansenii* cell suspension stored at -80 °C to the HS medium, followed by static cultivation at 30 °C for two days. The statically grown culture was then shaken vigorously to release attached cells from the cellulose pellicle. The resulting cell suspension (3 mL) was inoculated in a semi-capped glass vessel (250 mL) containing 100 mL of the modified HS medium, as described below, and then statically incubated at 30 °C for 10 days in duplicate experiments (Wu et al. 2014). After cultivation, BC pellicles were removed from the culture medium, washed in tap water and purified in 0.1 M NaOH for 2 h to remove the retained cells. Subsequently, the hydrated films were weighed. After oven drying at 60 ° C for 6 hours, they were weighed again.

2.6 Calculations of yield and production efficiency of BC

The yield of the biosynthesis process and the conversion ratio in BC were calculated as presented by Li et al. (2015) using Eq. (1) and Eq. (2), respectively:

$$\text{Yield } [\%] = \frac{m_0}{C} \text{ x } 100 \quad (1)$$

$$\text{Ratio of substrate conversion to BC (rBC) } [\text{g / L /h}] = \frac{m_{BC}}{\frac{V \text{ x } t}{2}} \quad (2)$$

Where m_0 is the dry mass of the film (g), C is the total mass of the carbon source [g], mBC is the hydrated mass of the film (g), V is the reaction volume (L) and t is the time of cultivation (h).

3. Results and discussion

3.1 Production of BC pellicles

The diagram in Figure 1 shows the process of BC production by the bacterium. Based on the hydrated and dry mass (g) of the BC films produced in the alternative media containing the rawhide sugar cane molasses (R), the molasses inverted by high temperature (IHT) and the molasses inverted by acidic and high temperature (IA/HT), the results for yield and the conversion efficiency of the substrate in BC were calculated for the 36 experiments and related to the standard HS medium. A low yield of BC was identified when treated molasses were used in the presence or not of the nitrogen sources. In the case of heat treatment, the inversion of the residual sucrose often produces toxic compounds during the Maillard reaction, while the acid treatment reduced considerably the pH and inhibited the microorganism growth, since the ideal pH is around 5 -7. Satisfactory results regarding the production of cellulose by using heat-pretreated molasses as the sole carbon source, as suggested by Tyagi and Suresh (2016) and by using 100% of the samples of rawhide molasses were not obtained in this work, in opposition to the finds obtained by Castro et al. (2011), since glucose, fructose or sucrose can serve as carbon sources for the production of BC. The importance of the presence of 25% of glucose in the media containing rawhide molasses to enable the production of BC under static conditions was evidenced. As described by Bae and Shoda (2005) the presence of extracellular polysaccharides as sucrose in the cultivation medium may be the possible reason for lower yields of BC in comparison to media containing other sugars.

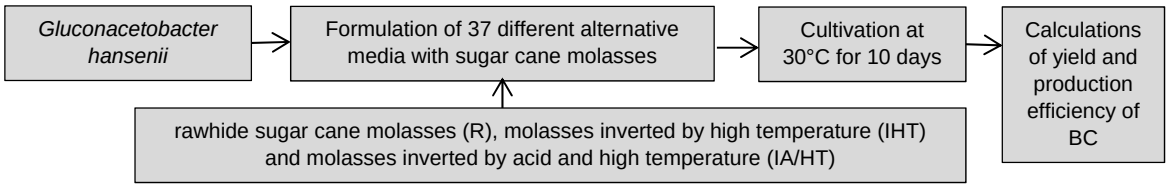

Figure 1 – Diagram of BC production

The results obtained for the 36-cultivation media tested in terms of dry, hydrated mass and yield are shown in Table 2. The alternative medium number 20 presented 73, 112 and 112% for dry, hydrated mass and yield, respectively, in relation to the standard HS medium, surpassing the results obtained by Tyagi and Suresh (2016). The characterization carried out with the molasses sample A showed 17.2% of sugars reducing sugars and 61.3% of total sugars, demonstrating the viability of the use of reducing sugars by the microorganism, becoming an excellent source of nutrients (Ahmad Sanadi et al, 2017), producing a ratio of substrate conversion to BC (rBC) equal to 1.6 g/L/h, surpassing the HS conversion by 74%, with a cost reduction of 54% over the standard medium. However, the cost and yield was exceeded by the alternative medium number 3, which used 75% of the rawhide molasses sample B and withdrew 100% of the synthetic nitrogen sources, showing 52, 59 and 65% in dry, hydrated mass and yield, respectively, relative to the HS medium. The characterization of molasses sample B showed 10.8% of reducing sugars and 60.8% of total sugars reducing sugars, which allowed a rBC equal to 1.4 g/L/h, surpassing in 52% the conversion in HS medium and a cost reduction of 72% over the composition of the alternative medium 20 and 82% over the standard medium.

The differences in the responses obtained when molasses samples A and B were used were due to the increase in acidity, starch, color, moisture and ashes (30.4, 25.0, 11.4, 31.4, 44.9%, respectively), which hinder the sugar crystallization process and probably act as impurities, reducing the utilization of sugars by the microorganism (Rein, 2013). The use of molasses C showed contamination of the fermentation medium, not allowing the evaluation of the response to the use of reducing sugars by the microorganism, probably due to

microbiological contamination of the molasses itself. The addition of crude molasses favored BC production, since the sugars (total sugars sugars) and the other parameters evaluated (Table 3) during the characterization favored the evaluation of BC production, considering that the sugars (glucose, fructose and sucrose) present in molasses are fundamental for microorganisms to develop BC, reducing production costs on an industrial scale.

Table 2 – Dry, hydrated weight and yield of BC pellicles obtained

Culture Medium	Dry weight (g/L)	Hydrated weight (g/L)	Yield (g/L)
1	11.08	0.17	8.50
2	7.80	0.10	5.00
3	16.85	0.28	14.00
4	13.53	0.21	10.50
5	Not Detected	Not Detected	Not Detected
6	8.23	0.14	7.00
7	11.27	0.22	11.00
8	Not Detected	Not Detected	Not Detected
9	6.53	0.06	3.00
10	12.59	0.16	8.00
11	6.34	0.06	3.00
12	Not Detected	Not Detected	Not Detected
13	Not Detected	Not Detected	Not Detected
14	6.74	0.26	13.00
15	3.27	0.02	1.00
16	3.50	0.03	1.50
17	Not Detected	Not Detected	Not Detected
18	5.18	0.23	11.50
19	4.23	0.02	1.00
20	19.16	0.36	18.00
21	Not Detected	Not Detected	Not Detected
22	Not Detected	Not Detected	Not Detected
23	Not Detected	Not Detected	Not Detected
24	11.87	0.20	10.00
25	10.18	0.20	10.00
26	Not Detected	Not Detected	Not Detected
27	9.71	0.20	10.00
28	11.67	0.26	13.00
29	9.84	0.16	8.00
30	5.20	0.06	3.00
31	4.50	0.05	2.50
32	Not Detected	Not Detected	Not Detected
33	Not Detected	Not Detected	Not Detected
34	Not Detected	Not Detected	Not Detected
35	Not Detected	Not Detected	Not Detected
36	Not Detected	Not Detected	Not Detected
37	Not Detected	Not Detected	Not Detected

Table 3 – Characterization of molasses samples used in the fermentation media for BC production

Molasses	Soluble solids (°BRIX)	Polarization (°Z)	Purity (%)	Reducing sugars (%)	Total reducing sugars (%)	Acidity (%)	Starch (%)	Color (U.I)	Moisture (%)	Ashes (%)
A	76.5	38.2	49.9	17.2	61.3	2.4	10.0	99.800.0	24.1	4.4
B	67.8	48.1	71.0	10.8	60.8	3.1	12.5	111.160.0	31.6	6.3
C	76.9	39.4	51.3	17.4	58.7	2.6	10.0	80.112.5	15.6	8.0

We emphasize the interference of substances such as starch, ashes and colored compounds. These substances come from the raw material, excess calcium oxide in the treatment of sugarcane juice and produced during the heating of molasses, causing a rise in color and impairing the performance of microorganisms, as they act as contaminants and competitors of nutrients.

4. Conclusions

Promising results for BC production using sugarcane molasses were demonstrated and confirm this industrial residue of plants as an alternative carbon and nitrogen source that reduces BC production cost. The amount of glucose, fructose and/or sucrose available in the alternative culture media containing crude molasses allowed the production of biocellulose. The physical-chemical characterization of the molasses samples allowed to evaluate the availability of the nutrients in biotechnological processes, and can also avoid contaminations that could interfere in the yield of the process.

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