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Production of Nanocellulose in Molases of Cane under Static Conditions and Surface Aeration

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Abstract

Objective: To evaluate the nano-cellulose production for *Gluconacetobacter xylinus* in presence of molasses of cane under static conditions and / or of intermittent air flow. **Methods:** Anova factorial was realized where they involved as incidental variables in the process the condition of aeration (static Culture without aeration and static culture with aeration), and as variable of response in the cellulose production (represented like wet weight, dry weight, compressibility and thickness). Aso applied an analysis of comparison of averages and one proves t-test, with the software Staph graphics centurion version XVII.I. **Findings:** Across this study, it was possible to verify that the bacterium *G. xylinus* in static conditions with superficial aeration and using molasses as source of carbon, a concentration of 103 g L^{-1} was obtained of Bacterial Cellulose, approximately 20%, more than in the static culture without aeration. Likewise, the commercial value of the way of culture used (Max Mza) was minor than that of other previous studies where used reagents of analytical degree (costlier) while we used molasses and reagents of industrial degree (cheaper). **Applications:** This work contributes for further study on the effect of the molasses and other Sources of carbon on the cellulose production in such a way that the production increases and that the costs diminish in order to climb the process. Likewise, the application of aeration as superficial way allows the synthesis by culture more efficient, and therefore the production of the biopolymer increases.

Keywords: Bacterial Nanocellulose, Cane Molasses, Culture Medium, Surface Aeration

1. Introduction

It is important to find a material that allows the replacement of vegetable-type cellulose in many applications; hence, the bacterial nanocellulose has been explored over the years with excellent results using different carbon sources. The gram-negative bacterium *Gluconacetobacter xylinus*, strict aerobic, produces in static cultures a cellulose film supernatant on the surface, allowing it to be at the air-liquid interface and thus obtain a greater amount of oxygen; promoting an increase in moisture retention and facilitating bacterial growth[1]. The bacterium synthesizes cellulose from a wide range of carbon sources, can metabolize glucose via the pentose phosphate pathways and the Krebs cycle. Cellulose biosynthesis occurs in the cytoplasmic membrane of the bacteria, involving some reactions that generate a micro fibril that excreted to the outside, forming branched fibers with a proximal length between 180 and 960 nm[2]. The bacterial cellulose given its high purity, degree of polymerization and structure is used in various industrial applications such as the stabilization of emulsions, the manufacture of materials of high aqueous absorption, as a document repairer, food additive, chromatographic techniques, and temporary skin for therapy of burns, dental implants and high sensitivity diaphragms for hearing aids[3].

The production of Bacterial Nanocellulose (BC) by *G. xylinus* is done in crops from economic substrates such as corn liquor, sucrose, beet, fruit juices, molasses, among others. In this work, cane molasses was used as the main carbon source because it is considered as an important alternative

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due to its composition, besides it reduces costs to biotechnological processes. A determining factor in the production of bacterial cellulose is oxygen, where it has been shown to act as a limiting substrate in the initial stages of cultivation[4].

The work presented deals with the evaluation of cellulose production in molasses medium, with a reactor of cylindrical configuration, at room temperature and acidic pH. It was developed in two stages; in the first, all the variables annexed to the process were evaluated in a static culture strategy, and in the second stage, the variables were evaluated but with a static culture strategy with intermittent aeration; observing the way in which the carbon source used together with the aeration and the general conditions of the culture which influence the synthesis of BC. The main scope of this study is the establishment of a culture medium where molasses is used as a carbon source under surface aeration conditions in a *G. xylinus* culture and screen for any increasing cellulose production by at least 10% which can serve as basis for further studies. In the search for a stable process for its production, in such a way, that economic interest in the microbial product (BC) can be generated and a positive social contribution can be presented to the development of the Department of Sucre and the Caribbean coast, aimed at the generation of employment.

2. Material and Methods

The experimental procedures were carried out in the Microbiology Research Laboratory, Microorganisms Biology Research Group (GIBM), Department of Biology and Chemistry, Faculty of Education and Sciences, University of Sucre.

Culture media for the maintenance of *Gluconacetobacter xylinus* IFO 13693 the medium of [5] was used; it was cultivated at 30°C under static conditions until the appearance of a BC layer on the surface of the culture. The composition of the liquid culture medium was as follows: Diluted molasses, ammonium, citric acid, supplemented with magnesium, phosphate, calcium and potassium salts. An initial concentration of molasses in solution of 13.3% was used according to previous studies that show that in concentrations below and above this value, the microbial growth is inhibited and BC production decreases. The pH of the medium was adjusted to 5.6 with 1.0 N NaOH or 10% HCl (v/v), and all culture media were sterilized in an autoclave at 121°C for 20 min. The maintenance of G. xylinus is done every 15 days.

2.1 Culture Conditions

Colonies of *G. xylinus* IFO 13693 were inoculated in 500 mL of liquid culture medium in an Erlenmeyer flask of 2 L capacity and 1.0% (v/v) ethanol was added, which were incubated at 30°C for 24 h in shaking at 125 rpm. Subsequently, after purity control, by observing the typical morphological characteristics in fresh preparations and Gram stains, the Erlenmeyer pre-inoculum flask with greater turbidity and formation of small cellulose fibers was selected, which was transferred to an adequate volume of culture medium and incubated at 30°C for 24 h under agitation at 125 rpm. This activated inoculum was transferred to the 36 cylindrical plastic bioreactors of 20 cm in diameter and 20 cm in height, with a final volume of 2 liters of medium in each bioreactor, with plastic cap and holes covered with cotton plugs and sterile gauze at 30°C for 1.5, 3, 7, 14, 21 and 28 days under static conditions and intermittent air flow (air was supplied in the surface area of the cultures intermittently through an air compressor, 3HP, 50 L, 110V mains voltage, 60Hz mains frequency, 1700 W input power, 3 HP motor power, 115 PSI pressure, 140 lx min capacity, 3400 RPM, 50 mm cylinder diameter, 35 Kg weight, and aeration was controlled with flow meter of air (Flowmeter LPM) Cole Parmer®, during 12 intermittent hours (2VVM) All the experiments were carried out in triplicate.

2.2 Measurement of Bacterial Nanocellulose (BC) Production

To establish the weight of each cellulose film, they were removed from the bioreactor at the different culture times and washed with 0.1 N NaOH at 30°C for 20 min, in order to dissolve the bacterial cells. To remove other impurities, the BC was washed with distilled water several times and partially dried with absorbent paper before being measured its wet weight. Subsequently, the cellulose was dried at 60 ± 10°C (220°C laboratorio oven, 28 liter Jouan, model: EU28) until the weight reached a constant value[6], which corresponded to the dry weight of the BC.

2.3 Experimental Design

A Factorial Anova was performed where the aeration condition (Static culture without aeration and static culture with aeration) was involved as variable variables in the process, and as a response variable the cellulose production represented as (wet weight, dry weight, compressibility and thickness), then a comparison analysis

of means and a t-test was applied, with the software Staphgraphics centurion version 17. The experiment was carried out in two phases, in the initial phase several experiments were carried out, in order to choose the concentration of molasses more suitable according to previous studies.

3. Results

In this study, cellulose production was evaluated under static and intermittent airflow conditions using cane molasses (13.3% w/v) as the main carbon source and industrial grade reagents in the culture medium. It was found that the production of cellulose at lower concentrations and more than 13.3% was not satisfactory, So, it was considered that it worked with a concentration of approximately 55 to 65% of sucrose, being a source of low cost and renewable carbon. In the same way, the source of nitrogen that was used comes from ammonium and in less quantity of molasses, and minimum amounts of bio elements and vitamins of the B complex contained in the molasses. The bioreactors used for BC production in preliminaries were of cylindrical shape with different diameters and height. The bioreactor used in this study was a cylindrical plastic with a diameter of 20 cm and a height of 20 cm with a final volume of 2000 mL with plastic cap that had 9 holes of 1.5 cm in diameter each and covered with a gauze and a cotton plug in the static experiment; In the experiments with aeration, a bioreactor of equal dimensions and materials was used, but with a special design consisting of a plastic cover, covered by an aluminum cover, with holes for the air to enter the surface area, Through a stainless steel tube with small holes, a hole for the air outlet with connection to a hose that went to a water source and prevents contamination of the crop, the aeration of the crop was carried out intermittently with a flow of 2 vvm in the surface area during 12 intercalated hours. It is possible to observe the results in Table 1 and 2, where production is evident. In the same way, properties such as compressibility of the film and pH for the static and static condition with surface aeration were determined.

Determination of the compressibility of synthesized cellulose. Once washed and dried with absorbent paper, the cellulose films were subjected to the application of a weight of 2000 g for 30 seconds[7]. Where P2 is the wet weight after having applied the weight of 2000 g and P1 is the wet weight obtained before applying the 2000 g.

3.1 Area-volume Ratio of the Bioreactor

The effect of the geometry of the bioreactor was determined using two configurations of bioreactor (conical and cylindrical) and determining the relative thickness limit (height of the film = height of the liquid). It is shown in Figure 1.

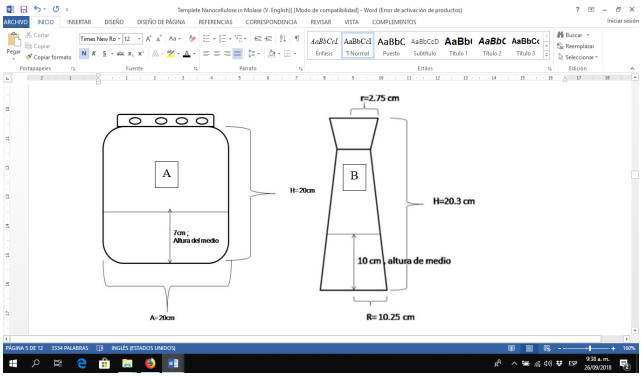

Figure 1. A: Diagram of culture vessel A (cylindrical plastic bioreactor), 2000 mL of culture medium, static and static + aeration; B: conical bioreactor, 2000 mL of culture medium, static.

The onset of BC synthesis was observed as the formation of a very thin layer on the surface of the culture medium under both conditions during the first 36 hours of cultivation, and over time a thicker film was formed. It is important to observe in the results that intermittent aeration was chosen, because with the continuous aeration for 24 hours initially and then 12 (data not shown), saturation of the surface area and the medium with (oxygen from the air) occurred, which inhibited the growth and consequently the production, in addition this continuous aeration transcended in the production costs of BC. The results indicate that there was a considerable increase in the production of wet BC between days 14 and 21 with a percentage of around 51% in the static culture, in contrast to what was observed in the culture of static aeration whose increase began to appear between days 7 and 14 with a percentage of 55%, the average speed of formation of BC was different during the 28 days of cultivation with values that oscillate between 0.027 g L^{-1} h^{-1} and 0.63 g L^{-1} h^{-1} for the static harvest, while for static cultivation with aeration, the speed remained almost constant with an average value of 0.70 g L^{-1} h^{-1}. In relation to dry cellulose, a production of 24.4 g L^{-1} of cellulose synthesized in static aerated culture and 13.0 g L^{-1} of BC in static culture after 7 days was observed. It was found that between days 7 and 14 there was increased cell growth,

for both strategies, of culture examining the thickness of the cellulose film. Maximum average values of 34.3 mm were reached, with a standard deviation of 1.15 and a coefficient of variability of around 3.36% for the experiment with aeration, which indicates little difference in the results obtained for each period, obtaining cellulose films with a maximum thickness of 12 mm after 7 days of cultivation. When comparing these values with the experimental study, maximum average thicknesses of 8.1 mm were obtained at 7 days, in a volume of 2000 mL, a culture medium, where the salts used were industrial grade, as the main source of carbon, cane molasses and with some growing conditions (Static-Aeration). All this represents an important result since the aeration was supplied intermittently for 12 hours, which was reflected in the values obtained that doubled the values of the static experiment whose maximum thickness was 4.1 mm.

3.2 Percentage of Compressibility

The percentage of compressibility of the cellulose was reduced to a measure in which the synthesis process was carried out due to the loss of water in its structure, being a pressure of 2000 g in a time of 30 seconds, however from the On day 14, the cellulose was presented by the percentage of compression over time, with a slight drop on day 28, this was due to the water content that the film reached in the final time and which made it more compact so that the volume of loss was less and therefore its compressibility also lower.

3.3 Effect of the Geometry

The effect of the geometry of the bioreactor in the production of cellulose was studied, the two areas were related, one of them with the same bioreactor configuration, but with different culture conditions, where it can be inferred that in the bioreactor configuration it is shown in Figure 1, The space occupied by the culture medium corresponded to 35% of the total space, unlike the conical bioreactor Figure 1B where the space occupied by the culture medium corresponded to 50%, now according to the results of the relationship between the area and the volume of culture medium for both bioreactors it could be inferred that the area-volume relationship is important because it shows a higher yield in the amount of cellulose obtained per area in the experiment with volumes of 2000 mL of static aeration, which did not happen with the static experiment of 2000 mL and the static one in the reactor that showed much lower yields.

4. Discussion

The results found in this study lead to consider a quite considerable production in relation to the amounts of cellulose obtained in previous studies[8], therein also used molasses in the culture medium (17% molasses) and the amount obtained was of 1.97 g L^{-1} after 6 days, due to the fact that at higher concentrations of molasses BC production decreases what was evident in this study, for a static culture and in a conical reactor configuration, or Erlenmeyer. Likewise, the weight of dry cellulose represented between 5% and 9% of the total weight of the wet cellulose, a value close to that found by other authors[9]. Figure 2 shows the wet and dry bacterial cellulose films (molasses medium) in static culture.

Figure 2. Wet and dry bacterial cellulose films (molasses medium) in static culture.

There was a greater production of cellulose for the aerated culture, a condition that was maintained during almost the entire process, However, it is important to note that if the aeration had been direct and not to the surface area where the cellulose film formed from day 3 it prevented the arrival of air to the culture medium, the production would have been greater. However, dry weight data were obtained higher than those found in the literature[9-11] who also worked with molasses, where it was assumed that the medium at its most part was composed of water. It is one of the most important properties of BC, when it has been used in biomedical applications, in the correct maintenance of moisture in wounds, indispensable for its application in modern therapy because the penetration of the active substances in the wound is facilitated by a humid environment. This also allows an easy dressing change, without pain or damage to the newly formed skin[12]. The thicknesses had an increase according to the expected, according to the time of the crop and the wet weight reached by cellulose.

In Tables 1, 2, it was observed that the thicknesses reached in the static culture are lower than those reached in the static culture with aeration are. These values are related to those obtained by those who worked with volumes of

similar medium (1600 mL), in a static culture, with a geometrical configuration of the rectangular bioreactor and Hestrin-Schramm culture medium 1954[13]. For this study, the main carbon source was glucose, which proved to be quite efficient in the production of cellulose. Over the time, a decrease in compressibility was evidenced during the cultivation time, this property is very important for the future applications that can be given to the cellulose because it is an important parameter that allows to control the final mechanical properties of the cellulose films[14].

The optimal pH range for cellulose production and growth of *G. xylinus* is from 4.5 to 5.5, as established[15], because the synthesis of cellulose and the growth of the bacteria are hindered in media that are above of 6.0 and below 4.0[14]. In this experimental study, the pH was maintained in the useful buffer zone, 5.6 – 4.6 due to the use of the citrate phosphate buffer, which showed a good buffering capacity during the process; this mechanism for pH control has a large number of advantages among which is not including additional risks of contamination. In the first days there was an abrupt decrease of the pH due to the production of gluconic acid in the initial stages during the catabolism of glucose[16] of equal has There have been studies where the effects of different pH between 4 and 8 in the culture medium HS (Hestrim-Schram) obtaining a maximum yield at a pH of 7[17].

Regarding the concentration of *Gluconacetobacter xylinus* cells, we found that the amount of viable cells was higher in the static culture with aeration due to the oxygen supplement on the surface layer of the medium, which made up for the lack of this important reagent for the bacterial cellulose synthesis process during the cultivation time. In addition, the increase in cell concentration coincides with the results obtained from wet weight, in the same way as expressed in previous studies[11]. Since at the end of the culture time, the production of bacterial cellulose is not so accelerated in a clear relationship with cell growth, which begins to decrease due to a limitation of nutrients in the culture medium, taking into account that cellulose films are considered as products of the secondary metabolism of cells[11].

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Table 1, 2 shows the cell growth has an exponential growth phase marked from day 7 to 14, and a decrease in the number of cells from day 15 onwards is observed until the end of the incubation time.

Table 1. Cellulose production in static

Time (days)	Production of wet cellulose (g)	Production of dry cellulose (g)	Compressibility (%)	pH	Thickness (mm)	UFC/mL
1.5	5	0.5	100	5.2	1.0	5×10^8
3	75	6.5	84	4.9	2.2	3×10^9
7	120	11.0	68	4.8	3.3	2.5×10^{10}
14	225	21.0	44	4.8	8.0	2.3×10^{10}
21	455	50.0	30	4.7	19.0	2.15×10^{10}
28	780	75.0	18	4.6	24.0	1.8×10^{10}

Table 2. Cellulose production in static–aeration

Time (days)	Production of wet cellulose (g)	Production of dry cellulose (g)	Compressibility (%)	pH	Thickness (mm)	UFC/mL
1.5	50	5	100	5.5	2.5	5.0×10^8
3	110	10	100	5.3	3.5	3.2×10^9
7	250	25	84	5.1	7.5	5.0×10^9
14	540	55	62	5.1	11.5	3.5×10^{10}
21	655	70	44	5.0	24.0	3.0×10^{10}
28	960	86	38	5.0	37.0	2.75×10^{10}

The production of cellulose obtained with economic culture medium (Max-MZA) used in this experiment and with the cylindrical bioreactor configuration in static conditions was compared with the results obtained in other studies[18], where no significant difference was found, data that contrasts with the amount of cellulose that was obtained in the experiment with aeration, where a significant difference was evidenced; relating it to the data reported in[19], where a rectangular bioreactor was used and with a similar amount of medium 1600 mL, and where a maximum yield of 1.93 g L^{-1} was obtained, which shows the efficiency of the conventional cylindrical bioreactor that was used in this experiment and also was satisfactory for the interest of establishing an industrial level production.

Different sources of carbon have been used for the production of bacterial cellulose, also using different cultivation strategies and different bioreactor configuration. In this study, cane molasses was used as the main carbon source in the Max MZA liquid culture medium, which also contains industrial grade salts. The molasses is a by-product of the sugar industry that has a large biotechnological use, it has a composition rich in sugars (sucrose, glucose and fructose), and in this work we obtained results tending to observe the behavior of the consumption of sugars during the time of cultivation and its influence on the production of bacterial cellulose.

The production process of the BC was favored in the interfacial area, where the oxygen diffusion of the gas phase has a greater effect. According to this it can be noted that the consumption of sucrose at the end of the culture time for the static experiment corresponds to an amount of 79%, and in the static-aeration experiment the concentration decreased up to 82%.

5. Conclusions

Different biological systems have the ability to synthesize cellulose, but it was found that Bacteria *G. xylinus* in static conditions with surface aeration and using molasses as a carbon source, 103 g L^{-1}, approximately 20%, more than in static culture without aeration. The dry weights obtained are 50% higher than those reported in the literature, despite the fact that the cultivation strategy was static, and the molasses used was only diluted, showing the importance of surface aeration, which supplements the decrease of oxygen during the cellulose production process. In relation to the amount of cells in the culture medium, an exponential phase growth is observed until day 14, after which a decrease tending to stability in the number of colonies is observed, caused by factors such as the decrease of nutrients and metabolites produced together during the process. The dry weight was gradually increasing during the culture time, which in the end showed a difference between the two strategies of around 44.8 % for the 28 days.

6. References

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