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Sisal Fiber Hydrolyzate for Biomass, Lipid and Carotenoids Production by *Rhodotorula mucilaginosa*

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The use of lignocelulosic materials to obtain substrates used in the cultivation of microorganisms that produce substances of interest has been intensively studied. Due to the large amounts of cellulose and lignin, sisal fiber has potential to be used in the production of hydrolyzates, which can be used as substrate to obtain biocomposites from the biomass produced, however, one of the main problems is the presence of growth production inhibitors during the hydrolysis process. In this context, the present research aimed to produce biomass, lipid and carotenoids using sisal fiber hydrolyzate. *Rhodotorula mucilaginosa* cells were inoculated in the sisal fiber hydrolyzate. Cultures were incubated in an orbital Shaker at 30 °C and 200 rpm. During the time interval of 120 h, biomass and reducing sugars present in the culture were quantified. Overall, 4.98 $g.L^{-1}$ of biomass, 19.28 $g.100g^{-1}$ of lipids and 0.48 $mg.L^{-1}$ of carotenoids were produced in the sisal fiber hydrolysate. In the lipid profile, oleic and linoleic were predominant fatty acids and the sisal fiber hydrolyzate was shown to be a interesting medium for the production of lipids and carotenoids using yeast *Rhodotorula mucilaginosa*.

1. Introduction

The use of waste and materials of low commercial value as raw material for the production of biocomposites has been increasingly consolidated. These materials have a very characteristic composition, since they have in their biochemical composition basically cellulose, lignin and hemicellulose in proportions that vary for each type of material and time/place of collection (Canilha et al., 2012).

A plant mostly composed of lignocellulosic materials is sisal (*Agave sisalana*), a cultivar of great economic interest in hot climates and arid regions such as Northeastern Brazil, with plantations concentrated mainly in the states of Bahia and Paraíba. Sisal fiber contains about 90 % fermentable sugar-generating materials (Debnath et al., 2010).

An alternative use of fiber is its use for the production of biocomposites using yeasts, since some microorganisms are able to convert a source of carbon, when in excess, into biocomposites inside their cells (Beopoulos; Nicaud; Gaillardin, 2011).

These biocomposites include carotenoids, which are pigments obtained almost exclusively by chemical processes. However, biotechnological production has been gaining prominence, standing out due to the possibility of using inexpensive substrates, independence of environmental conditions and need for less space for production (Zeni et al., 2011).

Carotenoids have a significant industrial importance, and can be produced by several organisms such as bacteria, fungi and plants. Several wastes, such as whey and potato extract, have been studied for the synthesis of carotenoids by yeasts (Marova et al., 2012) and the optimization of cultivation conditions has been one of the strategies used to increase the production of carotenoids and therefore the process viability (Santos et al., 2016).

The use of an inexpensive raw material as a substrate for the production of substances of interest in the industry is a good alternative for adding value to these materials. Thus, this research aimed to produce biomass, lipids and carotenoids using sisal fiber hydrolyzate as substrate.

2. Material and method

2.1 Sisal fiber

Sisal fiber was obtained in Cuité-PB and cut into small pieces to facilitate hydrolysis.

2.2 Sisal fiber hydrolysate production

About 50 g of sisal fiber and 500 mL of 5.5 % sulfuric acid solution were used. Hydrolysis was performed in autoclave under pressure of 1 atm for 1 h. At the end of the hydrolysis process, the filtrate (hydrolyzate) was collected and the waste was discarded.

2.3 Treatment of hydrolyzate

The hydrolyzed liquor from sisal fiber was subjected to an adsorption process to remove inhibitors. A 5 % active carbon concentration was used in the mass/volume ratio of the liquor (Villarreal, 2005).

2.4 Microorganism

R. mucilaginosa CCT 3892 yeast was used, obtained from the André Tosello Foundation, Collection of Tropical Cultures of São Paulo. The yeast was acquired in the activated form. For growth and maintenance, colonies were incubated in Petri dishes with YMA medium (Yeast, Malt, Agar) composed of 10 $g.L^{-1}$ glucose, 3 $g.L^{-1}$ of yeast extract, 3 $g.L^{-1}$ of malt extract, 5 $g.L^{-1}$ of bacto peptone and 20 $g.L^{-1}$ of agar. Petri dishes were incubated in oven at 30 °C. For maintenance, yeasts were stored on plates in refrigerator at 4 °C.

2.5 Pre-inoculum

Sterile distilled water (5 mL) was added to a Petri dish containing the *R. mucilaginosa* yeast, scraping the cells to form a suspension. Then, 5 mL was transferred to a 500 mL Erlenmeyer flask containing 200 mL of the medium used for the synthetic culture (Yeast Malt medium). After adding the inoculum, the Erlenmeyer flask was taken to the "Shaker" incubator at 30 °C and 200 rpm.

2.6 Cultivation

After 24 h from the start of the pre-inoculum, the medium was centrifuged and the supernatant discarded, 50 mL of sterile distilled water were added and the cell concentration was determined in a Neubauer chamber to calculate the volume of inoculum corresponding to $1x10^7$ cells. mL^{-1}. This cell volume was inoculated into 300 mL Erlenmeyer flasks containing 100 mL of sisal fiber hydrolyzate. Erlenmeyer flasks were incubated on Shaker at 30 °C and 200 rpm for 120 h. The culture was carried out in triplicate.

2.7 Analytical Methods

2.7.1. Cell Growth

During the time intervals of 0, 3, 6, 9, 12, 24, 48, 72, 96 and 120 h, 2 mL aliquots of the medium were collected. The biomass was quantified by measuring the turbidity of sample diluted by spectrophotometry at 600 nm with a standard absorbance curve versus dry cell concentration (Aksu and Eren, 2007).

2.7.2 Yield coefficients

Parameters productivity in biomass (P_x), and substrate conversion in biomass ($Y_{X/S}$) were determined.

2.7.3 Substrate consumption

During the established time intervals, 1 mL aliquots of the fermented product were collected to measure the content of sugars using High Performance Liquid Chromatograph (HPLC).

2.7.4 Extraction, quantification and fatty acid profile of lipids

Total lipid extraction was performed using the Bligh and Dyer method (Bligh and Dyer, 1959), modified by Manirakiza et al. (2001).

2.7.5 Fatty acid profile determination

The oil extracted was transesterified according to IUPAC 2.301 standard method (IUPAC, 1987). Chromatographic profiles were recorded, and the percentage of compounds was determined against a

calibration curve using a gas chromatograph (GC) coupled to a mass detector (Shimadzu® GCMS-QP2010, Kyoto, Japan) equipped with a Durabound DB-23 30 m x 0.25 mm x 0.25 µm column. The injector and detector temperature were set to 230 °C, and the column temperature was 90 °C. The elution gradient in the column followed three different ramps: 90 to 150 °C (10 °C/min), 150 to 200 °C (5 °C/min) and 200 to 230 °C (3 °C/min), over a total of 34 min. Helium was used as carrier gas.

2.7.6 Determination of total carotenoids

Total carotenoids were measured 96 h after the inoculum, the time of the highest biomass production. An aliquot of 5 mL of the fermented product was collected and centrifuged, the supernatant was discarded and the biomass was used for quantification of total carotenoids by spectrophotometry at wavelength of 450 nm (Rodriguez-Amaya and Kimura, 2004).

3. Results and discussion

3.1 Thermogravimetric characterization of sisal fiber

Table 1 shows the result for the thermogravimetric characterization of sisal fiber.

The moisture content obtained in the thermogravimetric characterization of sisal fiber was similar to that of Lima et al. (2013), which obtained 5.7 %. Regarding the lignin content, corresponding to 27.6 %, the content of the present study was well above that reported by these authors (13.5 %). The content of hemicellulose (13.21 %) was lower than that presented by these authors in the sisal fiber characterization. The high ash content (16.0 %) characterizes sisal fiber as an excellent source of minerals, indispensable nutrients, both in yeast growth and in the accumulation of biocomposites. These changes in the ash and lignin contents can be justified by some factors such as soil type, climate and other aspects related to the climatic conditions of sisal cultivation.

Table 1 - Thermogravimetric characterization of sisal fiber

Component	Quantification (%)
Moisture	5.05
Lignin	27.6
Hemicellulose	13.21
Cellulose	27.35
Ash	16.00

3.2 Biomass produced and carbon source consumption

The growth curve of the *R. mucilaginosa* yeast in the sisal fiber hydrolyzate is shown in Figure 2.

According to Figure 1, the maximum biomass, equivalent to 4.98 $g.L^{-1}$, was produced 96 h after the inoculum. This result is similar to that found by Reyna-Martinez et al. (2015), which produced 6.27 $g.L^{-1}$ when they cultured *R. mucilaginosa* yeast in a synthetic medium based on glucose, yeast extract and peptone. The biomass produced in this study was slightly lower than that obtained by Marova et al. (2012), which produced 7.70 $g.L^{-1}$ of dry biomass of *R. mucilaginosa* using medium based on potato extract.

Cheng and Yang (2016) obtained approximately 6 $g.L^{-1}$ biomass using a mixture of cane molasses and residue supplemented with yeast extract as a substrate at pH 4. The addition of a nitrogen source to the culture medium may have had significant influence on the growth of *R. mucilaginosa* yeast thus, the biomass produced in the present study has potential to reach much higher levels, since no complementary substance, only the crude residue, was used.

Approximately 5.0 g of carbon source was consumed, resulting in a biomass sugar conversion coefficient of 0.93 ($g.g^{-1}$) of *R. mucilaginosa* yeast. Initially, there was a rapid decrease in the concentration of reducing sugars, coinciding with the logarithmic phase of yeast growth. After 24 h, there was practically no reduction of the carbon source, although the microorganism development had not yet ceased, this may possibly be justified by the presence of enzymes in the yeast capable of hydrolyzing disaccharides and other compounds, releasing glucose and other reducing sugars in the medium.

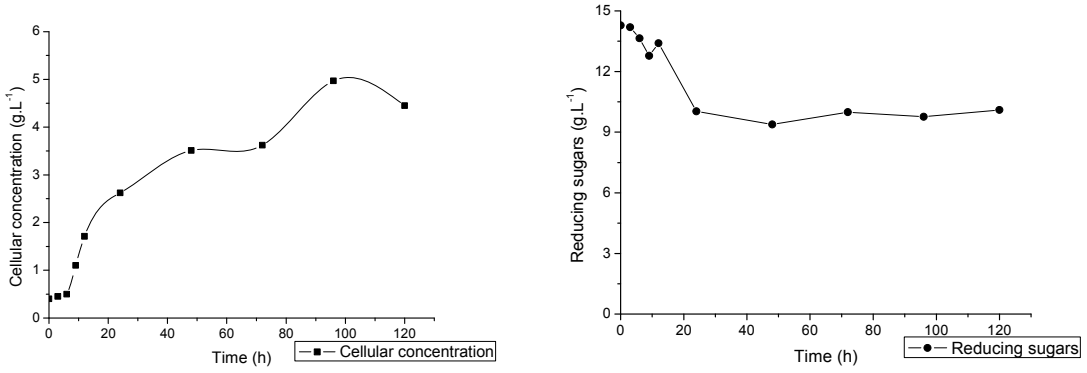

Figure 2 - Growth of R. mucilaginosa yeast in hydrolysed sisal fiber and consumption of carbon source

3.3 Lipids and carotenoids production

Table 2 shows the results for the lipids and carotenoids production and the respective yield coefficients
The lipid production by *R. mucilaginosa* yeast cultivated in the sisal fiber hydrolyzate was similar to that obtained by Reyna-Martínez et al. (2014), which produced 20.84 g.100g^{-1} lipids in dry biomass, using *R. mucilaginosa* yeast and a synthetic medium based on glucose, yeast extract and peptone. The lipid productivity with respect to the carbon source (0.21 g.g^{-1}) was higher than that obtained by Zhao et al. (2011), of 0.11 g.g^{-1} using artichoke tubers as medium extract. In relation to the carotenoid production, 0.48 mg.L^{-1}, equivalent to 98.6 µg.g^{-1}, was obtained in relation to the *R. mucilaginosa* yeast biomass. This production is similar to that obtained in one of the conditions of the study by Lopes et al. (2017), who investigated the influence of different cellular rupture methods on carotenoids extraction, when acetic acid was used as the chemical breaking agent, and 93.2 µg.g^{-1} of carotenoids were obtained. The carotenoid production of the present study was much higher than that of Naghavi et al. (2013), who obtained 0.29 mg.L^{-1} using *R. mucilaginosa* yeast and a synthetic culture medium. In this study, each gram of the carbon source used was converted into 0.21 mg of carotenoids, and this yield could be improved by optimizing some parameters influencing accumulation, such as pH, cultivation temperature and light incidence.

Table 2- Lipids and carotenoids production and respective yield coefficients

Products	Quantification	Yield coefficients	Quantification
Lipids (g.100g^{-1})	19,28±0,26	$Y_{L/S}$ (g.g^{-1})	0,21
Carotenoids (mg.L^{-1})	0,48±0,07	$Y_{C/S}$ (mg.g^{-1})	0,11

3.4 Fatty acids profile

Table 3 shows the fatty acids profile of the oil extracted from the biomass produced in the sisal fiber hydrolyzate.
The fatty acids profile of the extracted oil is very similar to that obtained from oleaginous yeasts as a source of saturated and unsaturated fatty acids, but with a predominance of unsaturated fatty acids. This result is consistent to Papanikolau and Aggelis (2011), because oleaginous yeasts are able to produce lipids with fatty acids profiles similar to those of vegetable oils, with saturated and monounsaturated fatty acids, mainly those containing from 16 to 18 carbon atoms in the chain. No short-chain fatty acids were detected, since the smaller chain was C14:0 of myristic acid. The predominant fatty acids were oleic acid (C18:1n9c) and linoleic acid (C18:2n6c). The presence of linolenic acid (C18:3n3) and linoleic acid, both belonging to the omega family, present nutritional and medicinal properties that deserve attention.
In the lipid profile of the oil obtained by Gientka et al. (2016), using *R. mucilaginosa* yeast, oleic acid (C18: 1) was also predominant, being responsible for 36.6 % of the total fatty acids. The percentage of linoleic acid (C18:2) was equivalent to 15.8 %, but both were lower than those detected in the present study.

Table 3 - Fatty acids profile of the oil extracted from the biomass produced in the sisal fiber hydrolyzate

Fat acid	%
Myristic acid, (C14:0)	1.02
Pentadecanoic acid (C15:0)	0.49
Palmitic acid (C16:0)	14.89
Palmitoleic acid (C16:1)	0.52
Heptadecanoic acid (C17:0)	1.22
Cis-10-heptadecenoic acid (C17:1)	0.48
Stearic acid (C18:0)	10.60
Oleic acid (C18:1n9c)	41.99
Linoleic acid (C18:2n6c)	21.03
Alfa -linolenic acid (C18:3n3)	5.61
Arachidic acid (C20:0)	0.55
Behenic acid (C22:0)	0.66
Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA)	0.96
Total	100.00

4. Conclusion

The sisal fiber hydrolyzate proved to be an interesting substrate for biomass, lipids and carotenoids production. The fatty acids profile of the lipids produced, predominantly long-chain fatty acids, especially oleic and linoleic acids, characterizes the oil obtained as high quality, making it potentially applicable by the industrial sector.

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