academicJournals

Vol. 9(24), pp. 1586-1594, 17 June, 2015
DOI: 10.5897/AJMR2015.7530
Article Number: A1FBE4954035
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Polyhydroxybutyrate production by *Spirulina* sp. LEB 18 grown under different nutrient concentrations

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Received 10 April, 2015; Accepted 15 June, 2015

In response to the environmental problems caused by plastics of petrochemical origin, a reduction in the use of these materials and their replacement by biodegradable polymers have been sought. Polyhydroxybutyrate (PHB), a biopolymer of biological origin that belongs to the polyhydroxyalkanoates (PHAs), is similar to polypropylene in terms of its mechanical properties, thermodegradability and melting temperature. Various microorganisms, including cyanobacteria, can synthesize this biopolymer. The objective of this study was to stimulate biopolymer synthesis by *Spirulina* sp. LEB 18 that was grown under different nutritional conditions. Initially, the growth was conducted with *Spirulina* sp. LEB 18 without the adaptation of the inoculum. In these assays, the concentrations of the carbon, nitrogen and phosphorus sources were varied. The assay that showed the maximum concentration of biopolymers was reproduced with the adaptation of the inoculum for 45 days. There was an inverse relationship between the cell growth and biopolymer synthesis. The assay that contained 0.25 g L^{-1} sodium nitrate, 4.4 g L^{-1} sodium bicarbonate and 0.5 g L^{-1} potassium phosphate showed the maximum cell concentration (0.6 g L^{-1}) and a low biopolymer accumulation (13.4%). In addition, the assay that contained 0.05 g L^{-1} sodium nitrate, 8.4 g L^{-1} sodium bicarbonate and 0.5 g L^{-1} potassium phosphate produced a high biopolymer concentration (30.7%) and a low cell concentration (0.5 g L^{-1}). The adaptation of the inoculum increased the cell concentration by 7.0% and the biopolymer yield by 20.5%. The biopolymer production was more efficient in assays in which the nitrogen was restricted and had maximum carbon consumption.

Key words: Biopolymers, polyhydroxyalkanoates, microalgae, cyanobacteria.

INTRODUCTION

Over the past 50 years, plastics of petrochemical origin have been used in many applications. Plastics are an indispensable part of many industries, having replaced glass and paper packaging over the years (Khanna and Srivastava, 2005). Their versatility, technical properties and cost (1 kg of polypropylene costs approximately US $ 1.00) have led to their widespread use.

With the forecasted reduction in oil reserves and the

biological non-degradation nature of these materials, sources for alternatives to petrochemical plastics are receiving a large amount of attention (Panda et al., 2006). Polyhydroxyalkanoates (PHAs) are biopolymers, their thermal degradation characteristics, melting temperature and mechanical properties are similar to polypropylene and can act as an alternative to plastics of petrochemical origin (Bugnicourt et al., 2014). Polyhydroxybutyrate (PHB) is the most studied representative of the PHA group. PHB also presents characteristics such as biodegradability, thermoplasticity, processibility, hydrophobicity and biocompatibility with cells and tissues, which suggests attractive applications in the food, pharmaceutical and medical areas (Sharma and Mallick, 2005). When broken down by microorganisms, PHAs form water and CO_2, which can be reintegrated into nature and close the carbon cycle. These polyesters are produced by various prokaryotic microorganisms, such as cyanobacteria (Balaji et al., 2013).

Cyanobacteria are photoautotrophic aerobes that form part of the microalgae group. Their vital processes require water, carbon dioxide, and inorganic light. Photosynthesis is the main way to obtain energy by converting nutrients in the medium to cellular material and releasing oxygen to the environment (Pelizer et al., 2003). The *Spirulina* is a photosynthetic cyanobacteria and producer of various products such as biofuels and biopolymers (Nautiyal et al., 2014; Jau et al., 2005). The strain *Spirulina* sp. LEB 18 is studied since 2008 when it was isolated. Since then several studies have been conducted with this micro-organism, such as biomass, dyes, biosurfactants, biofuels, biopolymers and effluent treatment (Morais et al., 2015).

The search for alternatives for the production of biodegradable plastics involves the search for new processes and materials while making use of biotechnology, through the use of microorganisms and their metabolic products. The knowledge of these microorganisms is very important in the conversion of certain substances into others and in the possible use of substrates in obtaining viable products and by-products.

The substrate conversion efficiency is important and depends on the physiology and biochemistry involved in the synthesis of biopolymers. Among the various nutrients in the medium, the carbon source contributes more significantly to the overall cost of the production process and to the stimulation of the biopolymer synthesis. Nitrogen and phosphorus are also important in PHB synthesis and should be limited in such a way that the metabolic pathway of the microalgae is directed to the production of biopolymers and not other bioproducts. Thus, the objective of this study was to stimulate the production of biopolymers by *Spirulina* sp. LEB 18 by varying the nutritional conditions.

MATERIALS AND METHODS

Growth of *Spirulina* sp. LEB 18 without adaptation of the inoculum

In this study, *Spirulina* sp. LEB 18 isolated from Mangueira Lagoon (33º30'12"S, 53º08'58"W) was used. Zarrouk medium was used to maintain the inoculum of *Spirulina* sp. LEB 18 (Table 1) (Zarrouk, 1966).

The cultures were grown in 2.0 L Erlenmeyer type photobioreactors with a working volume of 1.8 L. The initial biomass concentration of the assay was 0.15 g L^{-1}, maintained at 30°C in a thermostated chamber in a single batch process. The illuminance was 59.2 µmol m^{-2} s^{-1}, with a 12 h light / 12 h dark photoperiod. The assays lasted 15 days, and at the end of the growth, the extraction of the biopolymers of microalgal biomass was conducted. The carbon ($NaHCO_3$), nitrogen ($NaNO_3$) and phosphorus (K_2HPO_4) sources of the Zarrouk culture medium were evaluated in three concentrations (Table 2).

Growth of *Spirulina* sp. LEB 18 with adaptation of the inoculum

The inoculum of *Spirulina* sp. LEB 18 used in this assay was kept for 30 days with 8.4 g L^{-1} of sodium bicarbonate, 2.5 g L^{-1} sodium nitrate and 0.5 g L^{-1} potassium phosphate. After this period, the inoculum was diluted with Zarrouk with no carbon, nitrogen and phosphorus source in a ratio of 1:1 and cultured for 15 more days. After 45 days of adaptation of the inoculum, the assay with concentrations of carbon, nitrogen and phosphorus selected as the best biopolymer producer (assay 7: 8.4 g L^{-1} sodium bicarbonate, 0.05 g L^{-1} sodium nitrate, and 0.5 g L^{-1} potassium phosphate) was started. After the adaptation, the assays were started in the same conditions of 2.1 item.

Monitoring of the growth and responses

Samples were collected aseptically every 24 h to monitor the cell concentration, which was calculated by measuring the optical density at 670 nm in a spectrophotometer (Q7980RM, Quimis, Brazil), with a calibration curve that relate the optical density and dry biomass for each microalgae (Martins et al., 2014). The pH of the cultures was monitored daily with a digital pH meter (pH221, Lutron, Brazil).

From the data on the cell concentrations, the kinetic parameters of the microalgae growth and biopolymer yield (ηbiopol, %) were determined. The maximum specific growth rate (μ_{max}, d^{-1}) was calculated by exponential regression of the log phase of the cell growth curve.

The generation time (tg, d) is the time required to double the biomass. The maximum cell concentration is the maximum concentration obtained. The biomass yield (P, g L^{-1} d^{-1}) is defined as the mass formed in a given volume per unit of time. P_{max} is the

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Table 1. Composition of the Zarrouk culture medium.

Reagent	Quantity
$NaHCO_3$	16.8 (g L^{-1})
K_2HPO_4	0.5 (g L^{-1})
$NaNO_3$	2.5 (g L^{-1})
K_2SO_4	1.0 (g L^{-1})
NaCl	1.0 (g L^{-1})
$MgSO_4.7H_2O$	0.2 (g L^{-1})
$CaCl_2$	0.04 (g L^{-1})
$FeS0_4.7H_2O$	0.01 (g L^{-1})
EDTA	0.08 (g L^{-1})
Solution A5	1 mL
Solution B**6**	1 mL

Solution A_5: 2.86 g L^{-1} of H_3BO_3; 1.81 g L^{-1} of $MnCl_2.4H_2O$; 0.222 g L^{-1} of $ZnSO_4.7H_2O$; 0.079 g L^{-1} of $CuSO_4$. $5H_2O$; 0.015 g L^{-1} of $NaMoO_4$. Solution B_6: 22.96 mg L^{-1} of NH_4VO_3; 96 mg L^{-1} of $K_2Cr_2(SO_4)_4.24H_2O$; 47.85 mg L^{-1} of $NiSO_4.7H_2O$; 17.94 mg L^{-1} of $Na_2WO_4.2H_2O$; 61.1 mg L^{-1} of $TiOSO_4.H_2SO_4.8H_2O$; 43.98 mg L^{-1} of $CO(NO_3)_2.6H_2O$.

maximum productivity value found during growth. The biopolymer yield was obtained by the dry weight ratio of the microalgal biomass and the dry mass of the biopolymer extracted.

Carbon, nitrogen and phosphorus consumption during growth

The carbon concentration in the medium was determined by analysis of the alkalinity (ASTM, 2011). The nitrogen concentration was determined using the colorimetric method, where 10.0 mL of the sample is centrifuged and 0.2 ml of the supernatant is removed. Next, 0.8 mL of sulfuric acid with salicylic acid reagent was added, and after 20 min, 19.0 ml of 2N NaOH was added. After cooling, the absorbance was determined at 410 nm. Through using a standard curve, which was accomplished prior to the assays, the nitrogen concentration results were found in the form of nitrate. From stoichiometric calculations, the molecular nitrogen concentration (g L^{-1}) in the culture medium (Cataldo et al., 1975) was determined.

The phosphorus concentration was determined by phosphate analysis using a colorimetric kit (PhosVer 3, Hach, USA). In this analysis, a 10 mL sample of the previously diluted medium is placed in contact with the phosphate Kit, and after 2 min, a reading of the absorbance is conducted at 890 nm. Through the standard curve, the phosphorus concentration (g L^{-1}) was determined and given in molecular phosphorus form. Carbon, nitrogen and phosphorus were analyzed after 0, 5, 10 and 15 days of culture.

Extraction of biopolymer by digestion

The microalgal biomass was centrifuged at 7500 rpm for 20 min at 20°C, and the supernatant was then discarded. The precipitate obtained in the centrifugation was stirred for 10 min with sodium hypochlorite (10.0%, v/v) and distilled water to give a final concentration of 4.0% (v/v). The centrifugation and stirring with sodium hypochlorite steps were repeated. The precipitate was then washed with distilled water, centrifuged and washed with acetone while stirring for 10 min. The precipitate was centrifuged to separate the liquid and taken to the oven (Quimis, Q317 M, Brazil) at 35°C for 48 h. The obtained biopolymer was then weighed (Martins et al., 2014).

Characterization of the extracted biopolymer

The samples were characterized for the initial degradation temperature (TD_0, °C), final degradation temperature (TD_f °C) and non-degraded material (IP, % w/w) by differential thermogravimetric analysis (DTG) (DTG-60, Shimadzu, Japan). Approximately 5.0 mg of the sample was heated from 25 to 500°C at 10°C min^{-1} under a nitrogen atmosphere. The initial and final temperatures of the degradation were determined by the first derivative of the DTG curves, and the degree of the non-degraded material was considered to be the amount of sample that remained at the end of the analysis.

RESULTS AND DISCUSSION

The assays performed without the adaptation of inoculum as well as those with prior adaptation showed cell growth during the 15 days of growth, with no lag phase and cell decline observed. The phase adjustment or lag phase is the period in which the cell synthesizes the enzymes necessary for the use of the components present in the medium. If the cells are pre-adapted to the environmental conditions of the assay, then the length of time of the induction phenomenon can be reduced or does not exist. In these assays, although the nutrient concentration is changed, the sodium nitrate, sodium bicarbonate and potassium phosphate used in the assays are the same sources of nitrogen, carbon and phosphorus as in the Zarrouk culture medium, respectively. Thus, *Spirulina* sp. LEB 18 showed no lag phase because it did not need to develop differentiated structures to metabolize the nutrients.

Among the assays performed without adaptation of the inoculum, it was observed that assay 7 (0.05 g L^{-1} $NaNO_3$, 8.4 g L^{-1} of $NaHCO_3$ e 0.5 g L^{-1} of K_2HPO_4) showed a maximum biopolymer yield (30.7%). In assay 7, a lower growth trend was observed, followed by assay 9, which had the second highest biopolymer yield (22.7%) (Figure 1). Assay 6 showed a growth curve that was superior to the others after 10 days of culture (Figure 1), reaching the final 15 days with a maximum cell concentration of (0.6 g L^{-1}) and biopolymer yield of 13.4% (Table 3).

From the growth curves in Figure 1 and Table 3, it was observed that the tests where the cell concentration is maximum, the yield of PHB was minimal. The same behavior was observed by Samantaray et al. (2011), when growing the cyanobacterium *Aulosira fertilíssima* to obtain PHB.

The maximum specific growth rate was between 0.07 d^{-1} (assay 8) and 0.1 d^{-1} (assay 1). Assay 8 was conducted with the maximum values for the carbon, nitrogen and phosphorus sources, while assay 1 showed the minimum

Table 2. Concentrations of nitrogen, carbon, phosphorus and C/N ratio added in the assays performed with *Spirulina* sp. LEB 18.

Assay	$NaNO_3$(g L^{-1})	$NaHCO_3$(g L^{-1})	K_2HPO_4(g L^{-1})	C/N
1	0.05	4.4	0.1	78
2	0.25	4.4	0.1	16
3	0.05	8.4	0.1	149
4	0.25	8.4	0.1	30
5	0.05	4.4	0.5	78
6	0.25	4.4	0.5	16
7	0.05	8.4	0.5	149
8	0.25	8.4	0.5	30
9	0.15	6.4	0.3	38
7a*	0.05	8.4	0.5	149

*Assay 7 with adaptation of the inoculum

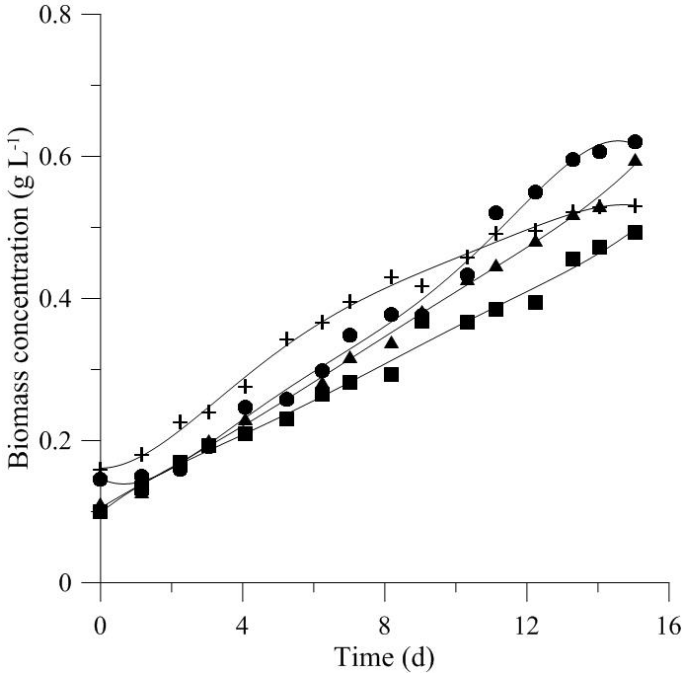

Figure 1. Cell concentration as a function of time for *Spirulina* sp. LEB 18 grown without adaptation of the inoculum. ● 0.25 g L^{-1} of $NaNO_3$, 4.4 g L^{-1} $NaHCO_3$ and 0.5 g L^{-1} K_2HPO_4 (Assay 6), ■ 0.05 g L^{-1} of $NaNO_3$, 8.4 g L^{-1} $NaHCO_3$ and 0.5 g L^{-1} K_2HPO_4 (Assay 7), ▲ 0.15 g L^{-1} of $NaNO_3$, 6.4 g L^{-1} $NaHCO_3$ and 0.3 gL^{-1} K_2HPO_4 (Assay 10), and + *Spirulina* sp LEB 18 grown with adaptation of the inoculum containing (+) 0.05 g $L^{-1}$of $NaNO_3$, 8.4 g L^{-1} $NaHCO_3$ and 0.5 g L^{-1} K_2HPO_4.

values. The minimum generation time was 5.9 days (assay 8). The minimum X_{max} found was 0.4 g L^{-1} in assay 9, which showed a low carbon consumption (2.4%, Table 3) and P_{max} (0.02 g L^{-1} d^{-1}). The low carbon consumption of assay 9 could have hampered the growth of the microorganism. Carbon is one of the most important chemical elements for the growth of the microorganisms and is responsible for the formation of organic nutrients of the 3 major classes: carbohydrates, proteins and lipids. These compounds provide energy for cell growth. A typical cell consists of approximately 50.0% carbon, which is the principal component of all of the classes of macromolecules (Markou et al., 2014).

The highest yield was observed in assay 7 (0.04 g L^{-1} d^{-1})

Table 3. Maximum cell concentration ($X_{max,}$ g L-1), maximum yield (P_{max}, g L^{-1} d^{-1}), maximum specific growth rate (μ_{max}, d^{-1}), generation time (tg, d) and biopolymer yield (ηbiopol, %) on day 15 for *Spirulina* sp. 18 LEB grown with different concentrations of sodium nitrate, sodium bicarbonate and potassium phosphate.

Assay	X_{max}	P_{max}	μ_{max}	tg	η_{biopol}
1	0.4	0.03	0.07	10.0	14.1
2	0.4	0.03	0.07	9.6	17.3
3	0.5	0.03	0.08	8.9	16.9
4	0.5	0.03	0.08	8.2	14.7
5	0.5	0.03	0.08	8.8	13.4
6	0.6	0.03	0.10	6.7	13.4
7	0.5	0.04	0.10	7.1	30.7
8	0.6	0.03	0.12	5.9	16.3
9	0.4	0.03	0.11	6.5	22.7
7a	0.5	0.03	0.07	8.6	38.6

followed by growth with the same concentration of carbon, nitrogen and phosphorus with adaptation of the inoculum (0.03 g L^{-1} d^{-1}). Comparing assay 7 with the others, it was found that the growth was conducted with the smallest maximum concentration of nitrogen, carbon (higher C/N tested) and maximum phosphorus. The microorganisms respond to nitrogen limitation by the preferential degradation of one or more macromolecules that contain this component, which results in the reduction of these nutrients and the accumulation of carbon reserve compounds such as lipids, including PHAs. The phosphorus component is essential to the metabolism of microalgal having direct influence on the formation of organic molecules such as nucleic acids (DNA and RNA), membrane phospholipids and ATP (Markou et al., 2014).

When repeating the conditions of assay 7 but performing adaptation of the inoculum for 45 days, an increase of 7.0% in the cell concentration and 20.5% in the yield of the biopolymers was observed. When Panda et al. (2006) conducted an inoculum adaptation of *Synechocystis* sp. PCC 6803 with glucose and an addition of 1.5 g L^{-1} of $NaNO_3$, 0.04 g L^{-1} K_2HPO_4 and 0.02 gL^{-1} of Na_2CO_3 in 150 ml flasks with 50 ml BG11 culture medium, at a temperature of 28°C with a 14 h light and 10 h dark photoperiod, they observed an increase of 29.0% in the PHB accumulation.

From the growth curves (Figure 1), it was observed that the inoculum-adapted assay had a higher development than the others until the 10th day of growth. After this period, the microalgae reduced its growth, finalizing the growth deceleration phase and entering the stationary phase. The deceleration phase occurs from the exhaustion of one or more components of the culture medium necessary for growth and from the accumulation of inhibitory metabolites. The instantaneous and specific growth velocities decrease until they cancel out each other at the end of the time (t_f), which is when the stationary phase begins.

This reduction in growth after 10 days occurred in parallel to the limitation of nitrogen that had already been 100% consumed at this time (Table 4). Thus, the microorganism reduced the biomass production by the limitation of the source of the nitrogen, and the biopolymer synthesis was stimulated, which occurred in assay 7a, where the maximum value was found (38.6%).

To better understand the PHB synthesis process by cyanobacteria, we will divide into three stages: photosynthesis, tricarboxylic acid cycle (TCA) and the synthesis of PHB. In the first stage, light energy is collected by breaking water molecules and releasing O_2, ATP, NADPH and glyceraldehyde-3-phosphate. It is suffering reactions to yield acetyl-CoA. The second stage is responsible for the use of acetyl-CoA in the formation of carbon skeletons and energy generation. The third step is the synthesis of PHB, which starts by the condensation of two acetyl-CoA molecules by the enzyme β-ketothiolase give acetoacetyl-CoA. This in turn is reduced to (R) -3-hydroxybutyryl-CoA reaction catalyzed by the enzyme 3-ketoacyl-CoA reductase NADPH dependent. The formation of PHB is terminated by polymerizing two or more molecules of (R) -3-hydroxybutyryl-CoA by the enzyme catalyzed reaction PHB synthase (Wang et al., 2013).

Then under balanced growth conditions, where all of the nutrients necessary for cell multiplication are available, the levels of free co-enzyme A (CoA) are high. Thus, it is possible to meet the great demands of the acetyl groups with the Krebs cycle, for the formation of carbon skeletons and energy generation. Free CoA has an inhibitory effect on the enzyme β-ketothiolase, which prevents the synthesis of PHB. When a certain nutrient becomes limiting to the multiplication of the microorganism, the demand for acetyl-CoA decreases. Thus, the

Table 4. Nitrogen concentration at time zero (X_{NO}, mg L^{-1}), nitrogen concentration at time 15 d (XN_{15}, mg L^{-1}), nitrogen consumption after 15 days (C_{N15}, % w/w), carbon concentration at time zero (X_{C0}, g L^{-1}), carbon concentration at time 15 days (X_{C15}, g L^{-1}), carbon consumption after 15 days (C_{C15}, % w/w), phosphorus concentration at time zero (X_{PO}, g L^{-1}), phosphorus concentration at time 15 days (X_{P15}, g L^{-1}) and carbon consumption after 15 days (C_{P15}, % w/w) in the *Spirulina* sp. 18 LEB assays.

Assay	X_{N0}	X_{N15}	C_{N15}	X_{C0}	X_{C15}	C_{C15}	X_{P0}	X_{P15}	C_{P15}
1	15.7	0.8	94.7	0.4	0.3	15.0	0.10	0.07	30.0
2	61.5	1.7	97.2	0.5	0.4	17.8	0.10	0.07	30.0
3	14.9	0.2	98.4	1.2	1.0	19.0	0.10	0.07	30.0
4	61.6	0.7	98.7	1.3	1.0	21.7	0.10	0.07	30.0
5	13.0	Nd	100	0.5	0.4	17.6	0.49	0.14	71.4
6	53.1	0.4	99.3	0.4	0.4	4.5	0.50	0.15	70.0
7	12.3	Nd	100	1.2	0.9	17.2	0.50	0.13	74.0
8	52.2	0.02	99.9	1.2	1.1	6.5	0.50	0.14	72.0
9	21.9	Nd	100	0.4	0.4	2.4	0.33	0.09	72.7
7a	15.7	Nd	100	1.2	0.6	50.0	0.50	0.29	42.6

*Nd: Undetectable.

free CoA level becomes reduced, decreasing the inhibition of the β-ketothiolase and triggering the synthesis of PHB (Laycock et al., 2013).

The process of reducing acetoacetyl-CoA to (R)-3-hydroxybutyryl CoA by 3-ketoacyl-CoA reductase enzyme is NADPH dependent, relationship between NADP and NADPH is necessary for the reaction. As previously mentioned, this relationship is maintained by photosynthesis, which produces O_2 and NADPH. It is understood then that for PHB accumulation of NADPH/ NADP ratio should be high, as observed by Hauf et al. (2013), which monitor the presence of NADP and NADPH in the cells of Synechocystis PCC6803 to obtain PHB obtained as the best response to relative high NADPH/NADP. The authors, as in this study, have obtained high C/N to be the best for obtaining PHB.

In the inoculum-adapted assay, biopolymer extraction was conducted during 5, 10 and 15 days of assays, obtaining a 25.2, 40.9 and 38.6% yield, respectively. The maximum biopolymer yield (40.9%) obtained at day 10 could be due to the microorganism being at the end of the deceleration phase and in the early stationary phase. In the deceleration phase, the microorganism reduces its growth and therefore can target its metabolism to bioproduct synthesis, in this case the biopolymers.

Sharma and Mallick (2005) grew the microalgae *Nostoc muscorum* for 42 days, periodically monitoring the PHB accumulation. The authors obtained a maximum PHB yield (8.6%) at day 21 of the growth, which is similar to that obtained in this study, at the end of the deceleration phase and in the early stationary phase. Panda et al. (2006) obtained a maximum PHB accumulation in the stationary phase (4.5 w/w) compared to the lag (1.8%) and Log (2.9%) phases.

In this study with *Spirulina* sp. LEB 18, there was a reduction in the concentration of the polymer after 15 days of growth compared to day 10. This reduction occurred because during the stationary phase, there is a balance between the growth rate and death rate of the microorganism as well as biochemical changes in the cell structure that stop the synthesis of the byproducts. According to Sharma and Mallick (2005), after the *Nostoc muscorum* reached the maximum biopolymer yield in the early stationary phase, there was a reduction in the buildup of the byproduct. The authors explain that this reduction is due to the use of PHB as a carbon source by the microorganism.

At the beginning of the growth, the pH of the assays without inoculum adaptation presented values between 9.9 and 10.3, and after 15 days, these values were 10.5 and 10.7. The minimum observed was pH 9.8 at day 3 of the culture (Assay 9). Assay 8 showed the maximum pH value (10.8), which was observed on day 15. According to Sharma and Mallick (2005), the maximum accumulation of PHB in the culture with the microalgae *Nostoc muscorum* grown in the BG-11 medium for 21 days was 8.9% (w/w) at pH 8.5, followed by 7.8% (w/w) at pH 9.5 and 7.2% at pH 10.5. In acidic pH, the authors did not have detectable amounts of PHB.

Assays 1, 2, 5 and 6, which had 4.4 g L^{-1} sodium bicarbonate solution, showed stable pH during the whole growth period. With the exception of assay 6, cultures 1, 2 and 5 showed pronounced consumption of carbon (15.0 and 17.8%), which justifies the pH stability (Table 4). In cultures that had higher concentrations of sodium bicarbonate (6.4 and 8.4 g L^{-1}), the pH increased by approximately 0.7 units.

The inoculum-adapted culture showed a pH that was superior to that of assays without adaptation, which ranged from 10.2 to 11.1, with a value of 10.3 on the first

day of growth and 10.4 on the last day. Sodium bicarbonate has a buffering action, which causes the pH to remain stable. The inoculum-adapted culture had the highest carbon source consumption (50.0%) (Table 4) and high pH stability compared to the other assays.

The assays conducted without inoculum adaptation presented nitrogen consumption between 94.1 and 100% (Table 4). With the maximum biopolymer yield (30.7%) compared to cultures without adaptation of the inoculum, assay 7 showed 100% nitrogen consumption before the 5th day. Panda et al. (2006) obtained 9.5% PHB in *Synechocystis* sp. PCC 6803, grown under a nitrogen limitation.

Assay 9 showed 0.00006 and 0.0 g L^{-1} of nitrogen on day 10 of the culture and a high concentration of biopolymers. The growth conducted with inoculum adaptation showed 100% nitrogen consumption before 10 days of assays, and the yield of biopolymers exceeded that of the assays that were not previously adapted. Samantaray et al. (2011), in *Aulosira fertilíssima* grow observed that the production of phb is connected to a source of nitrogen limitation in the medium. When this nutrient is limited, PHB production is stimulated. According to Koller et al. (2008), in the stationary phase, when the nitrogen limitation occurs, carbon flux is applied in three directions: power maintenance; PHB production and storage of the carbon source; and excretion of the intermediary metabolites of the cells.

When grown under nitrogen limitation, PHB synthase enzyme was found in the membrane of the cyanobacterium *Synechocystis* PCC6803 (Hauf et al., 2013). The PHB synthase is activated by acetyl phosphate. The second enzyme involved, called phosphotransacetylase, converts acetyl CoA to acetyl phosphate. It is regulated by the acetyl CoA concentration and the ratio of carbon to nitrogen (C/N) in the cell. Thus, the acetyl phosphate can act as a sign of C/N balance that affects the PHB synthesis metabolism (Kessler and Witholt, 2001). According to Lee (1996), the production of polyhydroxyalkanoates is most effective when nutrients such as nitrogen and phosphorus are limited but still present in the medium. The premature absence of nutrients can cause cell death and consequently will not produce PHB.

The maximum carbon consumption in cultures without adaptation of inoculum was 21.7% in assay 4, which contained the highest concentration of sodium bicarbonate (8.4 g L^{-1}). Assays 3 and 7, which also had 8.4 g L^{-1} of $NaHCO_3$ in the medium, showed 19.0 and 17.2% carbon consumption, respectively. Only assay 8, grown with 8.4 g L^{-1} of sodium bicarbonate, had a lower uptake (6.5%), where the difference in this experiment was the nitrogen concentration (0.25 g L^{-1}) and P (0 5 g L^{-1}).

The nutrients added to the culture medium do not act alone on the growth and bioproduct formation of the microorganisms. This interaction of nutrients can cause a change in the concentrations of these components, which can direct the use of the same substrate differently and can stimulate an increase or reduction in use by the cells.

The assay in which adaptation of the inoculum was conducted showed a carbon consumption of 50.0% of the total added. The previous adaptation of the inoculum to the condition in which the microalgae will be exposed during growth causes the cell to be stable in this nutritional status. Thus, the microorganism does not suffer stress due to the medium change, and in addition, it already has the intracellular components needed to metabolize these active nutrients. The culture that had the same concentrations of carbon, nitrogen and phosphorus but that had not been inoculum-adapted showed a 17.2% carbon consumption.

The assays that had the largest concentrations of phosphorus (0.3 and 0.5 g L^{-1}) had the maximum consumption values (70.0 and 74.0%). Among the cultures without adaptation, assay 7 had the highest concentration of biopolymers (30.7%) and phosphorus consumption.

Sharma and Mallick (2005) kept *Nostoc muscorum* for 21 days in a medium that contained different carbon sources and later started growth with a phosphorus deficiency. The authors observed that the maximum yield of PHB was obtained at time zero, when the phosphorus concentration was higher. In cultures with *Synechocystis*, Wang et al. (2013) observed that the quantity of phosphorus reached undetectable values and thus cell growth did not reach the concentration that was required to promote the production of PHB. Thus, the authors added 30.0 mg L^{-1} of potassium phosphate to the assay, and after three days of culture, the concentration of PHB was significantly increased. These results indicate that the absence of phosphorus does not favor the production of biopolymers. Phosphorus is an important nutrient for the production of ATP. Excess ATP causes a reduction in oxidative phosphorylation and an accumulation of reduced coenzymes (NADH), which leads to the formation of PHB, whose metabolic pathway reoxidizes these coenzymes (Dawes and Sênior, 1973).

The assay with the inoculum adaptation showed less phosphorus consumption (42.6%) when compared to the assays without adaptation (74.0%). In cultures that were performed with *Synechocystis* sp. PCC 6803 and phosphorus limitation, there was an increase of 11.0% in the concentration of the biopolymers compared to assays with no phosphorus limitation.

The biopolymers extracted from *Spirulina* sp. LEB 18 had an initial degradation temperature of 163.5 to 208.8°C (Table 5). The commercial PHB presented the TG_0 of 233.4°C. Samantaray et al. (2011), showed the initial temperature of PHB degradation to 174.0°C for *Aulosira fertilissima*. The polymer degradability characteristics depend on the chemical nature of the raw

Table 5. Initial temperature degradation responses (TG_0, °C), final degradation temperature (TG_f, °C) and non-degraded material (IP, %) of the biopolymers extracted from *Spirulina* sp. 18 LEB.

Assay	TG_0	TG_f	IP
1	189.1	332.2	31.4
2	163.5	322.7	30.9
3	179.3	355.8	33.3
4	168.4	324.1	32.7
5	183.3	333.5	40.2
6	178.8	324.0	36.2
7	203.6	305.0	33.2
8	185.6	337.5	36.7
9	189.6	337.3	40.2
7a	208.8	316.4	36.7
Commercial PHB	233.4	293.9	2.3

material offered as a carbon source, the environmental conditions for the operation of the process and the selected microorganism. Thus, the polymer characteristics can be directed during the process (Castilho et al., 2009).

The final degradation temperatures varied from 305.0 to 337.3°C. The assay in which adaptation of the inoculum occurred showed TG_0 and TG_F values of 208.8 and 316.4°C, respectively. Assay 7 and the inoculum-adapted assay showed initial and final temperature values closest to the commercial PHB degradation compared to the other cultures performed.

The profiles of the DTG curves of the extracted biopolymer samples showed little reduction in weight before the start of the degradation of the polymer. This reduction is due to the moisture of the samples, and the same was not true in the commercial sample, which had appropriate treatment and packaging to not absorb moisture.

The commercial PHB sample showed a constant weight after the final degradation temperature, because this sample has only a small amount of non-degraded material (2.3%). However, the same behavior is not observed in samples of biopolymers that were extracted from microalgae. In this sample, the presence of non-degraded material causes the weight to continue decreasing with increasing temperature even after the end of the biopolymer degradation. This finding can be confirmed by the high content of the non-degraded material contained in the samples, which reached 40.2% in assay 9.

Conclusions

In the growth of *Spirulina* sp. LEB-18 at different nutrient concentrations, it has been found that the microalgal growth was inversely related to the synthesis of the biopolymers. The maximum cell growth occurred in assay 6, with a concentration of 0.62 g L^{-1}, and the biopolymer yield was 13.4%. While in assay 7, the cell growth was 0.5 g L^{-1}, and the biopolymer accumulation was 30.7%. By making the reproducibility of the best conditions with prior adaptation to inoculation, an increase in the biopolymer yield of 20.5% was observed. Through the assays, the importance of the carbon source in the production of biopolymer was observed, with a maximum synthesis observed in the assay that had the highest consumption of carbon compared to the others.

Biopolymer production is more efficient when nutrients such as nitrogen and phosphorus are restricted but present in the medium. The initial and final degradation temperatures of the assays that had the maximum biopolymer yield were close to the value of commercial PHB (233.4 to 293.9°C). Biopolymers, especially polyhydroxyalkanoates, play an important role in the plastics market due to their biodegradability and the use of renewable resources in their production, which reduces environmental problems such as the pollution caused by plastics of petrochemical origin.

Conflict of interest

Author have not declared any conflict of interest.

ACKNOWLEDGEMENT

The authors would like to thank CNPq (National Council of Technological and Scientific Development), CGTEE (Company of Thermal Generation of Electric Power) and MCTI (Ministry of Science, Technology and Innovation) for their financial support of this study.

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