Algal Research 33 (2018) 231–238

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Efficacy of *Spirulina* sp. polyhydroxyalkanoates extraction methods and influence on polymer properties and composition

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ARTICLE INFO

Keywords:
Cyanobacteria
Biopolymer
Polyhydroxyalkanoate
Microalgae

ABSTRACT

The objective of this study was to evaluate the efficacy of methods used to extract polyhydroxyalkanoates from *Spirulina* sp. LEB-18 microalgae and to examine the influence of these methods on the purity, properties, and composition of the polymers. Polyhydroxyalkanoates were characterized by Fourier transform infrared spectroscopy (FTIR), molecular mass, degree of crystallinity, and monomer composition. The efficacy of the extraction methods varied, with polyhydroxyalkanoate accumulation.

between 6.10 and 9.80%, and degrees of purity between 63.51 and 93.62%. Using sodium hypochlorite in the initial stage of the extraction increased accumulation, while using methanol at the end of the process increased the purity of the polymers. The molecular mass and crystallinity index of the polyhydroxyalkanoates varied, showing that the extraction methods interfered with polymer properties. The composition of polyhydroxyalkanoates was also influenced by the extraction, with varying percentages of monomers identified. The copolymers of the polyhydroxyalkanoates obtained are formed by the monomers 11-hydroxyhexadecanoate, in a higher proportion, hydroxyheptanoate and hydroxytetradecanoate, demonstrating that *Spirulina* sp. LEB-18 is capable of producing medium and long chain polymers. The detection of these monomer blocks in the polyhydroxyalkanoate structure of this microalga is an important scientific novelty because these monomer blocks are constituents of new polymers. An indirect relationship ($R^2 = 0.8044$) was observed between the percentage of the 11-hydroxyhexadecanoate monomer and the degree of crystallinity of polyhydroxyalkanoates obtained by the different methods. This suggests that obtaining the polymer with medium- and long-chain monomers contributes to reduction of crystallinity.

1. Introduction

Polyhydroxyalkanoates (PHAs) are polyesters that are synthesized and accumulated intracellularly as granules by numerous microorganisms [1,2]. The properties of PHAs, including thermoplastic processability, absolute resistance to water, and complete biodegradability, suggest that PHAs can be a potential substitute for common plastics [3–5]. Despite these attractive characteristics, the use of PHAs in food packaging, biomedicine, pharmaceutical industry, and other applications is limited by high costs of production and extraction [2,6].

Cyanobacteria are a phylum of bacteria that can be used to obtain PHAs at a lower cost. This is possible because cyanobacteria have minimal nutrient requirements and possess photoautotrophic nature; these sole prokaryotes accumulate PHAs by oxygenic photosynthesis [7,8]. More than 100 cyanobacterial strains have been screened thus far; approximately 70% of these strains contain PHAs at concentrations ranging from 0.04 to 40% of dry cell weight under photoautotrophic growth conditions [9].

The method used to extract PHAs also affects the costs of obtaining, characteristics, and monomeric composition of the biopolymers, which, in turn, impacts their industrial application. The properties and application potential of these materials depend largely on monomer composition [6].

PHAs are comprised of monomers possessing a carbon backbone from 4 to 16 carbon atoms long, and a broad range of functional groups such as halogens, phenoxy, acetoxy, phenyl, cyano, and epoxy groups

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https://doi.org/10.1016/j.algal.2018.05.016
Received 13 December 2017; Received in revised form 17 May 2018; Accepted 18 May 2018

[5,10]. The method of extraction is very important for monomeric composition and can influence the properties of PHAs. Because it is a product accumulated in the cellular cytoplasm, PHAs are extracted from cells after production (culture) [1]. Extraction involves different procedures that ensure proper removal of the biopolymer from the interior of the cells. These include destabilization and/or cellular disruption, separation of the culture medium from the biomass, recovery of the biopolymer, and purification [2,7]. The methods used for extraction of PHAs include application of organic solvents; supercritical fluids; biological digestion (enzymes); application of mechanical methods, such as high-pressure homogenization and ultrasound; combined mechanical and chemical methods; and studies of spontaneous release of PHAs [5,6,11]. Extraction with organic solvents is employed most because of ease of application, low degradation, and high purity of the extracted product [12].

Because extraction methods are so important in obtaining PHAs, it is essential to study how these methods and efficiency of processing affect the molar mass, degree of crystallinity, monomer composition, and other characteristics of the final product. These polymeric characteristics are vital because they are directly related to the application and degradation time of the polymer [6].

Thus, the objective of this work was to evaluate and compare the efficacy of six different methods used to extract PHAs from the biomass of *Spirulina* sp. LEB-18 microalgae; we then correlated the results with the purity, composition, and properties of the obtained biopolymers.

2. Material and methods

2.1. Microorganism, culture medium, and cultivation conditions

In this study, we used *Spirulina* sp. LEB-18 isolated from the Mangueira Lagoon (33°30′12″S, 53°08′58″W; Rio Grande, Brazil). Zarrouk medium was used to maintain the inoculum of *Spirulina* sp. LEB-18. The medium constituents were 16.8 g L^{-1} $NaHCO_3$, 0.5 g L^{-1} K_2HPO_4, 2.5 g L^{-1} $NaNO_3$, 1.0 g L^{-1} K_2SO_4, 1.0 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.04 g L^{-1} $CaCl_2$, 0.01 g L^{-1} $FeS0_4{\cdot}7H_2O$, 0.08 g L^{-1} EDTA, and micronutrients [13].

Outdoor cultivation was conducted in raceway tanks with lengths, breadths, and heights of 2.20 m, 0.90 m, and 0.35 m, respectively; these tanks contained 240 L of *Spirulina* sp. LEB-18. The tank culture was agitated by submersible pumps 24 h per day with water temperature ranging from 37 to 26 °C. The volume of culture media was maintained by periodic addition of potable water to compensate for evaporation and maintain the volume at 240 L. Batch cultures were managed during the month of August (winter). The cultures were maintained under natural light, using a photoperiod of 12 h (light/dark), in the tank for up to 30 days; the initial cell concentration was 0.2 g L^{-1}. This period, cell concentration was determined by optical density using a digital spectrophotometer (BEL PHOTONICS UV-M51, Piracicaba – SP – Brazil) at the wavelength of 670 nm. Before the experiments, a standard growth curve of *Spirulina* sp. LEB-18 was generated and used to correlate optical density with the dry-weight biomass (Cell concentration g L^{-1} = 0.617 × Absorbance + 0.00125, R^2 = 0.9916).

The biomass produced in the pilot scale was removed, separated by centrifugation at 10000 rpm for 15 min (HITACHI CR22GIII, São Paulo – SP – Brazil), frozen, lyophilized, vacuum-packed, and stored until further testing.

2.2. Extraction methods

Six different methods were used to extract PHAs from the dry biomass (Table 1). Each extraction method was tested using 3 g of dry biomass. In the M 1 method, the biomass was washed with a solution of 4% sodium hypochlorite for 20 min at 45 °C and centrifuged. The supernatant was discarded, and the precipitate was washed with distilled water and re-centrifuged. The supernatant was discarded again, and acetone was added for 2 h at 45 °C to precipitate the biopolymer. The precipitate was dried in an oven at 35 °C for 48 h [11].

In the M 2 method, the biomass was washed with 4% sodium hypochlorite for 20 min at 45 °C and then centrifuged. The supernatant was discarded, and the polymer was extracted in hot chloroform for 3 h at 80 °C, followed by precipitation from the chloroform solution into chilled methanol. The methanol-chloroform mixture was decanted, and the precipitated polymer was separated by centrifugation. Then, the polymer was dissolved in chloroform and precipitated using evaporation of the solvent according to Samrot et al. [14] with modifications.

In the M 3 method, the biomass was suspended in methanol overnight at 4 °C to remove the pigments. The pellet obtained after centrifugation was dried at 60 °C, and the polymers were extracted in hot chloroform for 3 h at 80 °C. The PHAs was precipitated from the chloroform solution into chilled methanol. The methanol-chloroform mixture was decanted, and the precipitated polymer was separated by centrifugation. Then, the polymer was dissolved in chloroform and obtained via evaporation of the solvent [15].

Method M 4 was used according to Martins et al. [11] with modifications. First, the biomass was washed with 4% sodium hypochlorite solution for 20 min at 45 °C and then centrifuged. The supernatant was discarded, and the precipitate was washed with distilled water and re-centrifuged. The supernatant was discarded again, and chloroform was added for 3 h at 80 °C to precipitate the biopolymer; in this approach, chloroform was used instead of the acetone used in Method M 1. The precipitate was then dried in an oven at 35 °C for 48 h.

Method M 5 was used according to Penloglou et al. [16] with modifications. The dried biomass was suspended in methanol overnight at 4 °C to remove the pigments. The pellet, obtained after centrifugation, was dried at 60 °C, resuspended in distilled water, and incubated in an ultrasonic bath for 30 min. Then, the sample was centrifuged, and the supernatant was discarded. PHAs were extracted with hot chloroform for 3 h at 80 °C. Afterwards, the sample was filtered, and the polymer was obtained via evaporation of the solvent.

The M 6 method was similar to the M 5 method, but excluded the initial methanol-wash step. The dried biomass was resuspended in distilled water, incubated in an ultrasonic bath for 30 min, and centrifuged. PHAs were extracted with hot chloroform for 3 h at 80 °C. The sample was then filtered, and the polymer was obtained via evaporation of the solvent [16].

All extractions were performed in triplicate.

2.3. Accumulation of PHAs

The accumulation of PHAs was calculated gravimetrically, with respect to the initial mass of dry biomass employed in each extraction, according to Eq. (1).

$$Y = \frac{mp \times 100}{mb} \quad (1)$$

where

Y is the accumulation of PHAs expressed as a percentage;
mp is the mass of PHAs obtained in grams;
mb is the mass of dry biomass used in the extraction and expressed in grams.

2.4. Properties of PHAs

2.4.1. Fourier transform infrared spectroscopy (FTIR)

Samples of PHAs were qualitatively analyzed with Fourier transform infrared spectroscopy (FTIR; PerkinElmer Model Spectrum 100) between 4000 and 600 cm^{-1} using ATR accessory with a zinc selenide crystal.