Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioresource Technology 97 (2006) 1296–1301
BIORESOURCE TECHNOLOGY

Optimization of cultural and nutritional conditions for accumulation of poly-β-hydroxybutyrate in *Synechocystis* sp. PCC 6803

Bhabatarini Panda, Praveen Jain, Laxuman Sharma, Nirupama Mallick *

Agricultural and Food Engineering Department, Indian Institute of Technology, Kharagpur 721 302, India

Received 5 January 2005; received in revised form 27 May 2005; accepted 28 May 2005
Available online 19 July 2005

Abstract

Poly-β-hydroxybutyrate (PHB) accumulation in the unicellular cyanobacterium, *Synechocystis* sp. PCC 6803, was studied under various cultural and nutritional conditions. Under controlled condition, cells harvested at the stationary phase of growth depicted maximum accumulation of PHB, i.e., 4.5% (w/w of dry cells) as compared to lag (1.8%) or logarithmic (2.9%) phases of cultures. A temperature range of 28–32 °C and pH between 7.5 and 8.5 were preferred for PHB accumulation. Cells cultivated under regular light–dark cycles accumulated more PHB (4.5%) than those grown under continuous illumination (2.4%). Nitrogen and phosphorus starvation stimulated PHB accumulation up to the tune of 9.5 and 11% (w/w of dry cells), respectively. *Synechocystis* cells pre-grown in glucose (0.1%)-supplemented BG-11 medium when subjected to P-deficiency in presence of acetate (0.4%), PHB accumulation was boosted up to 29% (w/w of dry cells), the value almost 6-fold higher with respect to photoautotrophic condition. Fishpond discharges were found as suitable media for PHB accumulation in the test cyanobacterium.

Keywords: Carbon sources; N and P deficiency; Poly-β-hydroxybutyrate; *Synechocystis* sp. PCC 6803

1. Introduction

In the last 50 years, petrochemical-based plastics have become one of our most applied materials. Their versatility, outstanding technical properties and relatively low cost (1 kg of polypropylene costs about US$ 1.0) caused their success. However, it is well known that these plastic materials are not biologically degradable. Predicted end of oil reserves in near future and non-degradability of petrochemical-based plastics have paved the way for alternative sources of biodegradable plastics (Brandl et al., 1995).

Polyhydroxyalkanoates (PHAs), a group of biodegradable polymers of biological origin, have gained tremendous impetus in recent years. These are isotactic, highly crystalline and stiff polymers. Their glass to rubber transition temperature (T_g), melting temperature (T_m), and mechanical properties like Young's modulus and tensile strength are comparable with the isotactic polypropylene. Other properties of PHAs useful for specific applications are resistance to humidity, biocompatibility, piezoelectricity and optical purity (Lee, 1996).

In order to mass-produce PHAs, various wild type and recombinant bacteria have been studied under fermentation processes (Steinbuchel and Fuchtenbbusch, 1998). Nevertheless, the requirement of large amount of exogenous carbon supplementation and continuous oxygen supply made the fermentative production of bacterial PHB much more expensive than that of the

* Corresponding author. Tel.: +91 3222 283166; fax: +91 3222 282244.
E-mail address: nm@agfe.iitkgp.ernet.in (N. Mallick).

0960-8524/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2005.05.013

petrochemical-based plastics. For example, the commercially launched bacterial product from *Ralstonia eutropha* by Monsanto costs about US$ 15/kg. A cost reduction in PHA production could be possible by using cheap substrates such as molasses, whey, hemicellulose, palm oil, etc. (Reddy et al., 2003; Alias and Tan, 2005). Another potential production system may be cyanobacteria, which are oxygenic photoautotrophic prokaryotes. The advantages of using cyanobacteria in comparison to heterotrophic bacteria are enormous as these are oxygen evolving photoautotrophic organisms, so there is no need to supplement carbons for growth and oxygen in production units/area. Some of them can fix atmospheric nitrogen, so no need to provide nitrogen source(s) for those species. Moreover, cyanobacteria can successfully be cultivated in wastewaters due to their ability to use nitrogen and phosphorus from waste discharges. Therefore, in this report PHB accumulation in the culture of a model cyanobacterium, *Synechocystis* sp. PCC 6803 has been studied with an aim to establish the actual potential of the organism, and also how the accumulation is regulated by various factors such as pH, temperature, light–dark cycles, N and P status, and also by different carbon sources. Further, to explore the possibility of using biogenic wastewaters for cultivation as well as PHB production in cyanobacteria, special attempt has been made with fishpond discharges.

2. Methods

2.1. Organism and growth conditions

Axenic cultures of *Synechocystis* sp. PCC 6803 (source: Pasteur Collection of Cyanobacteria, Pasteur Institute, Paris, France) were grown in 150 ml Erlenmeyer flasks containing 50 ml of BG-11 medium (Rippka et al., 1979). The medium constituents were $NaNO_3$: 1.5 g, citric acid: 0.006 g, ferric ammonium citrate: 0.006 g, EDTA (disodium magnesium salt): 0.001 g, Na_2CO_3: 0.02 g, $MgSO_4 \cdot 7H_2O$: 0.075 g, $CaCl_2 \cdot 2H_2O$: 0.036 g, K_2HPO_4: 0.04 g, $MnCl_2 \cdot 4H_2O$: 1.81 mg, Na_2MoO_4: 0.039 mg, H_3BO_3: 2.86 mg, $CuSO_4 \cdot 5H_2O$: 0.079 mg, $Co(NO_3)_2 \cdot 6H_2O$: 0.04 mg and $ZnSO_4 \cdot 7H_2O$: 0.222 mg /l. The cultures were incubated in a temperature-controlled incubator at 28 ± 2 °C, pH 8.5, under a photoperiod of 14:10 h at light intensity of 75 μmol photon m^{-2} s^{-1} PAR. Cell dry weight was determined gravimetrically following Rai et al. (1991).

2.2. Extraction of poly-hydroxyalkanoates (PHA)

Biomass containing PHA was centrifuged and suspended in methanol (4 °C, overnight) for removal of pigments. The pellet obtained after centrifugation was dried at 60 °C and PHA was extracted in hot chloroform followed by precipitation with cold diethyl ether. The precipitate was centrifuged at 11,000*g* for 20 min, washed with acetone, and was dissolved again in hot chloroform following Yellore and Desia (1998).

2.3. Spectrophotometric assay of poly-β-hydroxybutyrate (PHB)

The spectrophotometric assay was performed as per Law and Slepecky (1961) with the help of a spectrophotometer (Specord S 100, Analytic Jena, Germany). The sample containing the polymer in chloroform was transferred to a clean test tube. The chloroform was evaporated and 10 ml of concentrated H_2SO_4 was added. The solution was heated in a water bath for 20 min. After cooling and thorough mixing the absorbance of the solution was measured at 235 nm against H_2SO_4 blank. To further confirm the presence of PHB, absorption spectra (200–1000 nm) of the sample as well as the standard (DL-β-hydroxybutyric acid, Sigma Chemical Co., USA) were taken in the Specord S 100 Spectrophotometer following acid digestion. These spectra were compared with the spectrum of crotonic acid (Sigma Chemical Co., USA).

2.4. Confirmation of PHB by gas chromatography (GC) and ^1H NMR

Gas chromatographic assay was performed following the propanolysis method of Riis and Mai (1988) using a GC (Clarus 500, Perkin–Elmer) in split mode (1:50, v/v), equipped with Elite-1 dimethylpolysiloxane capillary column (30 m × 0.25 mm × 0.25 μm) and flame ionization detector. The detection was made comparing the retention time of the standard, poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) [P(3HB-*co*-3HV)] (Aldrich, USA). Benzoic acid was used as the internal standard. ^1H NMR spectra of the standard P(3HB-*co*-3HV) and the extracted polymer from *Synechocystis* sp. PCC 6803 in $CDCl_3$ were obtained in Bruker 200 Spetrometer.

2.5. Impact of pH, temperature, light–dark cycles and carbon sources

Fifty milliliter of the medium was taken in 150 ml Erlenmeyer flasks. The pH was adjusted to different values, ranging from 5.5 to 10.5 (MES buffer, 4 mM for pHs 5.5 and 6.5, and Tris buffer, 4 mM for pH 7.5–10.5) before introducing the cells into the medium, and PHB accumulation was analysed as described above. PHB accumulation was also studied in cells grown at various temperatures, ranging from 16 to 36 °C (with an interval of 4 °C), and under continuous illumination vs. light–dark cycles (14:10 h). Impact of mixotrophy on PHB accumulation was studied by supplementing the