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RESEARCH ARTICLE - CHEMISTRY

Screening and Evaluation of Poly(3-hydroxybutyrate) with *Rhodococcus equi* Using Different Carbon Sources

Nadia Altaee[1,2] · Gamal A. El-Hiti[3] · Ayad Fahdil[1] · Kumar Sudesh[4] · Emad Yousif[5]

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Abstract A process has been developed for the production of poly(3-hydroxybutyrate) (PHB) with bacterium *Rhodococcus equi* using crude palm kernel oil (CPKO) as a carbon source. Such process will enable the production of biodegradable biopolymers to overcome some of the limitations associated with the use of petroleum-based plastics. A pure isolate was obtained from a fertile soil and was screened using the Nile Red stain for its ability for polyhydroxyalkanoates production. The isolate was identified by morphological characterization and biochemical tests. Identification by 16S rRNA has confirmed the species as *Rhodococcus equi*. Different carbon sources were used in an attempt to find the best one for the biosynthesis of PHB. The microscopic observations of PHB inside the bacteria were checked by the phase-contrast light microscope which showed a bright appearance. Moreover, the fluorescent microscope showed a bright orange fluorescence, and the transmission electron microscope showed white PHB granules with different sizes and different numbers within the bacteria cells. *Rhodococcus equi* gave the maximum cell dry mass as 1.43 g/L with the maximum PHB content as 38.1 % by weight when CPKO (1 %) was used as a carbon source.

Keywords *Rhodococcus equi* · Poly(3-hydroxybutyrate) · Crude palm kernel oil · Biodegradable biopolymers · Nile Red

✉ Gamal A. El-Hiti
gelhiti@ksu.edu.sa

✉ Emad Yousif
emadayousif@gmail.com

1 Department of Biotechnology, College of Science, Al-Nahrain University, Baghdad 10001, Iraq

2 Department of Horticulture and Garden Engineering, College of Agriculture, Al-Qasim Green University, Babil 51002, Iraq

3 Cornea Research Chair, Department of Optometry, College of Applied Medical Sciences, King Saud University, P.O. Box 10219, Riyadh 11433, Saudi Arabia

4 School of Biological Sciences, Universiti Sains Malaysia, 11800 Gelugor, Penang, Malaysia

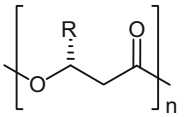
5 Department of Chemistry, College of Science, Al-Nahrain University, Baghdad 64021, Iraq

1 Introduction

The environmental problems resulting from the continuous use of petroleum-based plastic materials have increased over the years [1]. Many efforts concerned with finding a suitable substitute for such petroleum-based plastics have been made. PHB is one of the most common PHAs which can be used to replace petroleum-based plastics and in particular highly crystalline ones [1]. PHAs can act as storage compounds, in which energy and carbon can be accumulated. Also, they are biodegradable, ecofriendly, and biocompatible materials [1].

PHAs (Fig. 1) constitute a class of microbial polyesters that can be biosynthesized by several groups of prokaryotic microbes [2]. Typically, a PHA molecule consists of around 600 to 35,000 units of (*R*)-hydroxy fatty acid, which acts as a monomer [3]. The monomeric unit contains a substituent (R) in the side chain that is usually a saturated alkyl moiety [1,4]. PHAs can be classified into three main groups based on the number of carbon atoms within the monomeric unit as a short-chain length (3–5 carbons), medium-chain length (6–14 carbons), and long-chain length (>15 carbons) [5]. New modified types of PHAs, which contain different monomeric units, have been obtained [1,6].

Many researchers are keen to develop biodegradable polymeric materials as alternatives to the petroleum plastics



Polyhydroxyalkanoates (PHA)

R	*Carbon no.*	*PHA Polymer*
methyl	C_4	poly(3-hydroxybutyrate)
ethyl	C_5	poly(3-hydroxyvalerate)
propyl	C_6	poly(3-hydroxyhexanoate)
butyl	C_7	poly(3-hydroxyheptanoate)
pentyl	C_8	poly(3-hydroxyoctanoate)
hexyl	C_9	poly(3-hydroxynonanoate)
heptyl	C_{10}	poly(3-hydroxydecanoate)
octyl	C_{11}	poly(3-hydroxyundecanoate)
nonyl	C_{12}	poly(3-hydroxydodecanoate)
decyl	C_{13}	poly(3-hydroxytridecanoate)

Fig. 1 Polyhydroxyalkanoates (PHAs) chemical structures [1]

which are non-degradable by microorganisms [7]. PHB is one of the most widely studied and well characterized bioplastics and has mechanical properties similar to polypropylene [8]. It acts as a source for thermoprocessible, biodegradable, and biocompatible plastics [9,10]. PHB has various medical applications [11] and can be used in tablets, food packaging, tissue engineering, and microcapsules in therapy [12,13]. In addition, it plays an important role in the system of drug delivery [14,15].

Microbial strains can be screened rapidly by stains for demonstrating intracellular PHB granules for cells or colonies [16,17]. The colony staining method using Nile Blue can be used to detect PHB granules [18,19], but, Nile Red stain is the most sensitive stain for PHB granules detection [20]. Many substrates such as CO_2, H_2, O_2, sugar, vegetable oils, and others have a great impact on the production of PHB in terms of PHA biopolymer quantity and quality [21,22]. A wide range of Gram-positive and Gram-negative microorganisms have the ability to produce PHAs [23]. The genus *Rhodococcus* is a common aerobic Gram-positive bacterium which has an important role in the bioremediation of polluted environments and can be used in various biotechnology applications [24–26]. There are a few reports demonstrating the production of PHAs using several species of *Rhodococcus* such as *Rhodococcus fascians*, *Rhodococcus erythropolis*, and *Rhodococcus opacus* [27–29].

Recently, we have reported the recovery of PHB from *Rhodococcus equi* cells [29] as a contention of our interest in developing efficient procedures for the synthesis of various polymeric materials with potential industrial applications [30–34]. In this study we report a developed process for the production of biodegradable biopolymers using *Rhodococcus equi* in the presence of different carbon sources.

2 Experimental Procedure

2.1 Isolation of PHB Producing Bacteria

Rhodococcus equi was isolated from a soil sample at Gunung Lang, Perak, Malaysia. The soil sample was serially suspended in saline (0.9 % NaCl) on a tryptone soya agar (TSA) and incubated at different temperatures for 48 h. The best bacterial growth with visible colonies was seen at 30 °C. Therefore, 30 °C was chosen as the incubation temperature. The bacterial isolate was purified and cultured on nutrient agar slants. The culture was renewed monthly. For long-term storage, the bacterial isolate was preserved in glycerol at −20 °C. The isolate was qualitatively tested for PHB production by culturing the colonies on a mineral medium (MM) plate supplemented with the Nile Red stain [20] in comparison with *Cupriavidus necator* H_{16} (Riken Institute, Japan) as a positive control and *Ralstonia eutropha* PHB-4 (Riken Institute, Japan) as a negative control. The mineral medium was prepared according to a literature procedure [35]. The trace elements were described according the protocol reported by Kahar et al. [36].

2.2 Growth Curve Measurements

The growth curve was established by inoculation of three loops full of the bacterial culture from the tryptone soya agar plate. A flask that contained tryptone soya broth (TSB; 50 ml) was left in a shaker incubator (200 rpm) at 30 °C and bacterial broth (*c*a. 1 ml) was withdrawn aseptically every 3 h. The absorbance was measured on a Spectrophotometer U-5100 (Hitachi, Japan) at 600 nm at different times including at the start (0 h).

2.3 Morphological and Physiological Characterization

Phase-contrast light microscopy (Nikon Labophot-2 with ViS version 2.90 software) was used to study the morphology of the bacterial cells that had been grown on TSA at 30 °C for 48 h, to confirm the purity of the cultures. Also, the bacterial colonies were captured by the use of a digital camera.

2.4 Biochemical Tests

Standard techniques [37] were used to characterize the isolate along with other tests to characterize *Rhodococcus* species [38].

2.5 API Kit

Commercial API Coryne kit strips were obtained from BioMérieux-Vitek (Hazelwood, Missouri, USA) and used for the identification of isolates according to the manufac-