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Purification and characterization of two polyhydroxyalcanoates from *Bacillus cereus*

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ABSTRACT

This work aimed to study the potential of 155 strains of *Bacillus* sp., isolated from a collection of Tunisian microorganisms, for polyhydroxyalcanoates production. The strains were submitted to a battery of standard tests commonly used for determining bioplastic properties. The findings revealed that two of the isolates, namely *Bacillus* US 163 and US 177, provided red excitations at a wavelength of approximately 543 nm. The polyhydroxyalcanoates produced by the two strains were purified. Gas chromatography–mass spectroscopy (GC–MS), Fourier transformed infrared spectroscopy (FTIR), and gel permeation chromatography (GPC) were used to characterize the two biopolymers. *Bacillus* US 163 was noted to produce a poly methyl-3-hydroxy tetradecanoic acid (P-3HTD) with an average molecular weight of 455 kDa, a completely amorphous homopolymer without crystallinity. The US 177 strain produced a homopolymer of methyl-3-hydroxy octadecanoic acid (P3-HOD) with an average molecular weight of 555 kDa. Exhibiting the highest performance, US 163 and US 177 were submitted to 16S rRNA gene sequencing, and the results revealed that they belonged to the *Bacillus cereus* species. Overall, the findings indicated that the *Bacilli* from petroleum soil have a number of promising properties that make them promising candidates for bioplastic production.

1. Introduction

Bioplastics are a special type of biopolymer defined by the American Society for Testing Materials as "degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae" [1]. They are polyesters, produced by a range of microoganisms, such as *Bacillus*, *Pseudomonas*, *Aeromonas* [2], *Aeromonascaviae*, *Burkholderia sp.* [3], *Comamonas sp. EB172* [4], and fungi, such as *Rhizopus oryzae* [5], cultured under different nutrient and environmental conditions. These polymers, which are usually lipid in nature, are accumulated as storage materials (in the form of mobile, amorphous, and liquid granules), allowing microbial survival under stress conditions [6,7]. The number and size of these granules, monomer composition, macromolecular structure, and physico-chemical properties vary, depending on the producer microorganisms [8,9]. They can be observed as intracellular light-refracting granules or as electronlucent bodies that, in overproducing mutants, cause a striking alteration of the bacterial shape.

Polyhydroxyalkanoates (PHAs) are a class of biopolymers formed as naturally occurring storage polyesters by a wide diversity of microorganisms [10]. They are deposited as spherical intracellular inclusions with an amorphous hydrophobic PHA core which is mainly surrounded by proteins involved in PHA metabolism [11,12]. The weight of the polymer can range from 200 to 3000 kDa, depending on the organism and conditions under which it was produced [13]. PHAs can vary substantially in composition, as there are over 150 known constituents, resulting in a wide diversity of material properties. These bio-polymers also exhibit a crystallinity index ranging from 30% to 70% and a melting temperature of 50 °C to 180 °C, two thermoplastic material properties that make them valuable alternatives to oil-based plastics [10]. PHAs can be classified by chain length, with medium-chain-length PHAs (which have constituent C6—C14 chains) being produced mainly by *Pseudomonas* [14] and short-chain-length PHAs (which have constituent C3—C5 chains) being produced by a wide range of bacteria and archaea [14,15].

PHAs have a wide range of industrial applications particularly due to their desired properties, including biocompatibility, biodegradability, and low cytotoxicity to cells. They have, for instance, often been considered as efficient substitutes to petrochemically-based polymers in various fields and processes involving packaging and coating materials. Their compounding and blending properties have also broadened the scope of their

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performances as potential end-use applications [16]. These biopolymers have also been applied in the production of packaging materials, such as films, boxes, coating, and fibers, as well as of foam materials, biofuels, medical implants, and drug delivery carriers.

Due to their valuable properties, cost-effectiveness and eco-friendliness, PHAs have been extensively employed in large-scale applications involving biodegradable packaging materials [17]. They have been manufactured for non-woven materials, polymer films, sutures, and pharmaceutical products used in surgery, transplantology, tissue engineering, pharmacology [18], urological stents, neural- and cardiovascular-tissue engineering, fracture fixation, treatment of narcolepsy and alcohol addiction, drug-delivery vehicles, cell microencapsulation, support of hypophyseal cells, or as precursors of molecules with anti-rheumatic, analgesic, radiopotentiator, chemopreventive, antihelmintic or anti-tumuoral properties (those containing aromatic monomers or those linked to nucleosides) [7,19–24].

In the medical field, PHAs offer a distinct advantage over silicon, a conventional polymer often associated with malign effects, including the induction of cancer cell growth [25]. In order for PHAs to gain access as biomaterial substitutes to silicon, they need to fulfill five key criteria required for application in tissue engineering, namely biocompatibility; support of cell growth and adhesion; guidance and organization of cells; promotion of cell growth, passage of nutrients, and waste products; and biodegradability without the production of harmful compounds [26].

Considering the promising properties and attributes of *Bacillus* strains, the present study was undertaken to investigate and evaluate 155 *Bacillus* strains, designated US 100 to US 255, isolated from Tunisian petroleum soils, in terms of their viability and potential for the production of polyhydroyalcanoates. The strains were screened for a number of traits, namely red excitation in agar plates and culture media, at wavelengths between 280 nm and 543 nm. The bioplastics produced were purified and characterized by GC–MS, GPC, and FTIR. Two stains, namely US 163 and US 177, which exhibited high levels of bioplastic production were submitted to further 16S ribosomal RNA (rRNA) gene sequencing, and were identified as belonging to the *Bacillus cereus* species.

2. Materials and methods

2.1. Strains

Bacillus strains were purchased from the Tunisian Collection of Microorganisms of Centre of Biotechnology of Sfax.

2.2. Screening of Bacillus strains in agar plates for PHA productions

A staining solution of Nile blue A was prepared by dissolving 0.05 g of Nile blue A in l00 m1 of ethanol. Colonies on the agar plate were stained with 5 ml of the staining solution. After 20 min, the staining solution was removed from the agar plate, and the plate was left for a period of time sufficient to dry the surface [27]. The Nile blue A stained colonies were irradiated with a short wave ultraviolet light at 520, 320, 360, and 280 nm from a Mineralight UV lamp [27].

2.3. Screening of Bacillus strains in culture medium for PHA productions

Heat-fixed smears of bacterial cells were stained with the Nile blue A solution (1%; w:v) at 55 °C for 10 min in a coplin staining jar. The slides were then washed with tap water and 8% aqueous acetic acid for 1 min to remove excess of stain. After that, the stained smear was washed and blotted dry with bibulous paper, remoistened with tap water, and covered with a glass cover slip. The preparation was examined using a confocal microscope with an episcopic fluorescence attachment. A red excitation method that provided an excitation wavelength of approximately 543 nm was used [28].

2.4. Growth kinetics of Bacillus strains

Bacillus inoculum preparation was performed by transferring the microorganism from the stock solution to Luria Bertani (LB) agar plates and subsequent incubation for 24 h at 37 °C. A loopful of cells was then transferred from the LB agar plates to 100-ml conical flasks containing 50 ml of sterile LB media and incubated for 24 h at 37 °C and 250 rpm. This culture was used as the inoculum. Fermentation was carried out in 250-ml Erlenmeyer flasks containing 50 ml of the sterile production medium. The latter was inoculated with 5% (v/v) of 24-h old *Bacillus* culture. A sample was taken every 2 h, and OD was measured by a spectrophotometer at 600 nm. The medium used for growth and maintenance (LB-agar) contained (g/l): peptone, 10; yeast extract, 5; NaCl, 10; and agar, 17 (pH 7). Bacterial cells in the agar slants were incubated for 24 h at 37 °C.

2.5. Product characterization

2.5.1. Gas chromatography

Both lyophilized cells and the extracted pure PHA were submitted to methanolysis [29]. Benzoic acid was used as an internal standard. After fermentation, the culture broth was concentrated by centrifugation at 4000 rpm for 20 min. The residues were filtered and freeze-dried. The PHAs were extracted from the dried cell through esterification, which consisted of the following reagents: 0.29 g of benzoic acid, 3 ml of concentrated 98% H2SO4, and 97 ml of methanol. During extraction, 1 ml of the esterification solution and 1 ml of chloroform were added to the tubes containing 10–14 mg of the samples. The mixed samples were heated for 4 h at 100 °C. Afterward, 1 ml of distilled water was added to the cooled solution, and the mixture was vortexed for 1 min. The mixture was then left overnight to separate into two layers. The bottom layer, which contained dissolved PHA, was used for subsequent analysis [29].

PHA content and composition were determined by Agilent 19091S-433 gas chromatography equipped with a fused HP-5MS 5% Phenyl Methyl Siloxane column (length 30 m; diameter 250 μm; and film thickness 0.25 μm) (Agilent, USA). The resulting 0.4 ml of methyl esters were injected into the gas chromatography column. This was initially performed at 100 °C for 3 min, and the temperature was then increased at a rate of 8 °C/min to reach 220 °C, which was maintained for 5 min before the end of analysis. A PHA standard mixture containing various long-chain-length monomers (kindly provided by Dr. Lobna Jlaiel was used for PHA monomer band identification.

2.5.2. FT-IR spectroscopy

FT-IR spectra were recorded using a JASCOFT/IR430 spectrometer (JASCO Corp., Japan) over the 400–4000 cm^{-1} range at a spectral resolution of 4 cm^{-1}. The PHA was directly extracted using chloroform. Initially, the bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The lipids were then removed from the cell pellet-using methanol (10 times the volume of cell pellets), and the cells were incubated for 1 h at 95 °C. The suspension was then filtered to fully remove methanol, and the sediment granules were incubated in an oven at 65 °C till becoming dry. Chloroform was added to the dried granules and incubated at for 10 min 95 °C. After cooling, the solution was gently mixed overnight and then filtered to get the debris. Finally, the PHA was precipitated