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Characterization of medium chain length polyhydroxyalkanoate produced from olive oil deodorizer distillate

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ABSTRACT

Olive oil deodorizer distillate (OODD) was used for the first time as the sole substrate for polyhydroxyalkanoates (PHA) production by the bacterium *Pseudomonas resinovorans* in bioreactor cultivation. A PHA content in the biomass of 36 ± 0.8 wt% was attained within 19 h of cultivation. A final polymer concentration of 4.7 ± 0.3 g L^{-1} was reached, corresponding to a volumetric productivity of 5.9 ± 0.2 g L^{-1} day^{-1}. The PHA was composed of 3-hydroxyoctanoate (48.3 ± 7.3 mol%), 3-hydroxydecanoate (31.6 ± 2.6 mol%), 3-hydroxyhexanoate (12.1 ± 1.1 mol%) and 3-hydroxydodecanoate (8.0 ± 0.7 mol%) and it had a glue-like consistency that did not solidify at room temperature. The polymer was highly amorphous, as shown by its low crystallinity of 6 ± 0.2%, with low melting and glass transition temperatures of 36 ± 1.2 and −16 ± 0.8 °C, respectively. The polymer exhibited a shear thinning behavior and a mechanical spectrum with a predominant viscous contribution. Its shear bond strength for wood (67 ± 9.4 kPa) and glass (65 ± 7.3 kPa) suggests it may be used for the development of biobased glues.

1. Introduction

Deodorizer distillates are among the major byproducts generated by the vegetable oil refining industry [1,2]. They are usually mixed with other byproducts in the neutralization step of the refining process, resulting in a low market value material that has negative impacts if discharged into the environment [1]. The production of vegetable oil deodorizer distillates is expected to increase as a result of the intensification of edible oils consumption by the growing human population [1]. Hence, it is important to search for alternative routes for the valorization of this byproduct to avoid its negative environmental impact and the costs associated with its disposal or treatment.

Vegetable oil deodorizer distillates are complex mixtures mainly composed of free fatty acids (FFA) (>50 wt%) that include palmitic, oleic and linoleic acids. Other compounds are also present in significant amounts, such as squalene, tocopherols and sterol esters [1,2]. The high FFA content (>50%) of deodorizer distillates renders them a great potential for use as substrate for microbial cultivation and production of value-added bioproducts, thus adding value to the overall refining process. This approach has already been attempted for the production of biosurfactants by the bacterium *Pseudomonas aeruginosa* MR01 [3]. Other FFA-rich substrates have also been described as suitable substrates for the production of polyhydroxyalkanoates (PHA) [4–6], but the use of vegetable oil deodorizer distillates has not been explored so far.

PHA are biodegradable and biocompatible polyesters composed of hydroxyacids that occur as intracellular carbon and energy reserves in many bacteria. Polymers with different physical–chemical, thermal and mechanical properties can be produced, ranging from rigid and brittle thermoplastics to elastomers. Medium chain length PHA (mcl-PHA) are elastomeric polyesters composed of monomers with 6–14 carbon atoms, such as 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD), that have low melting (38–62 °C) and glass transition (−52 to −25 °C) temperatures and low crystallinity values [7]. Depending on their composition, mcl-PHA can range from rubber-like to glue-like polymers, as they become enriched in longer chain monomers [4–6], mcl-PHA are synthesized by a wide range of Gram-negative bacteria, mainly of the Genus *Pseudomonas* [7,8]. The polymers' monomer composition is dependent on the producing strain, as well as on medium composition, carbon source and cultivation conditions.

Due to their tacky behavior at room temperature, mcl-PHA might be considered as alternative materials for the development of new biobased adhesives to replace the commercial

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petrochemical-derived adhesives and glues. Solvent-based adhesives and/or glues, such as polyvinyl acetate (PVA), epoxies, polyurethane, etc., are extensively used in many areas of application due to their good adhesion capacity to different materials, low price and fast curing times. However, most of them are non-biodegradable and hazardous products, whose production and use poses environmental and public health concerns [9]. Thus, alternative biobased materials, such as soy protein [10], soy flour-based [11], sea cucumber protein [12] and frog protein [13], are currently being investigated as potential substitutes of the conventional ones or as building blocks on glue manufacturing. mcl-PHA have, so far, not been tested for such area of application.

In previous work, the ability of *P. resinovorans* to grow on olive oil deodorizer distillate (OODD) was demonstrated in batch shake flask assays [14]. In this study, OODD was used for the first time as the sole substrate for the fed-batch bioreactor cultivation of *P. resinovorans* for the production of mcl-PHA aiming at exploring the potential properties of this polymer. The produced polymer was extracted from the biomass, purified and characterized in terms of its composition, molecular mass distribution and thermal properties, as well as its rheological properties and shear bond strength on wood and glass materials.

2. Material and methods

2.1. Biopolymer production

Biopolymer production was carried out by cultivation of *Pseudomonas resinovorans* NRRL B-2649 in a 10 L bioreactor (BioStat B-Plus, Sartorius, Germany), with a working volume of 8.0 L. A 10% (v/v) inoculum was used. The mineral medium [15] was supplemented with OODD (20 g L^{-1}) as the sole substrate. The temperature was maintained at 30 ± 1 °C and the pH-value was controlled at 6.8 ± 0.2 by the automatic addition of 2 M NaOH. The air flow rate was kept constant (1 vvm – volume of air per volume of cultivation broth per minute) and the dissolved oxygen level (DO) was maintained at 30% air saturation by the automatic adjustment of the stirring rate (400–800 rpm). An OODD pulse of 15 g L^{-1} was supplied to the culture after 4 h of cultivation. Samples (15 ± 5 mL) were periodically withdrawn from the bioreactor for biomass, PHA and OODD quantification.

2.2. Analytical techniques

Cell dry mass (CDM) and OODD concentration in the broth were determined as described by Cruz et al. [16]. PHA content and composition were determined by gas chromatography (GC), according to the method described by Lageveen et al. [17], with slight modifications. Briefly, 2–3 mg of dried cells were hydrolysed with 1 mL 20% (v/v) sulphuric acid in methanol solution (Sigma–Aldrich, HPLC grade) and 1 mL benzoic acid in chloroform (1 g L^{-1}) (Sigma–Aldrich. HPLC grade), at 100 °C, during 3.5 h. Benzoic acid was used as internal standard. Pure copolymer solutions, containing poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), poly(3-hydroxyhexanoate-*co*-3-hydroxyocatnoate) and poly(3-hydroxyocatonate-*co*-3-decanoate-*co*-3-dodecanoate) (Sigma–Aldrich), were used as standards in concentrations ranging from 0.325 to 5.0 mg mL^{-1}. The resulting methyl esters were analyzed by GC (Varian CP-3800) coupled with a flame ionization detector (FID) (CTC Analytics, Switzerland) in a BR89342 WCOT fused silica column (60 m × 0.53 mm). Helium was used as carrier gas with a flow rate of 1 mL min^{-1}. Split injection was used at 280 °C with a split ratio of 10. The oven temperature program was as follows: 40 °C; 20 °C min^{-1}, until 100 °C; 3 °C min^{-1}, until 175 °C and, finally, 20 °C min^{-1}, until 220 °C. The detector temperature was set at 250 °C. All analyses were performed in duplicate.

2.3. Biopolymer extraction

PHA was recovered from dried biomass (~10 g) by Soxhlet (250 mL) extraction with chloroform, at 70 °C, for 24 h. Afterwards, the solution was filtered with 0.45 μm pore size filters (GxF, GHP membrane, PALL) to remove cell debris, and precipitated in cold ethanol (1:10, v/v) under strong stirring. The polymer was collected by centrifugation (7012 × *g*, 20 min), dried at room temperature and stored at 4 °C.

2.4. Biopolymer characterization

2.4.1. Composition

Polymer composition and purity were evaluated by GC analysis, using the modified Lageveen et al. [17] method described in Section 2.2.

2.4.2. Molecular mass

Weight average ($\bar{M}_w$) and number average ($\bar{M}_n$) molecular mass were determined using a Size Exclusion Chromatography (SEC) apparatus (Waters), equipped with a solvent delivery system composed of a model 510 pump, a Rheodyne injector and a refractive index detector (Waters 2410), according to the procedure described by Cruz et al. [16], additionally using butylhydroytoluene as internal standard. The polydispersity index (PDI) was given by the ratio between ($\bar{M}_w$) and ($\bar{M}_n$).

2.4.3. Thermal properties

The thermal properties of the polymers were determined by differential scanning calorimetry (DSC), as described by Morais et al. [18]. The glass transition temperature (T_g, °C) was taken as the midpoint of the step-transition and the melting (T_m, °C) temperature was estimated from the endothermic peak. The crystallinity (X_c, %) of the polymer samples was estimated as the ratio between ΔH_m associated with the detected melting peak and the melting enthalpy of 100% crystalline poly(3-hydroxybutyrate), P(3HB), estimated as 146 J g^{-1} [19].

2.4.4. Apparent viscosity and viscoelastic properties

The apparent viscosity and the viscoelastic properties of the mcl-PHA were accessed using a controlled stress rheometer (HAAKE MARSIII, Thermo Scientific) equipped with a plate-plate serrated geometry (diameter 20 mm), with a gap of 1 mm, at a temperature of 20 °C. Flow curves were determined using a steady state flow ramp in the range of shear rate from 10^{-3} to 10 s^{-1}. Frequency sweeps were conducted for frequency ranging from 10^{-2} to 10^{2} Hz, with a constant shear stress within the linear viscoelastic region.

2.4.5. Shear bond stress tests

The polymer's adhesive capacity was assessed using wood (AKI store, Portugal) and glass (Deltalab S.L.) strips (2.6 cm × 7.6 cm × 0.22 cm and 2.6 cm × 7.6 cm × 0.11 cm, respectively). Prior to the tests, the wood strips were placed in a desiccator with silica gel, at room temperature, for 24 h, for moisture removal. The shear bond strength of the biopolymer in wood–wood and glass–glass joints was determined, according to Kim and Netravali [9], with some modifications. Briefly, the mcl-PHA (~70 mg) was spread homogenously at the end of the strips in a superficial area of 5.0 ± 0.2 cm^2. The specimens were overlapped and hand-pressured for 10 s. The thickness of the mcl-PHA layer was determined with a digimatic micrometer (Mitutoyo Corporation). Two commercial binder clips, with an applied tension of 24.1 ± 4.8 kPa, were placed in the joint area of the strips in order to improve the gluing process

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