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Conversion of fat-containing waste from the margarine manufacturing process into bacterial polyhydroxyalkanoates

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ABSTRACT

A fat-containing waste produced from the margarine manufacturing process was tested as a low cost carbon source for cultivation of different polyhydroxyalkanoates (PHAs) producing bacterial strains, including *Cupriavidus necator*, *Comamonas testosteroni* and several *Pseudomonas* strains. The margarine waste was mainly composed of free fatty acids (76 wt.%), namely mystiric, oleic, linoleic and stearic acids. In preliminary shake flask experiments, several strains were able to grow on the margarine waste, but *C. necator* reached the highest PHA content in the biomass (69 wt.%). This strain was selected for batch bioreactor experiments, wherein it reached a cell dry weight of 11.2 g/L with a polymer content of 56 wt.%. The culture produced 6.4 g/L of polyhydroxybutyrate, P3(HB), within 20 h of cultivation, which corresponds to a volumetric productivity of 0.33 g_{PHA}/L h. The P3(HB) polymer produced by *C. necator* from the margarine waste had a melting point of 173.4 °C, a glass transition temperature of 7.9 °C and a crystallinity of 56.6%. Although the bioprocess needs to be optimized, the margarine waste was shown to be a promising substrate for P(3HB) production by *C. necator*, resulting in a polymer with physical and chemical properties similar to bacterial P(3HB) synthesized from other feedstocks.

1. Introduction

In the margarine manufacturing process, vegetable oils or animal fats are modified by hydrogenation, rearrangement and fractionation, and blended with a mixture of water, brine and powdered ingredients [1]. Additives (e.g. vitamins, emulsifiers, salt, flavours) are included in the blend to improve the quality of the product and enhance flavour. The mixture is subjected to temperatures of 50–60 °C, resulting in the formation of an emulsion (margarine) that is pasteurized and packed [1,2]. According to IMACE, International Margarine Association of the Countries of Europe (www.imace.org), in 2012, the European production of margarine was 2440 Mton, while worldwide it reached 9374 Mton. Around 1% of this production results in the generation fat-containing wastes that require adequate disposal or treatment [3,4]. The fat-containing wastes are usually removed from the aqueous effluents produced by the plant in a gravity separator [3]. Though having a low commercial value, it is commonly sold to oil-recycling companies.

Fat-containing materials (e.g. tallow generated from the food industry) have been proposed as feedstocks for the production of value-added products, namely polyhydroxyalkanoates (PHA) [5]. The use of such waste materials is a valuable strategy to improve the producing industries sustainability and economical viability, by converting a waste into high-value products. PHAs are hydroxyalkanoic acids that are synthesized by many microorganisms as intracellular carbon and energy reserve materials or reducing-power storage materials [6]. These polymers possess physical characteristics similar to traditional plastics and have received extensive attention mainly due to their biodegradability and biocompatibility [6,7]. Polyhydroxybutyrate, P(3HB), is the most widely studied and best characterized PHA. It is a homopolymer of 3-hydroxybutyrate, which has mechanical properties similar to polypropylene [6].

Due to their properties, PHAs are used as packaging materials, biomedical devices and in the food industry (e.g. edible packaging, flavour delivery agent) [8,9]. However, PHA commercial applications have been limited by their high production cost that is mainly related to the high price of the carbon source [5]. In this context, the use of inexpensive renewable feedstocks is currently

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being evaluated since their use can reduce the overall production costs in about 40–50% [10]. Fatty acids and vegetable oils, as well as wastes and by-products rich in oils or fats, have been reported as suitable for PHA production by a wide range of microorganisms, including *Cupriavidus necator*, *Comamonas testosteroni* and *Pseudomonas* sp. [11–16]. *C. necator* is a well known P(3HB) producer able to accumulate high amounts of polymer (up to 80 wt.%) from plant and waste oils [13,15,17]. *C. testosteroni* has been reported to synthesize medium chain length PHA (mcl-PHA) during cultivation on vegetable oils, accumulating up to 87.5 wt.% [18]. Several *Pseudomonas* sp., including *P. citronellolis*, *P. oleovorans*, *P. resinovorans* and *P. stutzeri* have also been described to synthesize mcl-PHA from tallow, fatty acids and biodiesel co-product stream [11,12,14,19].

Oils and fats can be used by many microorganisms, in the presence of extracellular lipase that induces their enzymatic hydrolysis into free fatty acids that are transferred through the cell membrane and metabolized via β-oxidation pathway to produce PHA monomers [15]. In *C. necator*, the synthesis of P(3HB) involves three enzymes and their encoding genes: (1) condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, catalyzed by β-ketothiolase (encoded by phaA gene); (2) reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by the NADPH-dependent enzyme acetoacetyl-CoA reductase (encoded by phaB gene); (3) polymerization of (R)-3-hydroxybutyryl-CoA monomers catalyzed by PHA synthase (encoded by the phaC gene) [6,20]. Another type of PHA biosynthetic pathway is exhibited by *Pseudomonas* species that derive 3-hydroxyacyl-CoA from the intermediates of fatty acid β-oxidation pathway, enoyl-CoA, 3-ketoacyl-CoA, and/or S-3-hydroxyalcyl-CoA [20].

In this work, a fat-containing material waste generated by the margarine manufacturing process was, for the first time, tested as carbon source for the production of bacterial PHA. The margarine waste was characterized in terms of its physical and chemical properties. Several bacterial strains were screened for their ability to grow and produce PHA using the margarine waste as the sole carbon source and the highest PHA yielding strains was selected for bioreactor cultivation. The resulting PHA polymer was characterized in terms of its chemical and thermal properties.

2. Materials and methods

2.1. Margarine waste characterization

The fat-containing waste from the margarine manufacturing process was supplied by FIMA, SA – Unilever, Portugal. Two margarine waste samples, supplied by the manufacturer at different times, were analyzed to determine their composition and assess their variability. The samples were characterized in terms of density, pH, water content, inorganic compounds content and the composition in organic compounds (total carbohydrates and lipids). All analyses were performed in duplicate. The margarine waste was a solid material at ambient temperature and it had to be melted by placing at a temperature of 50 °C.

The margarine waste analyzed for its carbon, hydrogen, nitrogen and sulphur content, using the elemental Analyzer Thermo Finnigan-CE Instruments (Italy), model Flash EA 1112 CHNS. The water content of the margarine waste was determined as the weight loss by a 2 mL sample upon lyophilization, in a percentage basis. The total carbohydrates content was determined using the phenol-sulphuric acid method [21], using glucose solutions (0–200 mg/L) as standards. The total sugar content was expressed as percentage of sugar in the margarine waste sample. To determine the total content in inorganic compounds and their composition, 1 mL of margarine waste was subjected to pyrolysis at 550 °C, for 24 h. The resulting ashes were dissolved in 20 mL 2.3 M H_2SO_4. The solution was analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES), (Horiba Jobin-Y, France, Ultima), for quantification of aluminium, calcium, iron, magnesium, phosphorus, potassium and sodium.

The margarine waste content in glycerine, mono-, di- and triglycerides was determined by on-column gas chromatography (GC) (Trace GC Ultra), according to the European norm EN 14105 (Thermo Fisher Scientific Inc.). Standard solutions (Biodiesel Consumables Kit EN), containing glycerin, monolein, diolein, triolein, butanetriol (IS1) and tricaprin (IS2) were used at the concentration specified in the European norm. IS1 (80 μL), IS2 (100 μL) and of N-methyl-N-(trimethylsilyl)triluoroacetamide (MSTFA reagent) (100 μL) were added to 100 mg of margarine waste and the mixture was vigorously shaken. After 15 min, 8 mL of n-heptane were added, and the mixture was used for GC analysis.

The free fatty acid content of the margarine waste was determined by automatic titration (TIM 86J Titration Manager) of a solution of margarine waste sample (0.1–0.5 g) in isopropanol (30 mL) with 0.1 M NaOH. The fatty acid composition of the margarine waste was determined by direct transesterification of the lipids to the corresponding methyl esters, according to a modified Lepage and Roy method [22]. The methyl esters were quantified by GC, with a Thermo Trace GC ULTRA gas chromatograph, equipped with a flame ionization detector and a split/splitless injector, according to European norm EN1403PTV (Thermo Fisher Scientific Inc.). Methyl heptadecanoate was used as internal standard.

2.2. Microbial cultivation experiments

The bacterial cultures used in this study were *Pseudomonas oleovorans* strains NRRL B-14682, NRRL B-14683, NRRL B-778 and NRRL B-3429, *P. resinovorans* strains NRRL B-2649 and NRRL B-4205, *P. citronellolis* NRRL B-2504, *P. stutzeri* strains NRRL B-775 and *P. stutzeri* NRRL B-2461, *Comamonas testosteroni* NRRL B-2611 and *Cupriavidus necator* DSM 428. All *Pseudomonas* strains and *C. testosteroni* were offered by the National Center for Agricultural Utilization Research, USA, and C. *necator was* purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany.

Luria Bertani (LB) medium (bactotryptone, 10.0 g/L; yeast extract, 5.0 g/L; NaCl, 10.0 g/L), pH 7.0, was used for reactivation of the cryopreserved bacterial cultures. Solid LB medium was prepared by adding agar (15 g/L). A nitrogen-limited mineral medium, with the composition described by Freitas et al. [23] was used for all shake flasks and bioreactor experiments. The mineral medium was supplemented with the margarine waste (20 g/L) as the sole carbon source. The margarine waste was autoclaved separately and added while hot (~50 °C) to the mineral medium. Prior to culture inoculation, the shake flasks containing the medium supplemented with the melted margarine waste were placed in an orbital shaker (at 30 °C, 200 rpm, 24 h) to obtain an homogenous mixture. The stability of the mixture thus obtained was confirmed by no phase separation being observed after leaving the flasks at rest for several days. In the bioreactor experiments, the hot melted margarine waste was added to the mineral medium and the mixture was stirred (400 rpm) until homogenous mixtures were obtained prior to culture inoculation.

The cultures were reactivated by inoculating LB agar plates with a sample of the cryopreserved microorganisms and incubation at 30 °C for 24 h. Afterwards, isolated colonies were inoculated into 50 mL liquid LB and incubated in an orbital shaker at 200 rpm and 30 °C, for 72 h. 10 mL of the cultures thus obtained were used as inocula for the 100 mL shake flask cultivations. In all shake flask experiments, the cultures were incubated under the same