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Synthesis and characterization of poly(3-hydroxyalkanoates) from *Brassica carinata* oil with high content of erucic acid and from very long chain fatty acids

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ABSTRACT

Pseudomonas aeruginosa produced medium chain length poly(3-hydroxyalkanoates) (mcl-PHAs) when grown on substrates containing very long chain fatty acids (VLCFA, C > 20). Looking for low cost carbon sources, we tested *Brassica carinata* oil (erucic acid content 35–48%) as an intact triglyceride containing VLCFA. Oleic ($C_{18:1}$), erucic ($C_{22:1}$), and nervonic ($C_{24:1}$) acids were also employed for mcl-PHA production as model substrates. The polymers obtained were analyzed by GC of methanolyzed samples, GPC, ^{1}H and ^{13}C NMR, ESI MS of partially pyrolyzed samples, and DSC. The repeating units of such polymers were saturated and unsaturated, with a higher content of the latter in the case of the PHA obtained from *B. carinata* oil. Statistical analysis of the ion intensity in the ESI mass spectra showed that the PHAs from pure fatty acids are random copolymers, while the PHA from *B. carinata* oil is either a pure polymer or a mixture of polymers. Weight-average molecular weight varied from ca. 56,000 g/mol for the PHA from *B. carinata* oil and oleic acid, to about 120,000 g/mol for those from erucic and nervonic acids. The PHAs from erucic and nervonic acids were partially crystalline, with rubbery characteristics and a melting point (T_m) of 50 °C, while the PHAs from oleic acid and from *B. carinata* oil afforded totally amorphous materials, with glass transition temperatures (T_g) of −52 °C and −47 °C, respectively.

1. Introduction

Agriculture and the industries connected frequently produce food surplus and byproducts that can be used as low-cost substrates in fermentation processes. One of the possible practical applications of this concept would be the production of bacterial polyesters by fermentation from such substrates, specifically the production of the biosynthetic polymers generically known as poly(hydroxyalkanoates) (PHAs).

PHAs are a family of aliphatic polyesters of 3-hydroxyacids generally produced in the form of intracellular granules with function of energy and carbon reserve material from a wide variety of bacteria in the presence of nutrient-limiting conditions and in the presence of excess carbon sources. In general, short chain length PHAs behave as semi crystalline, thermoplastic polymers and are commonly produced by microorganisms of the species *Ralstonia eutropha* and *Alcaligenes latus*, while medium chain length PHAs, typically produced by fluorescent pseudomonads (such as *Pseudomonas aeruginosa* and *Pseudomonas oleovorans*), are more amorphous compared to short chain length PHAs (scl-PHAs) and exhibit elastomeric properties [1–4] depending on the composition of the side chain.

While the substrates conventionally and commonly used as carbon sources for the PHA production by fermentation are glucose and various carbohydrates, the fatty acids (FAs) obtainable from triacyl glycerols (TAGs) have attracted the researchers' attention because they seem to be a better fermentation substrate with respect to carbohydrates from an energetic point of view. Furthermore, it is to be taken into account that TAGs are renewable materials. For fermentative purposes it is desirable to directly use the starting materials in the triglyceride form as substrates rather than the corresponding FAs, which must be previously obtained by means of an additional saponification step.

As noted above, savings in the fermentation process for PHA production could derive from the use of food reserves cheaply obtained from agriculture, taking into account that for the production of

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"bio-based" products by fermentation the substrate cost represents about 28–50% of the total costs [5,6]. As a consequence, the researchers' attention has more and more been focused on cheap fermentable starting materials to be used as substrates for PHA production, such as fats, oils, molasses, and serum.

The first report of production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P3HB-co-3HHx) from olive oil (mainly consisting of oleic acid triglyceride, $C_{18:1}$) by means of *Aeromonas caviae* dates back to the early nineties [7]. Later, Fukui and Doi [8] reported use of olive oil, corn oil (mainly rich in TAGs of linoleic acid, $C_{18:2}$) and of palm oil (rich in TAGs of palmitic acid, $C_{16:0}$, as well as in oleic acid) for the production of P3HB and P(3HB-co-3HHx) from *R. eutropha*.

Kahar et al. [9] described the use of soybean oil (mainly rich in triglycerides of linoleic and oleic acids) with *A. caviae*, and Loo et al. [10] studied the possibility of obtaining scl-PHA from genetically engineered *R. eutropha* using raw palm oil and acid palm oil.

Cromwick et al. [11] were the first to demonstrate, in 1996, the use of an intact TAG (tallow, i.e. animal fat) for the synthesis of mcl-PHA by means of *Pseudomonas resinovorans*. Later, the synthesis of mcl-PHAs by means of *P. resinovorans* starting from other oils and fats was reported, and it was shown that the composition of the repeating units of the biopolymer reflects the composition in FAs of the oil or fat employed for the synthesis [12]. The literature of the field also includes studies on the synthesis of mcl-PHA from pure glycerol or from the production residues of glycerol-rich biodiesel [13]. The main obstacles towards the use of these or other residues are their variable amounts and the presence of unfermentable components. These elements unavoidably affect the productivity of the fermentation and the process line. For instance, the high cell density fermentation, which is critical to reach high yields of PHAs, is difficult to obtain with agricultural products and by-products containing diluted concentrations of substrates such as sugars and glycerol.

In some countries of the Mediterranean area [14], such as in Italy [15], particularly in Sicily, the production, collection, and pressing of *Brassica carinata* are being tested experimentally, with the aim to create a new chain of production for biofuels. *B. carinata* is a plant of the Brassicaceae family, a family comprising herbaceous plants with big leaves some of which are of a vital importance for economy and human food, such as the various species of cabbage and cauliflower (*Brassica oleracea*) and rapeseed (*Brassica napus*). *B. carinata* or *Abyssinian mustard* (or *Abyssinian cabbage*) is a plant originating from the Ethiopian plateau and has been recently introduced in Sicily for production of oil for biodiesel starting from the seeds. The major component of such oil is the triglyceride of erucic acid ($C_{22:1}$, a higher homologue of oleic acid). The growing of *B. carinata*, in the experimentation stage, turned out to be a quite valid choice, as such plant is suitable to be grown in crop rotation with wheat, thus assisting in improving the quality and protein content of the latter. In addition, the concerned culture does not take away land to the food cultures and protects the land fertility.

The widespread and cheap production of *B. carinata* oil, intended for the production of biodiesel, specially for use in agricultural machinery, brings about as a consequence a wide availability of said starting material at low cost, also in view of further uses, both as an alternative choice and as a means to dispose of the production surplus.

Our previous studies [16,17] have shown that a microorganism of the Pseudomonadaceae family, *P. aeruginosa* ATCC 27853, is able to employ saturated FAs as carbon sources. However, the same studies have also shown that the PHA production does not occur when the FA has more than 15 or 20 carbon atoms in the case of odd- or even-numbered FAs, respectively.

The aim of the present study is to investigate on the capability of *P. aeruginosa* ATCC 27853 to grow and synthesize PHAs from unsaturated VLCFA longer than C20, or from *B. carinata* oil. VLCFA were erucic acid ($C_{22:1}$) and its upper homologue nervonic acid ($C_{24:1}$). The latter has lately been the object of several studies, since it has been successfully employed in the treatment of some neurological affection, such as senile dementia, Alzheimer's disease, and multiple sclerosis.

With reference to the latter starting material, researchers of the Institute of Plant Biotechnology of the Canadian National Research Council have recently informed that they have genetically transformed *B. carinata* to have it to produce nervonic acid in an amount of up to 40% of its total oil content. The development of such technology may represent an economically efficient source for the industrial scale production of nervonic acid for pharmaceutical or nutraceutical applications. According to what is proposed by the present study, a further application of nervonic acid may consist in its use as a substrate for the production of biodegradable and biocompatible plastics.

Table 1
PHA production from *P. aeruginosa* cultured on *B. carinata* oil, oleic, erucic, and nervonic acids.

Substrate	Dry cell weight (mg/L)	PHA content (% dry cell weight)	PHA yield (mg/L)
B. carinata oil	1000	5.0	50
Oleic acid	380	15.0	57
Erucic acid	866	9.3	81
Nervonic acid	416	10.0	42

2. Materials and methods

2.1. Growth conditions

P. aeruginosa ATCC 27853, grown in Luria Bertani (LB) broth at 37 °C, was used throughout the experiments. For PHA production, cells were harvested by centrifugation, washed twice in phosphate-buffered solution (PBS, Sigma) and subsequently transferred in *E** medium, containing the following: 1.1 g/L $(NH_4)_2HPO_4$, 5.8 g/L K_2HPO_4, 3.7 g/L KH_2PO_4, 10 mL/L $MgSO_4$ 0.1 M, supplemented with 1 mL/L of a microelement solution (MT stock). MT stock contained the following: 2.78 g/L $FeSO_4{\cdot}7H_2O$, 1.98 g/L $MnCl_2{\cdot}4H_2O$, 2.81 g/L $CoSO_4{\cdot}7H_2O$, 1.67 g/L $CaCl_2{\cdot}2H_2O$, 0.17 g/L $CuCl_2{\cdot}2H_2O$, 0.29 g/L $ZnSO_4{\cdot}7H_2O$. *B. carinata* oil with erucic acid content of 35–48% (a kind gift of Consorzio di Ricerca Gian Pietro Ballatore, Zona Industriale Dittaino, Assoro, Enna, Italy) at a final concentration of 0.4% and oleic, erucic (Sigma–Aldrich, Milano, Italy) and nervonic (Polichimica, Sondrio, Italy) acids at a final concentration of 5 mM, were added as carbon sources. The pH was adjusted to 7.0 and the medium was autoclaved. Exponential-phase cells, precultured in 500 mL of LB broth cultures, were inoculated in 1 l *E** medium batch cultures until a final concentration of 3×10^8 cells/mL (OD_{540} = 0.3). Nitrogen deprivation was obtained by inoculation of exponential-phase cells in modified

Table 2
Comonomer composition (mol%) of PHAs obtained from various carbon sources, determined by GC.[a]

Substrate	C	O	$O_{:1}$	D	$D_{:1}$	Δ	$\Delta_{:1}$	$T_{:1}$	$T_{:2}$	$T_{:3}$
B. carinata oil	3	34	3	32	3	10	1	9	2	3
Oleic acid	4	55		27		8		6		
Erucic acid	3	43		36		10		8		
Nervonic acid	4	28		43		14		11		

[a] C = 3-hydroxyhexanoate; O = 3-hydroxyoctanoate; $O_{:1}$ = 3-hydroxy-5-octenoate; D = 3-hydroxydecanoate; $D_{:1}$ = 3-hydroxy-7-decenoate; Δ = 3-hydroxydodecanoate; $\Delta_{:1}$ = 3-hydroxy-6-dodecenoate; $T_{:1}$ = 3-hydroxy-5-tetradecenoate; $T_{:2}$ = 3-hydroxy-5,8-tetradecadienoate; $T_{:3}$ = 3-hydroxy-5,8,11-tetradecatrienoate.