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Production of Bacterial Polyesters from Some Various New Substrates by *Alcaligenes eutrophus* and *Pseudomonas oleovorans*

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Poly(3-hydroxy alkanoate)s (PHA)s are bacterial polyesters that have, due to their biodegradability and biocompatibility, attracted considerable industrial interest. All the substrates used in feeding *Alcaligenes eutrophus* and *Pseudomonas oleovorans* have been reviewed as far as we know, and some more new substrates or mixtures have been used in PHA production by microorganisms. *Alcaligenes eutrophus* was fed with 4-pentenoic acid, 2-hydroxy ethyl methacrylate (HEMA), corn oil acids, linseed oil acids and limonene as well as mixtures of acetic acid and glucose or lactose. Either HEMA as a sole carbon source or the mixture of glucose did not produce polyester; limonene as a sole carbon source gave few dry cells and very few mgl^{-1} Poly (3-hydroxy butyrate-co-3-hydroxy valerate)(PHBV) containing 5 mol-% of hydroxy valerate (HV) units. Poly(3-hydroxy butyrate), (PHB), was obtained from corn oil acids and the mixture of glucose (15 gl^{-1}) and acetic acid (2.5 gl^{-1}); Poly (3-hydroxy butyrate-co-3-hydroxy valerate) (PHBV) was obtained in moderate yield from 4-pentenoic acid as a sole carbon source and the rest of the substrates above. *Pseudomonas oleovorans* was fed with linoleic acid, laurel seed oil acids, corn oil acids, laurel leaf oil, rose oil and limonene. Medium chain length polyesters were obtained from linoleic acid, corn oil acids and laurel seed oil acids, but the others did not give any detectable polyester. The polymers obtained were characterized by size exclusion chromatography, ^{1}H and ^{13}C NMR, FT-IR, thermal analysis and fast atom bombardment-mass spectrometer techniques.

Key Words: Bacterial polyesters. *A. eutrophus* and *P. oleovorans*. Limonene, linoleic acid, 4-pentenoic acid. Laurel seed-, corn-, linseed-oil acids, rose oil and laurel leaf oil.

Introduction

Poly (3-hydroxy alkanoates)(PHA)s are a class of naturally occurring polyesters that accumulate as inclusion bodies in many diverse bacteria, with the general structure shown below [1-4]:

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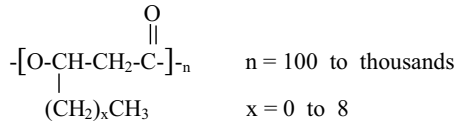


Alcaligenes eutrophus has the ability to synthesize short chain-length (scl) polyesters in which x = 0 or 1, whereas *P. oleovorans* produces medium chain-length (mcl) polyesters in which x = 2 to 8 [5,6]. It has recently become of industrial interest to evaluate these polyesters as biodegradable thermoplastics for a wide range of agriculture, marine
and medical applications. Because the physical and mechanical properties of these copolymers can change considerably as a function of the monomers composition and distribution, it is desirable to incorporate different types of repeating units into the polymer in order to produce materials with specific requirements for practical applications [7,8]. In that regard, various substrates were used in feeding bacteria to produce polyester. To the extent of our knowledge, substrates used in feeding bacteria and the type of PHA formed by *A. eutrophus* and *P. oleovorans* are listed in Tables 1 and 2, respectively. Table 3 contains a list of substrates that do not produce polyester.

Table 1. List of substrates used in the production of polyesters by *A. eutrophus*.

Substrate	Type of PHA obtained
Acetic acid, D-gluconic acid, adipic acid, lactic acid, malic acid, citric acid, phenyl acetic acid, alanine, phenyl alanine[9], 4-hydroxy hexanoic acid[10], palm oil[11],oleic acid[11, 12], glucose[13], fructose[13,14], saccharose, butyric acid[14], lactic acid[14], vernonia oil(saponified)[15], glucose + ethylene glycol(or propylene glycol)[16].	P(3HB)
Glucose + propionic acid[13], pentanoic acid + butyric acid[17], Acetic acid + propionic acid[18], oleic acid + nonanoic acid[12].	P(3HB-co-3HV)
4-hydroxy butyric acid + butyric acid[17], γ-butyrolactone, $\gamma-$ butyrolactone + fructose(or butyric acid)[19],4-chloro butyrate,	P(3HB-co-4HB)
1,4-butane diol, 4-chloro benzoate + benzoate[20]. 5-chloro benzoate, 5-chloro benzoate + pentenoate[20], 5-chloro pentanoate + pentanoic acid[21].	P[3HB-co-3HV-co-5HV]

Abbreviations: P(3HB): Poly-3-hydroxy butyrate, P(3HV): Poly-3-hydroxy valerate, P(4HB): Poly-4-hydroxy butyrate, P(5HV): Poly-5-hydroxy valerate.

Experimental

Materials. Glucose, acetic acid, 2-hydroxy ethyl methacrylate (HEMA), lactose, glycerol, limonene and solvents were purchased from Merck AG; 4-pentenoic acid was purchased from Aldrich Chemicals. Corn oil, and linseed oil, laurel seed oil, laurel leaf oil, rose oil were the extracts of the related plants grown in Turkey.

Bacterial strain and culture conditions. P. oleovorans (Deutsche Sammlung von Microorganismen und zell kulturen GmbH.,DSM # 1045) and *A. eutrophus* (DSM # 428) were grown in 3 l flasks or 10 l

fermenter at 30 °C in E-2 medium as described elsewhere [6,12]. Growth medium was prepared to provide 20 mM solutions of each carbon substrate as a sole carbon source or a mixture of another substrate.

Table 2. List of substrates used in the production of polyesters by *Pseudomonas oleovorans and Pseudomonas putida.*

Substrate	Type of side chains in the PHA obtained
4-hydroxy hexanoic acid[10], n-octane[22-27], undecane, dodecane[23,24], caproic acid[25], heptanoic acid, nonanoic acid[6,25], hexane, heptane, nonane[23,24,27], decane[27], decanoic acid[6,25,29*], levulinic acid[30*].	Saturated alkyls
Nonene[23,24], octene, decene[23,24,27], glucose, fructose, glycerol[29], undecenoic acid[31,32*,33], 7-octenoic acid[33], hazelnut, sesame, olive, hamci (anchovy) oily acids[34], linseed oily acids, tall oily acids[35*], tallow, lard, butter, olive, sunflower, coconut, soybean oils[36], side oleic acid[12,19,36].	Unsaturated side chains
5-p-tolyl valeric acid, 5-p-ethyl valeric acid, 5-p-biphenyl valeric acid, 8-4'-tolyl octanoic acid[37], 3-phenyl propionic acid, 3-hydroxy 3-phenyl propionic acid, 5-phenyl valeric acid[38], 9-phenyl nonanoic acid, 11-phenyl undecanoic acid, 9-p-tolyl nonanoic acid[39*], 6-phenyl hexanoic acid, 7-phenyl heptanoic acid[39*,40*], 8-phenyl octanoic acid[40*], 5-phenoxy valeric acid, 9-phenoxy nonanoic acid[41], 11-phenoxy undecanoic acid[41,42], 6-phenoxy hexanoate, 8-phenoxy nonanoate[42], 6-p-methyl phenoxy hexanoic acid, 8-p-methyl phenoxy octanoic acid, 8-m-methyl phenoxy octanoate, 8-o-methyl phenoxy octanoate[32,43*], 2',4'-dinitro phenyl valeric acid, 4'-nitrophenyl valeric acid[44].	Phenyl containing side chains
5-, 6-, 7-methyl octanoic acids[45], 6-, 7-, 8-methyl nonanoic acids, 9-methyl decanoic acid[46].	Methyl branched side chains
6-bromo hexanoic acid, 8-bromo octanoic acid, 11-bromo undecanoic acid[47], chlorides and fluorides of some alkanoic acids[48-50].	Halogene containing side chains

**P. putida* was used.

Saponification of the oils: The following procedure described in reference [15] was used for the hydrolysis of corn oil, linseed oil and laurel seed oil. A 500 mL round bottomed flask was charged with 100 mL methanol and 4.95 g (0.124 mol) sodium hydroxide. The mixture was refluxed until the sodium hydroxide had dissolved. To the hot alkaline solution was added 0.02 mol of the oil. The resulting brownish solution was refluxed with continuous stirring for 30 min, after which the hot mixture was slowly transferred into a beaker that contained about 50 g water and 50 g ice. The resulting semi-solid or waxy-oily acids were filtered and air dried (yield 95%).

Table 3. The list of substrates used in feeding *P. oleovorans* and *A. eutrophus* but not produced any detectable polyester.

Substrates which do not produce polyester
Esculin, maltose, N-acetyl glucose amine, arginine, tyrosine[9], 1,3-octadiene, 1,4-octadiene, 2,2-dimethyl heptane, 2,2-dimethyl octane, 2'-octanone[23], 11-amino undecanoic acid, 8-hydroxy octanoic acid, 10-hydroxy decanoic acid, 11-cyano undecanoic acid, 11-ethoxy undecanoic acid, 6-ethoxy hexanoic acid, hexane-, heptane-, octane-, nonane-, decane-, dodecane-dioic acids[32], 2,6-dimethyl hexanoic acid[46], 2-, 3-, 4–methyl-, 3,4-dimethyl-, 2,6-dimethyl-, 2-,4-,6-trimethyl phenoxy-valeric, -heptanoic, -decanoic acids[41], octyne, octanol, suberic acid, 1-bromo octane, octyl amine, 1-,2-,7-,8- octane tetrol[25], 2-, 3-, 4-, 5-methyl nonanoic acids[51].

Polymer characterization. NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC 200L instrument at 200 MHz for ^{1}H and 50.32 MHz for ^{13}C . The deuterated solvent used was $CDCl_3$containing tetramethyl silane (TMS) as a reference.

Thermal analysis was carried out for 8-10 mg samples on a Du Pont 910 Differential Scanning Calorimeter (DSC). The polymer samples were heated at a rate of 20 °C/min from –100 °C to 130 °C or from -50 °C to 200 °C.

Methanolysis and Gas Chromatography. The methanolysis reaction was carried out in chloroform/methanol/sulfuric acid (1 ml/0.85 ml/0.15 ml) at 100 °C for 140 min following a procedure described previously [46]. The methyl esters obtained were assayed by gas chromatography and mass spectroscopy (GC-MS analysis) using a Hewlett Packard HP 5890 gas chromatograph with He carrier gas [34].

Molecular weight measurements. Molecular weights were determined by gel permeation chromatography, GPC, with a Waters model solvent delivery system with a model 410 refractive index detector, and with 2 ultrastyragel linear columns (HRI and HT6E) in series. Tetrahydrofuran or chloroform was used as the eluent at a flow rate of 0.1 mL/min. Sample concentrations of 2-3 mg/mL and injection volumes of 150 mL were used. A calibration curve was generated with six polystyrene standards having molecular masses of 3×10^6, 233×10^3, 22×10^3, 2150, 580 and 92 Daltons.

Results and Discussion

PHAs from *A. eutrophus*

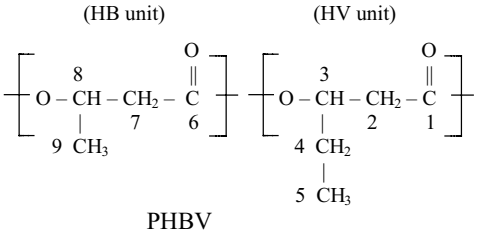
A. eutrophus produced scl-PHAs from 4-pentenoic acid, linseed oil acids, corn oil acids, lactose, glucose + acetic acid mixture, limonene and glucose + limonene. However, it did not produce PHAs from hydroxyethylmethacrylate (HEMA) or the mixture of acetic acid. The results and conditions of PHA syntheses from these substrates, the copolymer analysis of scl-PHA obtained by this method and some thermal analyses are listed in Table 4.

4-Pentenoic acid was recently used as a sole carbon source for feeding *Rhodospirillum rubrum* in order to obtain PHBV copolymer containing unsaturated repeating units of 14-30 mol-% [52]. However, wholly

saturated PHBV copolymer was obtained in 0.3 gL^{-1} of polymer yield when *A. eutrophus* was fed with 4-pentenoic acid.

Table 4. PHA Production by *A. eutrophus.*

Run no of scl-PHA obtained	Substrate, (gl^{-1}	Time (h)	Dry cell (g)	Polymer (g) type	T_g T_m (°C)
427	(2.5) 4-pentenoic acid	24	1.6	(0.3) PHB-V 20 80	-20 90,105,130 -10
748	(2.5)Linseed oil acid	36	40	(1.0) PHB-V 90 10	
79	(2.5) Corn oil acid	72	2.0	(0.9) PHB	
65	(15) Lactose	45	1.7	(0.2) PHB-V 97 3	
742	(15) Lactose + (1.0) Acetic acid	45	1.7	(0.15) PHB-V 97 3	
188	(15) Glucose + (1.0) Acetic acid	24	2.2	(0.2) PHB-V 80 20	115
189	(15) Glucose + (2.5) Acetic acid	24	1.2	(0.3) PHB	
261	(2.5) Limonene	24	1.2	(0.01) PHB-V	
249	Limonene + glucose (1.5) + (7.5)	24	1.4	(0.64) PHB	
562	(2.5) HEMA	24	-	-	
546	HEMA + glucose 2.5 2.5	24	-	-	



PHBV

The ^1H NMR spectrum of the copolymer has the characteristic signals of HB and HV units: δ_{ppm}: 0.9, t (CH_3-5), 1.3, d (CH_3-9), 1.6, m (CH_2-4), 2.52, m (CH_2-2 and CH_2-7), and 5.22, m (CH-8 and CH-3). There was no signal for 3-hydroxy pentenoic acid units [53]. Thermal analysis of PHB obtained from 4-pentenoic acid indicated lower glass transition (T_g) and melting transition (T_m) than PHB. As listed in Table 4, there are two T_g's at –10 and –20 °C and three T_m's at 90, 105 and 130 °C.

Linseed oil acids gave scl-poly(3-hydroxy alkanoate)s, PHBV in high yields containing few percent of HV units while corn oil acids gave pure PHB. Mol ratio of HV units to HB units of PHBV obtained from linseed oil was calculated as 10 to 90 using ^{13}C NMR spectrum of the polyester.

Lactose as a sole carbon source and the mixture of acetic acid and lactose led to PHBV containing a small amount, 3mol-% , of HV units. The mixture of glucose and acetic acid also gave scl PHA. Interestingly,

a higher amount of acetic acid in the mixture led to pure PHB while a lower concentration of the acid produced PHBV copolymer with 20 mol-% of HV units (see run no. 188 and 189 in Table 4).

Limonene produced a few dry cells and a few milligrams of PHBV containing 5 mol-% of HV units. PHB was only obtained when *A. eutrophus* was co-fed with glucose. HEMA and the mixture of glucose also did not produce any polyester.

PHAs from *P. oleovorans*

P.oleovorans produced medium chain length mcl-PHAs containing unsaturated side chains from linoleic acid, corn oil acid and laurel seed oil acids, but laurel leaf oil, rose oil, limonene and the mixture of limonene and octanoic acid did not because of their terpenoid structures. Results and conditions of mcl PHA production from these substrates are listed in Table 5. PHAs containing unsaturated side chains were analyzed using the GC-MS technique. Table 6 contains the copolymer structure analysis results obtained from GC-MS spectra. They contain mainly PHO, PHD and 7-29 mol% of unsaturated units. Because of the long side chains (indicated as "others" in Table 6), has lower T_m's at 13 and 36 °C and T_g's at around –50 °C. Molecular masses varied from 58K Dalton to 67K Dalton. Thermal analysis results and some of the molecular masses of the PHAs obtained are presented in Table 5.

Table 5. PHA Production by *P. oleovorans*.

Run no. of mcl-PHA obtained	Substrate	Time	Dry cell (g/l)	Polymer	$M_n \times 10^4$	M_w/M_n	T_g T_m (°C)
322 PHA-linoleic	Linoleic acid	18	1.0	0.5			
343 PHA-corn	Corn oil acid	24	2.3	1.9	5.8	2.30	-50 13
349 PHA-laurel seed	Laurel seed oil acid	24	2.0	0.26	6.7	2.39	-50 36
362	Laurel leaf oil	72	-	-			
378	Rose oil	72	1.0	-			
242	Limonene	72	-	-			
239	Limonene+octanoic acid		0.60	-			

Table 6. Copolymer composition of the PHAs obtained from laurel seed oily acids, corn oily acids and linoleic acid by *P. oleovorans*.

Mcl-PHA	Copolymer composition, mol-%			
	PHO	PHD	Other	Unsaturated units
PHA-linoleic	40	26	5	29
PHA-laurel seed	52	21	16	11
PHA-corn	58	27	8	7

Conclusion

A. eutrophus is the only suitable microorganism to produce PHB or PHBV copolymers whatever the substrates used. This microorganism accumulates saturated scl-PHAs when it feeds unsaturated substrates such as oily acids and 4-pentenoic acid. Lactose and its acetic acid mixture produced PHBV copolymer containing 97 mol-% PHB copolymer. Glucose and acetic acid mixtures were interesting; by varying acetic acid concentration, pure PHB or PHBV copolymer could be obtained. A natural product, limonene, gave a few milligrams of PHBV with 5 mol-% of HV units. HEMA as substrate did not yield any polyester. *P. oleovorans* produced mcl-PHAs from linoleic acid, corn and laurel seed oil acids. Mcl-PHAs obtained by *P. oleovorans* contained the same functionalities as their substrates. Functional groups of substrates can be inserted into PHAs using *P. oleovorans* but not *A. eutrophus*. Rose oil, limonene and laurel leaf oil cannot be considered to be a substrate to produce PHAs. Laurel leaf oil and limonene also did not grow bacterium. In conclusion, this work reports the fermentation results of some new substrates for PHA production from *A. eutrophus* and *P. oleovorans*.

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