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ORIGINAL ARTICLE

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Production of polyhydroxyalkanoic acids by *Ralstonia eutropha* and *Pseudomonas oleovorans* from an oil remaining from biotechnological rhamnose production

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Abstract Screening experiments identified several bacteria which were able to use residual oil from biotechnological rhamnose production as a carbon source for growth. *Ralstonia eutropha* H16 and *Pseudomonas oleovorans* were able to use this waste material as the sole carbon source for growth and for the accumulation of polyhydroxyalkanoic acids (PHA). *R. eutropha* and *P. oleovorans* accumulated PHA amounting to 41.3% and 38.9%, respectively, of the cell dry mass, when these strains were cultivated in mineral salt medium with the oil from the rhamnose production as the sole carbon source. The accumulated PHA isolated from *R. eutropha* consisted of only 3-hydroxybutyric acid, whereas the PHA isolated from *P. oleovorans* consisted of 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid, and 3-hydroxydodecanoic acid. The composition was confirmed by gas chromatography of the isolated polyesters. Batch and fed-batch cultivations in stirred-tank reactors were done.

Introduction

Since the detection of poly(3-hydroxybutyric acid) as a storage compound in *Bacillus megaterium* by Lemogine (1926), further investigations have shown that many bacteria are also able to synthesize aliphatic polyesters as bacterial intracellular storage products. In addition to 3-hydroxybutyric acid, which is a widespread constituent of bacterial polyhydroxyalkanoic acids (PHAs), more than 120 different hydroxyalkanoic acids have been detected as constituents of these bacterial polyesters in the last 70 years (Steinbüchel and Valentin 1995). PHAs are accumulated as insoluble inclusions in the cytoplasm when the bacteria are cultivated with a suitable carbon source in excess, and if another nutrient such as the nitrogen source limits the growth of the bacteria. PHAs produced by bacteria revealed chemical and physical properties similar to those of synthetic polymers produced by the chemical industry. These properties, in combination with their biodegradability and the possibility of producing them from cheap and renewable carbon sources, explain the interest of the chemical industry in PHAs. As suitable carbon sources for the accumulation of PHA by bacteria, renewable resources such as carbohydrates, lignite or CO_2 (Steinbüchel and Füchtenbusch 1998) or compounds derived from chemical processes (Füchtenbusch et al. 1996) may be used. Other potential carbon sources for the production of PHAs might be industrial waste products or residual compounds from biotechnological processes. Such substrates are of particular interest, since the disposal of these compounds causes extra costs due to waste management. Residual chemicals from commercial biotechnological rhamnose production may be suitable candidates.

The use of *Pseudomonas* species for overproduction of L-rhamnose via rhamnolipids (the chemical structures of some rhamnolipids are shown in Fig. 1) is described in the literature (Daniels et al. 1988; Giani et al. 1997). Rhamnolipid contents of 70–100 g/l culture solution can be achieved using inorganic salts ($NaNO_3$, trace elements) and vegetable oils such as soya or rape oil as fermentation raw materials. The microorganisms are inactivated by heat at the end of the fermentation. The culture solution containing rhamnolipids is concentrated by centrifugation. After catalytic hydrolyses, an emulsion is obtained, which comprises an aqueous phase containing L-rhamnose and the oily phase which can be used as raw materials for the production of PHAs.

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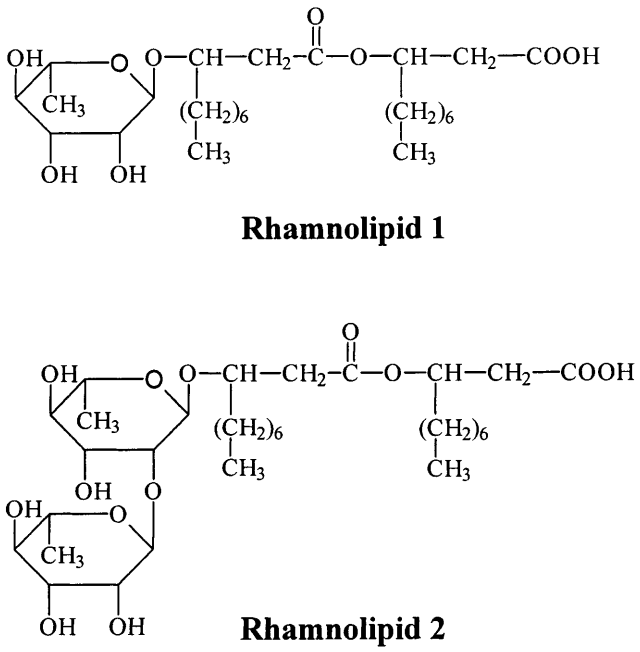

Fig. 1 Structural formulas of the rhamnolipids 1 and 2 of *Pseudomonas aeruginosa*. Data from Edwards and Hiachi 1965; Syldatk et al. 1985a, b; Gruber et al. 1993; Yamaguchi et al. 1976

The aim of this study was to identify bacteria which were able to use residual oil from rhamnose production as a carbon source for growth and accumulation of PHA. This would allow the biotechnological conversion of waste products from commercial rhamnose production into a useful and valuable product.

Materials and methods

Bacterial strains, media and growth conditions

The bacterial strains used in this study are listed in Table 1. To prepare cultures for the fermentation process, the cells were grown under aerobic conditions at 30 °C in nutrient broth (NB) or in mineral salts medium (MSM) according to the method of Schlegel et al. (1961), except for *Escherichia coli*, which was grown with residual oil as the sole carbon source. The concentration of ammonium was limited to 0.05% (mass/vol) to promote accumulation of PHA. To obtain solid media, 1.5% (mass/vol) agar was added. The submersed cultivations of *Pseudomonas oleovorans*, *P. putida*, and *Ralstonia eutropha* were done in 300-ml Erlenmeyer flasks equipped with two baffles and containing 100 ml MSM at 28 °C. The strains of *E. coli* were grown in Luria-Bertani medium at 37 °C, and all investigations on the accumulation of PHAs were done in Luria-Bertani medium supplemented with the residual oil.

Table 1 Bacterial strains used in this study. *ATCC* American Type Culture Collection, *DSMZ* Deutsche Sammlung für Mikroorganismen und Zellkulturen, *NCIMB* National Collection of Industrial and Marine Bacteria

Bacterial strain (plasmid)	Source of reference
Burkholderia cepacia	DSMZ 50181
Escherichia coli LS1298 (pBHR71)	Langenbach et al. 1997; Qi et al. 1998
Escherichia coli RS3097 (pBHR68)	Spiekermann et al. 1998
Nocardia corallina N° 724	Valentin und Dennis 1996
Pseudomonas oleovorans	ATCC 29347
Pseudomonas oleovorans (pVK101::PP1)	Timm et al. 1990
Pseudomonas putida KT2440	Worsey and Williams 1975
Ralstonia eutropha H16	DSMZ 428
Rhodococcus erythropolis	DSMZ 43060
Rhodococcus fascians pRF28	Desomer et al. 1990
Rhodococcus opacus MR22	DMSZ 3346
Rhodococcus ruber	NCIMB 40126, Anderson et al. 1990

Cultivation of *P. oleovorans* and *R. eutropha* in a 30-l stirred-tank reactor

Batch cultivations of *P. oleovorans* and *R. eutropha* were for 72 h and at 28 °C in a 30-l (UD30, Braun Melsungen, Germany) stirred-tank reactor in 20 l MSM with 2% (by vol) residual oil as sole carbon source and with 0.5% (mass/vol) ammonium chloride. The culture was inoculated with 5% (by vol) of a preculture grown in Erlenmeyer flasks with baffles, MSM and 1% (by vol) residual oil. The aeration rate was adjusted to 1 vvm and the stirring speed was varied at 200–300 rpm, depending on the oxygen demand of the culture.

Fed-batch cultivations were done as described above; however 0.5% (mass/vol) ammonium chloride and 2.0% (by vol) residual oil were fed three times during the cultivation period. As an indicator for the exhaustion of ammonia or of the carbon source, the oxygen concentration in the medium was measured. When the concentration of dissolved oxygen began to decrease, additional carbon source and ammonium chloride were fed to promote growth and accumulation of PHA. The aeration rate was adjusted to 1 vvm and the stirring speed was varied over 200–300 rpm, depending on the oxygen demand of the culture.

Analysis of accumulated PHA

For the analysis of PHA, 5–7 mg lyophilized cells or isolated polyesters were subjected to methanolysis in the presence of 15% (by vol) sulfuric acid suspended in methanol, and the resulting methyl esters of hydroxyalkanoic acids were analysed by gas chromatography according to the methods of Brandl et al. (1988) and Timm et al. (1990) employing a Perkin & Elmer model 8420 gas chromatograph equipped with a flame-ionization detector and a Permaphase PEG 25 Mx-capillary column (Perkin-Elmer, Überlingen, Germany).

Quantitative and qualitative analysis of carbon source

To analyse the composition of the complex residual oil and the consumption of individual compounds of the carbon source during the cultivation process, samples of the cultivation broth were taken and mixed with the same volume of chloroform. The organic phase was subjected to methanolysis as described above. The organic constituents of the residual oil were analysed by coupled gas chromatography/mass spectrometry on a Varian GC 3400 gas chromatograph with a SE 25 capillary column (length 25 m) coupled to a Finnigan MAT 8320 with data system SS 300.

Harvesting of the cells and PHA isolation

The cells were harvested by continuous flow centrifugation employing a CEPA Z41 centrifuge (Carl Padberg Centrifuges, Lahr/Schwarzwald, Germany). The harvested cells were washed twice with water to remove the carbon source and other media components. PHAs were extracted from lyophilized cells by chloroform in a soxhlet apparatus and subsequently precipitated in 10 vol ethanol. The precipitated PHAs were separated from the solvent by filtration, and the polymer was dried under a constant stream of

air. The precipitation was repeated three times in order to obtain highly purified PHA samples.

Chemicals

All chemicals used in this study were of analytical grade from Merck (Darmstadt, Germany). Residual oil from rhamnose production was kindly provided by Südzucker AG (Mannheim, Germany) and Aventis (Frankfurt am Main, Germany).

Results

Analysis of the residual oil by coupled gas chromatography/mass spectrometry

The composition of the complex residual oil was determinated by coupled gas chromatography/mass spectrometry. 3-Hydroxydecanoic acid (3HD) (62.1 mol%) and 3-hydroxyoctanoic acid (3HO) (9.1 mol%) were identified as the major constituents of the residual oil. In addition, lower concentrations of other 3-hydroxyalkanoic acids, as well as of saturated and unsaturated fatty acids were found (Table 2). The content of alkanes was less than 1% and was not further analysed in detail.

Table 2 Gas-chromatographic analysis of residual oil constituents

Compound	Concentration (mol%)
Alkanes	
C_8 to C_{18}	Less than 1
Saturated fatty acids	
C_{11}	1.79
C_{12}	1.86
C_{14}	0.94
C_{16}	7.9
C_{18}	6.9
C_{20}	1.24
C_{22}	0.93
Unsaturated fatty acids	
$C_{16:1}$	0.90
$C_{16:2}$	0.21
$C_{18:1}$	1.70
$C_{18:2}$	0.31
3-Hydroxyalkanoic acids	
3HHx	1.9
3HO	9.1
3HD	62.1
3HDD	0.9

[a] The presence of trace amounts of alkanes was confirmed by mass spectrometry

Growth experiments with selected bacteria

Screening experiments showed that several bacteria (Table 1) were able to use residual oil as the sole carbon source for growth and accumulation of PHAs on solid agar media. Staining of the colonies with sudan black suggested that these bacteria accumulated hydrophobic intracellular substances. Growth experiments showed that no growth inhibition occurred due to residual oil, even if this carbon source was provided at concentrations as high as 5% (by vol) in the medium. Therefore, obviously no toxic compounds are present in the residual oil.

Further investigations showed that *P. oleovorans* was most suitable to use the residual oil for growth and for the conversion to PHAs. In this strain, PHAs were accumulated as up to 61.7% of the cellular dry matter (CDM) with 3HD and 3HO as major constituents and 3-hydroxyhexanoic acid (3HHx) and 3-hydroxydodecanoic acid (3HDD) as minor constituents (Table 3). *P. oleovorans* harbouring the plasmid pVK101 ::PP1 with the PHA synthase operon from *R. eutropha* accumulated a blend consisting of a copolyester with a similar composition as described for the wild type and a homopolyester of 3-hydroxybutyric acid (3HB).

Cultivation experiments with *R. eutropha* H16 showed that this strain was also able to use residual oil as a carbon source for the accumulation of poly(3HB) with a high efficiency; the CDM of the accumulated polyester contributed to 38% PHA of the CDM (Table 3).

Also recombinant strains of *E. coli*, which harboured plasmids encoding for the poly(3HB) biosynthesis pathway of *R. eutropha* (pBHR68) or the PHA synthase 1 from *P. aeruginosa* (pBHR71), produced small amounts of PHAs mainly consisting of 3HO and 3HD or 3HB and 3HO, respectively (Table 3). It is worth

Table 3 Composition of polyhydroxyalkanoic acids (PHAs) accumulated by various bacteria. *n.d.* not detectable, *CDM* cell dry matter, *3HB* 3-hydroxybutyric acid, *3HHx* 3-hydroxyhexanoic acid, *3HO* 3-hydroxyoctanoic acid, *3HD* 3-hydroxydecanoic acid, *3HDD* 3-hydroxydodecanoic acid. The strains were cultivated in 100 ml mineral salts medium (MSM) containing 0.05% (mass/vol.) NH_4Cl or Luria-Bertani (for *E. coli*), respectively, for 96 h at 28 °C in Erlenmeyer flasks equipped with four baffles. The cultures were inoculated with 0.05 vol% of a NB or a Luria-Bertani (*E. coli*) preculture. The results of the polymer constituents were obtained from gas chromatography analyses of whole cells

Bacterial strain (Plasmid)	PHA content [% of CDM]	Composition of PHA [mol%]				
		3HB	3HHx	3HO	3HD	3HDD
P. oleovorans	61.7	n.d.	2.4	11.8	82.9	2.9
P. olevorans (pVK101::PP1)	49.1	6.1	16.9	12.9	61.2	2.9
P. putida KT2440	30.2	n.d.	14.2	39.3	37.7	5.8
R. eutropha H16	38.3	100.0	n.d.	n.d.	n.d.	n.d.
E. coli LS1298 (pBHR71)	4.2	n.d.	n.d.	20.9	79.1	n.d.
E. coli RS3097 (pBHR68)	6.3	97.1	n.d.	2.9	n.d.	n.d.

mentioning that the polyester accumulated by *E. coli* (pBHR68) contained small amounts of 3HO.

Measurement of the carbon source constituents over the cultivation period

The decrease of the major individual constituents of the residual oil by *P. oleovorans* was followed over a cultivation period of 72 h by analysing the fatty acids and 3-hydroxy fatty acids gas chromatographically in extracted cell-free supernatants. The results showed that *P. oleovorans* was able to use all fatty acids and 3-hydroxy alkanoic acids for growth and obviously also for PHA accumulation. Further investigations showed that the accumulation of PHA in *P. oleovorans* correlated in particular with the consumption of 3-HD as the major constituent of the residual oil. The growth period correlated with the degradation of fatty acids in the medium. During the time course of the experiment, the concentration of 3HD in the medium decreased from 30 g/l (mass/vol) at the beginning to 9.6 g/l at the end (Fig. 2). The consumption of the components of the residual oil over the cultivation period was approximately 78%. That 3HD was not completely utilized was most probably caused by the restriction of growth due to nitrogen limitation as well as by the fact that the cells already contained large amounts of the storage polyester at the final stage of the experiment.

With *R. eutropha* H16, which was investigated as a second example, similar results were obtained. The data suggested that this strain was also able to use all fatty acids present in the residual oil for growth. The accumulation of poly(3HB) coincided with the degradation of 3HD in the medium.

Isolation and characterization of the accumulated PHA

For the isolation of larger amounts of PHAs produced from residual oil and for characterization of the accumulated polyesters, batch and fed-batch cultivations with *R. eutropha* and *P. oleovorans* in a 30-l stirred-tank reactor were done. Cell densities of 5 g CDM/l or 6.3 g CDM/l in the case of batch cultivation, and of up to 15 g CDM/l or 16.4 g CDM/l in the case of fed-batch cultivation were obtained for *R. eutropha* and for *P. oleovorans*, respectively. The maximum polyester content of the CDM varied over 17.3–41.3% and depended on the strain used and on the cultivation method (Table 4). If *R. eutropha* was grown in MSM in the presence of residual oil as the sole carbon source, poly(3HB) was obtained, and 40 g of this homopolyester could be isolated from 295 g dry cells from one single batch of fed-batch cultivation experiment (Fig. 3). Under the same conditions, poly(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD) with 3HD as the main constituent was accumulated in cells of *P. oleovorans* and 35 g of this copolyester could be isolated from a fed-batch culture containing 278 g dry cells (Table 4).

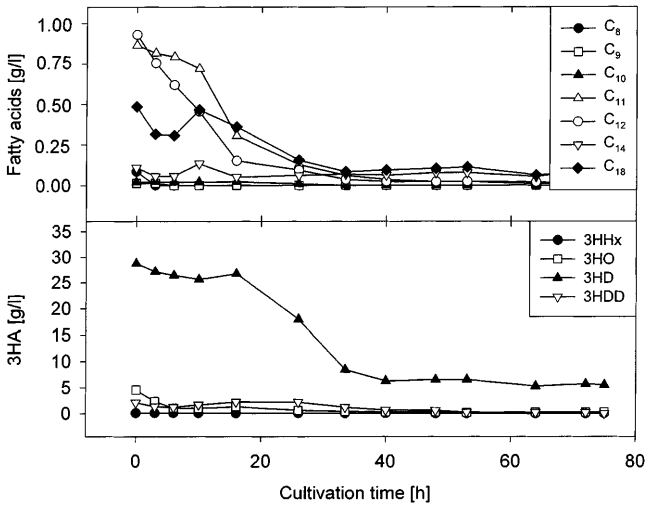

Fig. 2 Consumption of the major constituents of the residual oil during cultivation. The cultivation was done in 300-ml Erlenmeyer flasks equipped with two baffles and containing 100 ml mineral salts medium (MSM) broth at 28 °C for 72 h. The concentration of residual oil was 2.5% (by vol), and the NH_4Cl concentration was reduced to 0.05% (mass/vol) to promote polyhydroxyalkanoic acid (PHA) accumulation. The concentration of the constituents in the cultivation broth was determined by gas chromatography. C_9–C_{18}, length of the carbon chain of the fatty acid; *3HA* 3-hydroxyalkanoic acids, *3HHx* 3-hydroxyhexanoic acid, *3HO* 3-hydroxyoctanoic acid, *3HD* 3-hydroxydecanoic acid, *3HDD* 3-hydroxydodecanoic acid

Discussion

The aim of this study was to show that bacteria are able to use waste from an industrial rhamnose production process for growth and for conversion into an interesting biotechnological product. It was demonstrated that

Table 4 Composition of the polymers isolated after batch and fed-batch cultivation. *n.d.* not detectable, *CDM* cell dry matter, *3HB* 3-hydroxybutyric acid, *3HHx* 3-hydroxyhexanoic acid, *3HO* 3-hydroxyoctanoic acid, *3HD* 3-hydroxydecanoic acid, *3HDD* 3-hydroxydodecanoic acid

Bacterial strain	CDM [g/l]	Polyester content [% of CDM]	Composition of the polyester [mol%]				
			3HB	3HHx	3HO	3HD	3HDD
Batch cultivation							
P. oleovorans	5.0	17.3	n.d.	3.5	33.2	61.7	1.6
R. eutropha H16	6.3	19.7	100.0	n.d.	n.d.	n.d.	n.d.
Fed-batch cultivation							
P. oleovorans	16.4	38.9	n.d.	2.7	29.4	64.3	3.7
R. eutropha H16	15.4	41.3	100.0	n.d.	n.d.	n.d.	n.d.

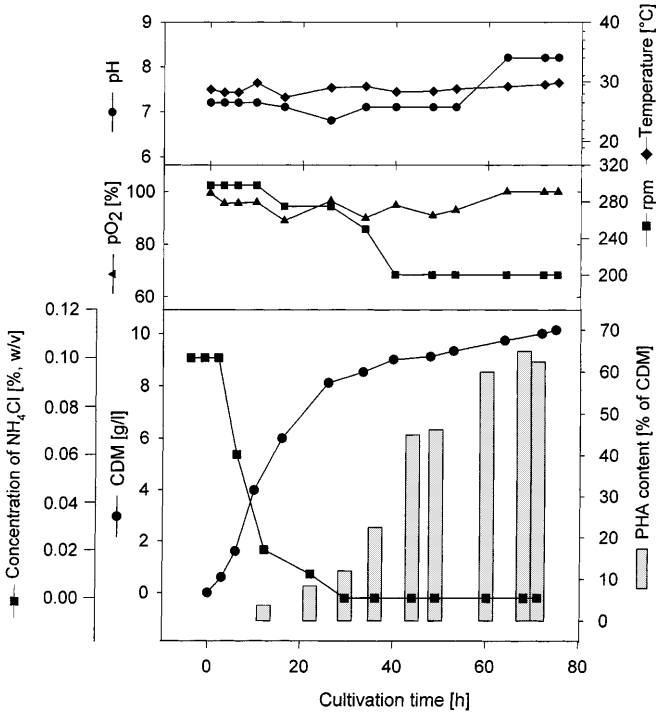

Fig. 3 Growth and polyhydroxyalkanoic acid (PHA) accumulation of *Pseudomonas oleovorans* on residual oil. The cultivation was done in a 30-l stirred-tank reactor containing 20 l mineral salt medium (MSM) at 28 °C for 72 h. The concentration of residual oil was 2.5% (by vol), and the NH_4Cl concentration was reduced to 0.05% (mass/vol) to promote PHA accumulation. The aeration rate was adjusted to 1 vvm and the stirring speed was varied at 200–300 rpm depending on the oxygen demand of the culture

the residual oil from the rhamnose production process could be converted into various PHAs.

Among the various bacteria studied, *P. oleovorans* and *R. eutropha* H16 were able to accumulate larger amounts of PHA when the residual oil was provided in excess; approximately 20–25% of the components of the residual oil were converted into PHA. Experiments in stirred-tank reactors with volumes up to 30 l outlined a practical way for the production of larger amounts of PHA from this substrate.

Using different bacterial strains, the production of PHAs containing different constituents was possible. With *R. eutropha* H16, a homopolyester of 3-HB was produced in batch or fed-batch cultivation experiments. With *P. oleovorans*, a copolyester consisting of medium chain length 3-hydroxalkanoic acids, poly(3HHx-co-3HO-*co*-3HD-*co*-3HDD), was produced from the residual oil. A recombinant strain of *P. oleovorans* harbouring plasmid pVK101::PP1, encoding the *R. eutropha* PHA operon, produced a blend of the homopolyester poly(3HB) and the copolyester poly(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD). The differences in the proportions of the concentrations of 3HO and 3HD in the analysis of whole cell lysates and the isolated polyester are due to the solubility of 3HD in the cytoplasmic membrane or in the cytoplasm of the cells (Tables 3, 4). Free 3HO and 3HD could obviously not be completely removed by washing the lyophilized cells with organic solvents. Further experiments must be done to optimize the growth of the selected bacteria in high cell density fed-batch cultivation experiments in stirred-tank reactors and to increase the yield of PHA.

That 3HD is the major constituent of the copolyesters accumulated by the two latter strains is due to the occurrence of large amounts of 3HD in the residual oil. 3HD, which is the predominant organic component of the residual oil, derives from the chemical cleavage of rhamnolipids into rhamnose and 3HD and remains in the waste after the separation of rhamnose.

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