Bacterial Production of PHA from Lactose and Cheese Whey Permeate

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Summary: Due to the large availability of agro-industry wastes containing potentially exploitable substrates, such as whey from dairy industry, a study on the bacterial conversion of lactose and whey permeate to poly(β-hydroxyalkanoate) (PHA) was undertaken. A first approach was carried out on culture collection strains. Among a number of strains tested, *Hydrogenophaga pseudoflava* DSM 1034 and *Sinorhizobium meliloti* 41 were found to grow on lactose and produce PHA. These findings suggested to investigate among a wider range of microorganisms by directly isolating new strains from soil. A number of soil bacteria were first isolated on a minimal medium containing lactose as unique carbon source and PHA-accumulating traits were then investigated. Three isolates, identified by 16S rDNA sequence analysis as *Sinorhizobium* sp., *Bacillus megaterium* and *Bacillus* sp., were selected for their efficient growth and PHA production using lactose as carbon source. The same strains were also tested for their ability to accumulate PHA by direct fermentation of whey and whey permeate. Our results suggest that production of the polymer from cheese whey or whey permeate may be possible, although further research is needed to determine whether these microorganisms have the potential for commercial production of such biodegradable polymers.

Keywords: bacteria; biopolymers; lactose; polyhydroxyalkanoates; whey

Introduction

Disposal concerns and environmental legislation are forcing plastic manufactures to consider biodegradable polymers as alternative materials. Polyhydroxyalkanoates (PHAs) are a family of biodegradable polyesters that have been proposed as potential substitutes of petrochemically produced plastics [1, 2, 3]. These biodegradable thermoplastic polymers can be produced from renewable resources by using bacteria [4, 5]. The best characterized PHA, poly((R)-3-hydroxybutyrate) [P(3HB)], is synthesized and accumulated by a variety of bacteria as carbon and energy storage compound. Interesting, PHA properties such as biodegradability, biocompatibility, piezoelectricity and non-linear optical activity gave rise to intensive research to use these polymers in high value added applications such as packaging material and as drug

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carrier systems, implant biomaterial and optical material. Several factors affect the economics of PHA production, especially the substrate cost [6, 7] and the ability to produce biodegradable polymers from inexpensive and renewable carbon sources. Since cheese whey is available in large amounts and only partially used as animal feed, its high production claims for an alternative way of disposal or for enhancing the added value of this material. Whey is a nutrient rich medium from which lactose can be generated in large volumes. Sweet whey contains approximately 5% lactose, 0,2% lactic acid and 1% proteins as well as fats, minerals and vitamins [8].

New ways to utilize this waste material are widely explored and one promising possibility could be its utilization as substrate for the microbiological production of biodegradable polymers such as PHAs. Of course, the utilization of cheap carbon sources as substrate for bacterial growth strongly requires the search for suitable microbial strains able to produce and accumulate these polymers.

In the present work we report results on the isolation of new PHA producing strains and comparison with known strains for bacterial conversion of lactose and whey permeate to PHA.

Materials and Methods

Bacterial strains

The culture collection microorganisms selected in this study were *Sinorhizobium meliloti* 41 [9], *Hydrogenophaga pseudoflava* DSM 1034 and *Paracoccus denitrificans* DSM 413 (from Deutsche Sammlung Mikroorganismen).

Isolation of bacteria from soil

Indigenous bacteria of non-sterile soil from the experimental station of the faculty of Agronomy (University of Padua) were isolated by standard methods [10] on nutrient agar, plate count agar or minimal salt medium containing lactose as carbon source.

Culture media and growth conditions

Sinorhizobium meliloti 41 was grown aerobically in YMB (K_2HPO_4 0.5 g/l, $MgSO_4 \cdot 2H_2O$ 0.2 g/l, NaCl 0.1 g/l, yeast extract, 0.4 g/l, mannitol, 10.0 g/l, pH 6.8) at 30°C. *Hydrogenophaga pseudoflava* DSM 1034 and *Paracoccus denitrificans* DSM 413 were grown aerobically in nutrient broth (meat extract 1.0 g/l, yeast extract 2.0 g/l, peptone 5.0 g/l, NaCl 5.0 g/l) at 35 and

30°C respectively. The soil isolates were grown in liquid nutrient broth or in minimal salts medium [11] containing lactose as sole carbon source. Media were solidified by the addition of 1.5% (w/v) agar. Liquid cultures were shaken at 150 rpm.

Preparation of media

To produce PHA batch fermentations were conducted in shaken flasks with aeration. The media used were a minimal salts medium (MSM) containing lactose as sole carbon source or a substrate directly prepared from cheese whey. Cheese whey permeate (CWP) was obtained by ultra filtration from an Italian dairy industry (Latterie Vicentine S.c.a.r.l.) with an initial lactose concentration of approximately 200 g/l. Whey permeate was diluted with water, adjusted to pH 7, sterilized at 110°C for 10 minutes and supplemented with $FeCl_3$ and vitamins.

Biomass and cellular protein measurement

To measure biomass, 10 ml of culture broth was centrifuged, suspended in 10 ml of distilled water, centrifuged again and the pellet was transferred to a pre-weighed aluminium dish and dried to constant mass at 80°C. Cell protein was determined by using the " Protein Quantification by Bradford Method" kit (Amresco-U.S.A.).

Analyses of PHA

The polyester was isolated from the cells by chloroform extraction. PHA samples were converted to the methyl ester of the single constitutive monomers that were quantified by gas chromatography with a silica fused capillary column AT-WAX (Alltech Italia s.r.l., Milano) and a flame ionization detector [12]. The gas carrier was helium, the injection port temperature was 250°C, the detector temperature was 270°C and the oven temperature was 150°C. The internal standard was benzoic acid, and the external standards were 3-hydroxybutyric acid (Sigma) and a P(HB-co-HV) copolymer (Biopol; Imperial Chemical Industries).

Lactose analyses

Residual lactose in culture supernatant was quantified by using a kit for the enzymatic determination of lactose (Boehringer Mannheim). Lactose was hydrolyzed to D-glucose and D-galactose in the presence of a β-galactosidase. D-galactose was oxidized by nicotinamide-adenine dinucleotide (NAD) to galactonic acid in the presence of the β-galactose dehydrogenase enzyme. The amount of NADH formed in the reaction is stechiometric with the amount of lactose and D-galactose respectively.

Results

Production of PHAs from lactose by different culture collection strains

We examined a variety of aerobic Gram-negative bacteria for their capability to use lactose as the sole carbon source for growth. *Sinorhizobium meliloti*, *Hydrogenophaga pseudoflava*, *Paracoccus denitrificans* were found to grow on mineral salts agar plates and in the same liquid medium that contained 1 g/l of lactose. Therefore, the ability of these strains to accumulate PHB in shake flasks with lactose as carbon substrate was evaluated (Table 1).

Table 1. Production of poly((R)-3-hydroxybutyrate) by the selected collection strains.

Bacterial strains	mg PHA/ mg cell proteins	Incubation time (h)
Sinorhizobium meliloti	1,02 ± 0,12	167
Hydrogenophaga pseudoflava	5,04 ± 0,45	48
Paracoccus denitrificans	2,67	48

In the case of *S. meliloti*, growth in modified YMB under nitrogen limited conditions and lactose as sole carbon source produced 14 %P(3HB) after 160 h. Additional lactose (13,0 g lactose/l) supplemented after 29 h (exponential growth phase) or 49 h (late exponential growth phase) resulted in an increase of PHA accumulation (Fig. 1a and b).

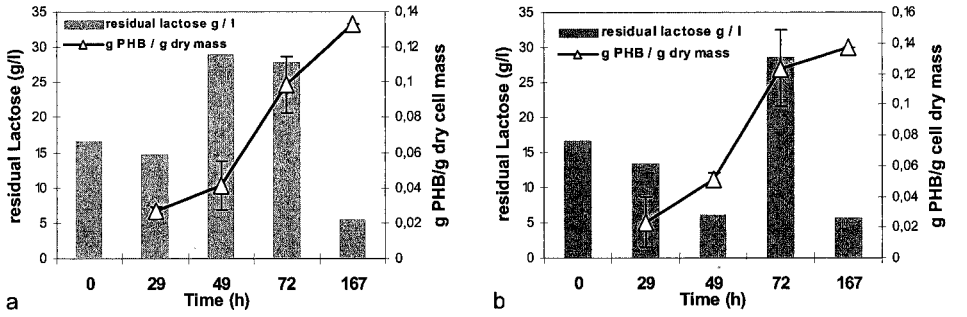

Figure 1. Poly((R)-3-hydroxybutyrate) content (g/g cell dry mass) of *Sinorhizobium meliloti* 41 and residual lactose in the medium. Modified YMB with lactose as carbon source (16,0 g/l) was used and additional 13,0 g lactose/l were added after 29 h (a) and 49 h (b) growth. Data are means of three replicates ± standard deviation.

Production of PHAs from cheese whey permeate by different culture collection strains

We examined the above selected strains for their capability to use directly modified cheese whey permeate as substrate for growth. *Sinorhizobium meliloti* and *Hydrogenophaga pseudoflava* showed a good growth rate on modified permeate plates and in the same liquid media. Table 2 shows results regarding the ability of these strains to accumulate P(3HB) in shaken flasks using this substrate.

Table 2. Accumulation of PHA by the selected collection strains in modified whey permeate.

Bacterial strains	Cell dry wt (g/l)	PHA % (wt/wt) of biomass (dry wt)	Incubation time (h)
Sinorhizobium meliloti	0,483 ± 0,038	3,5	96
Hydrogenophaga pseudoflava	0,375 ± 0,010	4,4	96

New isolation of soil bacteria able to use lactose and to accumulate PHAs

With the purpose to find new strains able to accumulate PHAs from lactose or whey, direct isolation of bacteria from soil was performed. A number of soil bacteria were first isolated on a minimal medium containing lactose as sole carbon source, and PHA-accumulating traits were then investigated. Colonies grown on lactose that showed PHA granules after Nile Red staining, were inoculated in MSM/lactose liquid media. All isolates were grown till early stationary phase and intracellular PHA was quantified by gas chromatography. Three isolates showed to

accumulate PHA in appreciable amounts and were therefore selected for further investigations. The selected strains (Isolate M, 7 and 1) were identified as *Sinorhizobium sp.*, *Bacillus sp.* and *Bacillus megaterium* by 16S rDNA sequencing (Table 3).

Table 3. Similarities between the 16S rDNA of the soil isolates used for poly((R)-3-hydroxy-butyrate) production and the 16S rDNA sequences found in

Soil Isolate	% of identical nucleotides	Microorganism with higher % similarity
Isolate M	97	*Sinorhizobium sp.*
Isolate 7	97	*Bacillus sp.*
Isolate 1	99	*Bacillus megaterium*

We examined these isolates for their capability to use modified cheese whey permeate as substrate for growth. The strains were found to grow on modified cheese whey permeate plates and in the same liquid media and to accumulate P(3HB) (Figure 2, 3 and 4).

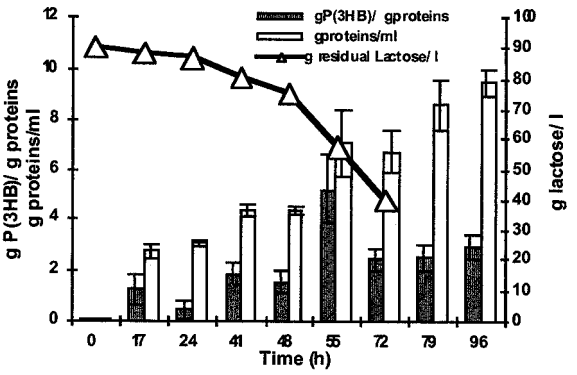

Figure 2. Poly((R)-3-hydroxybutyrate), protein content and residual lactose of a culture performed in modified whey permeate (6,15% lactose as carbon source) with the soil isolate identified as a *Bacillus megaterium* strain. Data are means of three replicates ± standard deviation.

The amounts of polymer produced in cheese whey permeate were found to be two-fold of those obtained in minimal medium using lactose as substrate (data not shown). *Sinorhizobium sp.* showed to accumulate, in such a condition, the highest amount of P(3HB).

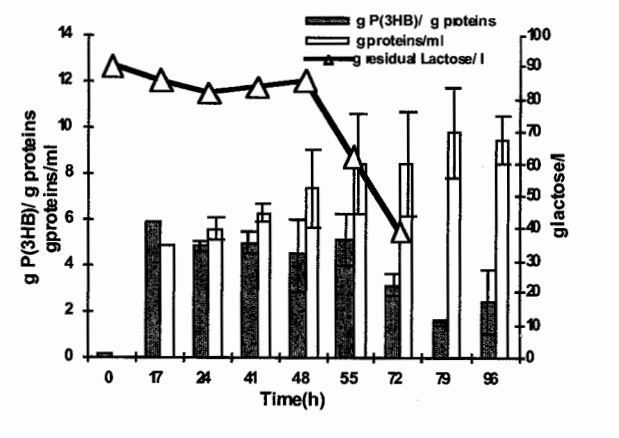

Figure 3. Poly((R)-3-hydroxybutyrate), protein content and residual lactose of a culture performed in modified whey permeate (6,15% lactose as carbon source) with the soil isolate identified as a *Bacillus* sp. strain. Data are means of three replicates ± standard deviation.

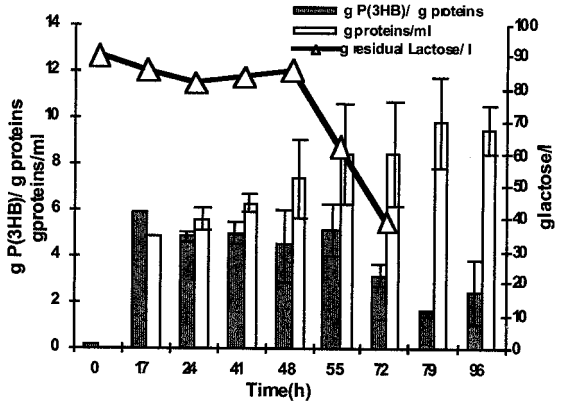

Figure 4. Poly((R)-3-hydroxybutyrate), protein content and residual lactose of a culture performed in modified whey permeate (6,15% lactose as carbon source) with the soil isolate identified as a *Sinorhizobium* sp. strain. Data are means of three replicates ± standard deviation.

Discussion

The efficient production of polyesters highly depends on the cost of the substrate used. Flask experiments showed that some bacterial strains can use lactose as carbon and energy source accumulating the excess carbon as polyhydroxyalkanoates. The same strains were shown to use modified cheese whey permeate to produce PHB. *Sinorhizobium meliloti* and other *Sinorhizobium sp.* seem to be good candidates to accumulate the polymer using lactose or cheese whey permeate. The problem with these strains regards the utilization of the carbon source in the accumulation of other polymers like exopolysaccharides (EPS). Since an efficient control of the carbon/energy flux is one of the requisites for economic polymer production, the possibility to shift this flux towards the accumulation of P(3HB) instead of EPS will bring new insight into polymer production. Such an approach could be performed by studying the regulation of both pathways and their interconnection [13].

It was reported that *Hydrogenophaga pseudoflava* DSM 1034 can accumulate not only P(3HB), but also copolymers with 4HB and/or 3HV monomers [14]. This strain was found here to produce P(3HB) from lactose confirming previous results [14] and was also found to synthesize the polymer during growth in modified cheese whey permeate. Therefore, we propose that *Hydrogenophaga pseudoflava* DSM 1034 is a good candidate for production of PHAs.

The reported results suggest that production of PHAs from cheese whey or whey permeate may be possible, although further research is needed in order (a) to look for further new strains by direct isolation (b) to determine whether the selected microorganisms have the potential for commercial production of biodegradable polyesters and (c) to improve the transformation efficiency of the carbon source.

Acknowledgment

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