Bioresource Technology 101 (2010) 7902–7907

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Polyhydroxyalkanoates production by engineered *Cupriavidus necator* from waste material containing lactose

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ARTICLE INFO

Article history:
Received 14 January 2010
Received in revised form 6 May 2010
Accepted 10 May 2010

Keywords:
Polyhydroxyalkanoates
Cupriavidus necator
Depolymerase gene disruption
Lactose
Cheese whey

ABSTRACT

Cupriavidus necator DSM 545 is a well-known polyhydroxyalkanoates (PHAs) producer, but unable to grow on lactose. The aim of this study was to construct a recombinant strain of *C. necator* that can use lactose-containing waste material such as cheese whey, to produce PHAs. One of the intracellular PHA depolymerases (*phaZ1*) of *C. necator* was chosen to insert the *lacZ, lacI* and *lacO* genes of *Escherichia coli*. This would have the effect to allow polymer production on lactose and, at the same time, to remove part of the PHA intracellular degradation system. Disruption of *phaZ1* was achieved by gene replacement after isolating a fragment of this gene and interrupting it with a cartridge containing the *lac* genes and a synthetic promoter. Growth and polymer production studies of the genetically modified (GM) strain mRePT in lactose, whey permeate and hydrolyzed whey permeate as carbon sources, were performed. Lower PHA degradation and higher yields were obtained compared to the wild-type strain. Inactivation of the putative depolymerase gene *phaZ3* on mRePT recombinant strain was also reported.

1. Introduction

Polyhydroxyalkanoates (PHAs) are accumulated in a number of bacteria as intracellular carbon and energy storage materials under limited nutrient conditions. PHAs have generated significant commercial interest due to their application as biodegradable thermoplastics and elastomers that can be produced using renewable resources (Khanna and Srivastava, 2005). The cost of the carbon source for growth of the producing strain and the PHA purification methods are factors that mostly influence the price of these polyesters (Choi and Lee, 1997). The use of cheap carbon sources, specially generated from industrial or agricultural by-products can provide a way to reduce the price (Koller et al., 2005; Solaiman et al., 2006). Cheese whey, a by-product of the dairy industry, is available in large amounts as co-product stream resulting from cheese manufacture and is rich in lactose, lipids and soluble proteins. Whey is not only a cheap raw material, but is a surplus product in the EU that causes disposal problems. From the feed stock milk, skimmed whey is generated after the casein is precipitated and the major part of lipids are removed. After removing 80% of water from the skimmed whey, the concentrate whey is ultra filtrated to generate whey permeate (the lactose fraction) and whey retentate (the protein fraction with considerable lactose residues). Certain components of the retentate such as lactoalbumin and lactoferrin fractions are of pharmaceutical interest, instead whey permeate (which contains 81% of the original lactose in milk) is a potential carbon source for the biotechnological production of PHAs. There are some reports describing the use of cheese whey as carbon source to produce different polyester members of the PHA family (Povolo and Casella, 2003; Koller et al., 2005), and on the production of PHA using recombinant *Escherichia coli* strains (Park et al., 2002; Nikel et al., 2005). *Hydrogenophaga pseudoflava* DSM1034 (Koller et al., 2007) and *Methylobacterium* sp. ZP24 (Yellore and Desai, 1998) were reported as wild-type microorganisms able to synthesize PHAs directly from lactose. Recently, Pantazaki et al. (2009) described the production of a heteropolymer from whey by *Thermus thermophilus* HB8.

One of the main shortages in the production of PHA has been the intracellular polymer degradation caused by its endogenous PHA depolymerases. Intracellular PHAs can be degraded when the bacteria require carbon and, the monomers energy released can be used to allow bacterial growth (Steinbüchel and Hein, 2001). The first sequence of an intracellular depolymerase (*phaZ1*) of *Ralstonia eutropha* was reported by Saegusa et al. (2001) and it was surprising that the protein sequence had no similarity to the extracellular depolymerases. The function of PhaZ1 *in vivo* was reported (Saegusa et al., 2001) and mutants in *phaZ1* of *R. eutropha* were generated. From these studies it was concluded that different intracellular PHA depolymerases must be present in *R. eutropha*. York et al. (2003) reported the identification of candidates for intracellular depolymerase genes from *R. eutropha* (*phaZ2* and *phaZ3*) and their characterization *in vivo*. The function of PhaZ2 and PhaZ3 was examined by generating *R. eutropha* H16 deletion

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doi:10.1016/j.biortech.2010.05.029

strains (York et al., 2003). When these modified strains were grown in rich medium, PhaZ1 was sufficient to account for intracellular PHA degradation. When cells that had accumulated ~80% (cell dry weight) PHA were subjected to PHA utilization conditions, PhaZ1 and PhaZ2 were sufficient to account for PHA degradation. PhaZ2 was thus suggested to be an intracellular depolymerase. The role of PhaZ3 remains to be established. During the last years, genome analysis of *R. eutropha* H16 and other related strains and the characterization of several depolymerases and oligomer hydrolases have revealed the existence of a complex poly-3-hydroxybutyrate (PHB) hydrolytic system in this microorganism (Pötter et al., 2004). Characterization of the regulatory system of these isoenzymes expression is just at the preliminary stages (Kessler and Witholt, 2001). So far, the only example of medium-chain-length PHA intracellular depolymerase was described in the genus *Pseudomonas* (de Eugenio et al., 2007; Sandoval et al., 2005). Recently the construction and characterization of a defined *phaZ* knockout mutant of *Pseudomonas putida* KT2442 was reported in order to develop an industrially useful strain for medium-chain-length PHA production (Cai et al., 2009).

Cupriavidus necator (formerly *Wautersia eutropha*) is a well-known PHA producer that accumulates PHB up to 80% of the cell dry mass from various carbon sources (Reinecke and Steinbüchel, 2009). However, *C. necator* cannot grow on the disaccharide lactose which is the main sugar contained in whey. To make use of this carbon source for PHB production we constructed a recombinant strain by inserting the *E. coli lac* operon in the chromosome of *C. necator*. On a previous study a mutant was constructed (Pries et al., 1990), but inserting these genes not in a specific place in the chromosome. In order to obtain a new mutant able to grow on lactose, and at the same time less able to degrade the intracellular PHB accumulated during growth, the choice of the target gene relapsed into *phaZ1*, one of the three PHA intracellular depolymerases of *C. necator* (Saegusa et al., 2001).

In this paper, we report the construction of *C. necator* recombinant strains and data related to their growth and polyester accumulation in lactose, hydrolyzed whey and directly in whey permeate. The final aim was to develop an industrially useful strain able to use cheap carbon sources and with an improved PHA production by removing the polymer degradation mechanism.

2. Methods

2.1. Bacterial strains, plasmids and growth conditions

The strains of *C. necator* and *E. coli* as well as plasmids used in this study are listed in Table 1. *C. necator* strains were cultured at 30 °C in minimal salt medium DSMZ81 (Deutsche Sammlung Mikroorganismen, http://www.dsmz.de/, Germany) with glucose as carbon source. *E. coli* strains were cultured at 37 °C in Luria–Bertani medium (LB) containing (g L^{-1}): yeast extract 5, tryptone 10, and NaCl 10. Antibiotics were supplemented as required at the following concentrations: for *C. necator* kanamycin 100 μg mL^{-1}, gentamicin 40 μg mL^{-1}, tetracycline 8 μg mL^{-1}; for *E. coli* tetracycline 10 μg mL^{-1}, kanamycin 50 μg mL^{-1} and ampicillin 100 μg mL^{-1}. Media were solidified by the addition of 1.5% (w/v) agar. Liquid cultures were shaken at 150 rpm.

2.2. Shake flask culture conditions for polyhydroxyalkanoates production

To produce PHA batch fermentations were performed in shake flasks with aeration. The media used were minimal salts medium DSMZ81 containing lactose as sole carbon source or a medium prepared from cheese whey permeate (see below). One-step growth process was used and 5% (v/v) of the seed culture were inoculated into a 500 mL flask containing 100 mL DSMZ81 medium supplemented with the various carbon sources. For each condition, three parallel shake flasks were arranged. Cheese whey permeate (CWP) was obtained by ultra filtration of cheese whey from an Italian dairy industry (*Latterie Vicentine* S.c.a.r.l., Italy) with an initial lactose concentration of approximately 200 g L^{-1}. The detailed compositions of whey permeate and retentate used for this study were described in a previous study (Koller et al., 2007).

Whey permeate was diluted with water, pH adjusted to 7, sterilized at 110 °C for 10 min and supplemented with 0.05 g L^{-1} $FeCl_3$ and 0.1 mg mL^{-1} vitamins before use. Whey permeate was hydrolyzed to glucose and galactose by adding MAXILACT LX 5000 (DSM Food Specialities, UK) under the conditions indicated by the manufacturer. The efficiency of lactose hydrolysis was monitored via HPLC as described in the next paragraph.

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
Cupriavidus necator DSM545	Glucose utilising strain	Schlegel and Gottschalk (1965)
Cupriavidus necator mRePT	$LacZ^+$ $LacI^+$, $LacO^+$, respective genes integrated into the chromosome inside *phaZ1*	This work
Cupriavidus necator mRePT-*gfp3*	$LacZ^+$ $LacI^+$, $LacO^+$, respective genes integrated into the chromosome inside *phaZ1*; *gfp* gene integrated inside *phaZ3*	This work
Escherichia coli JM109	*recA*, *supE*, *supF*, *lacY1*	Yanisch-Perron et al. (1985)
Escherichia coli S17-1	*E. coli* 294, *thi* RP4-2-Tc::Mu-km::Tn7 integrated into the chromosome	Simon et al. (1983)
E. coli XL1-Blue	*recA1 lac* [F′ *proAB* $lacI^q$ ZΔM15 Tn10 (Tet^r)] *thi*	Bullock et al. (1987)
Plamids		
pSUP102	Cm^r, Tc^r, harboring *mob* site	Simon et al. (1983)
pDG1	Ap^r, LacZ α-peptide	Giacomini et al. (1994)
pDRIVE	Km^r, Ap^r, LacZ α-peptide	Qiagen®, 2001
pRK2013	Km^r, Mob^+, Tra^+	Figurski and Helinski (1979)
pDRIVE-*phaZ1*	Ap^r, Km^r, a *phaZ1* fragment of *C. necator* subcloned in pDRIVE	This work
pSUP102-*phaZ1*	Tc^r, Mob^+, a *phaZ1* fragment of *C. necator* subcloned in pSUP102	This work
pSUP102-*phaZ1PT::lacZ*	Tc^r, Mob^+ pSUP102-*phaZ1*with *lacZ*, *lacI* and *lacO* genes and a synthetic promoter, into *SmaI*	This work
pRK415 *pnnrs::gfp*	*norS*, *gfp*	Yin et al. (2003)
pDrive-*phaZ3*	Ap^r, Km^r, *phaZ3*	This work
pSUP102-*phaZ3*	Tc^r, Mob^+, *phaZ3*	This work
pSUP102-*phaZ3::gfp*	Tc^r, Mob^+, *gfp*	This work
pDrive-*gfp*	Ap^r, Km^r, *gfp*	This work

2.3. Analytical procedures

For biomass measurements, 10 mL of culture was centrifuged, suspended in 10 mL of distilled water, re-centrifuged 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 Coomassie plus protein assay reagent kit from Pierce (Rockford, IL, USA) with bovine serum albumin as a standard.

PHB concentration was determined by the method previously developed (Braunegg et al., 1978): 3-hydroxyalkyl esters were quantified by gas chromatography with a silica fused capillary column AT-WAX (Alltech Italia s.r.l., Milano) and a flame ionization detector. The gas carrier was helium, the injection port temperature was 250 °C, the detector temperature 270 °C and the oven temperature 150 °C. The GC-temperature programme was: initial oven temperature 90 °C (maintained for 1 min), with increases of 5 °C min^{-1} to a final temperature of 150 °C (maintained for 6 min). The internal standard was benzoic acid, and the external standards were 3-hydroxybutyric acid (Sigma–Aldrich, Italy) and a P(3HB-co-3HV) copolymer (Biopol™; Imperial Chemical Industries, Great Britain).

Residual lactose, glucose and galactose in culture supernatants were quantified by using high pressure liquid chromatography. A Biorad model 2700 chromatographer equipped with a refractometer (Biorad model 1755) as detector, was employed. An Aminex column HPX 87C (Biorad, Italy) was used to separate sugars with an isocratic elution, carried out with 0.03% H_2SO_4. Lactose, glucose and galactose were separated at 65 °C with a carrier flux rate of 0.3 mL min^{-1} in a total time of 30 min. Standards of lactose, glucose and galactose (Sigma–Aldrich, Italy) were used for quantification.

2.4. Determination of β-galactosidase activity

A variable volume of cell suspension with an optical density at 600 nm (OD_{600nm}) between 0.8 and 1.0 was diluted with buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol; pH 7) to a final volume of 1 mL. Cell membranes were rendered permeable by a treatment with 0.1% SDS and chloroform. Reaction was started with the addition of 0.2 mL of 10 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The reaction was stopped by the addition of 0.5 mL of 1 M sodium carbonate after the solution became pale yellow. After centrifugation, absorbance at 420 nm was determined (Miller, 1972).

2.5. Transfer of DNA

Conjugation between *E. coli* and *C. necator* were performed on solidified nutrient broth medium (NB medium: meet extract 1 g L^{-1}, yeast extract 2 g L^{-1}, peptone 5 g L^{-1} and NaCl 5 g L^{-1}). After 24 h incubation at 30 °C, the cells were re-suspended in saline solution (9 g NaCl L^{-1}) and dilutions plated on selective media.

2.6. Isolation of DNA and manipulation

Plasmid and total DNA isolation, agarose gel electrophoresis and transformation of *E. coli* strains were carried out as described by Sambroock et al. (1989). All DNA-manipulating enzymes were used as described by the manufacturers.

2.7. Construction of recombinant C. necator strains

For *phaZ1* disruption two primers, SP-L and SP-R (5′-catcaagctgctcaaggatg-3′ and 5′-aagagatctacccgcagctg-3′), were designed on the sequence of *phaZ1* gene published in the Genebank that amplify a region of 918 bp (from nucleotide 302 to 1194 of the gene). The amplified fragment was cloned into the pDrive plasmid giving rise to plasmid pDRPT. A *Bam*HI–*Hind*III fragment containing the amplified *phaZ1* fragment was isolated from plasmid pDRPT and cloned between the *Bam*HI–*Hind*III sites of the suicide vector pSUP102 (Cm^r, Tc^r) (Simon et al., 1983) resulting in plasmid pSUP102-*phaZ1*. The amplified fragment of *phaZ1* gene inside pSUP102-*phaZ1* was interrupted by a cartridge containing *lacZ*, *lacI* and *lacO* genes and a synthetic promoter (Giacomini et al., 1994), based on the consensus of a number of naturally occurring promoters, introduced into the unique *Sma*I site present in the construct pSUP102-*phaZ1*. The resulting plasmid was named pSUP102-*phaZ1PT::lacZ*. The recombinant plasmid pSUP102-*phaZ1PT::lacZ* was transferred from the donor strain *E. coli* JM109 to the recipient *C. necator* by using the helper plasmid pRK2013 (Figurski and Helinski, 1979) through a three-parental mating. Conjugation between *E. coli* strains and *C. necator* was performed on solidified NB medium. After 24 h incubation at 30 °C, the cells were re-suspended in saline solution (9 g NaCl L^{-1}) and dilutions plated on selective media DSMZ81 containing lactose as the only carbon source. Colonies of *C. necator* able to grow in lactose and sensitive to Cm were selected. The resulted strain was named *C. necator* mRePT.

For *phaZ3* disruption two primers, PT-3L and PT-3R (5′-atgatcgaggcaggttatcg-3′ and 5′-tcgatggtcagcaatgaaac-3′) were designed on the sequence of *phaZ3* gene published in the Genebank that amplify a region of 974 bp (from nucleotide 146 to 1120 of the gene). The amplified fragment was cloned into the pDrive plasmid giving rise to plasmid pDrive-*phaZ3*. An *Eco*RI fragment containing the amplified *phaZ3* fragment was isolated from plasmid pDrive-*phaZ3* and cloned between the *Eco*RI site of the suicide plasmid pSUP102 (Cm^r, Tc^r) (Simon et al., 1983) resulting in plasmid pSUP102-*phaZ3*. The amplified fragment of *phaZ3* gene inside pSUP102-*phaZ3* was interrupted by a cartridge containing *gfp* gene, introduced into the unique *Stu*I site present in the construct pSUP102-*phaZ3*, resulting in the plasmid named pSUP102-*phaZ3::gfp*. The *gfp* gene used (714 pb) derives from plasmid pRK415*pnnrs::gfp* (Yin et al., 2003). The recombinant plasmid pSUP102-*phaZ3::gfp* was transferred from the donor strain *E. coli* JM109 to the recipient *C. necator* by using the helper plasmid pRK2013 (Figurski and Helinski, 1979) through a three-parental mating. As *C. necator* recipient strain the modified mRePT strain was used. Conjugation between *E. coli* strains and *C. necator* was performed on solidified NB medium. After 24 h incubation at 30 °C, the cells were re-suspended in saline solution (9 g NaCl L^{-1}) and dilutions plated on selective media DSMZ81 containing lactose as the only carbon source. Fluorescent colonies of *C. necator* mRePT able to grow in lactose were selected. The resulted strain was named *C. necator* mRePT-*gfp3*.

3. Results and discussion

3.1. Disruption of phaZ1 gene in C. necator with a cartridge containing a synthetic promoter and the lacZ, lacI and lacO genes of E. coli

In order to obtain a bacterial strain able to produce polyhydroxyalkanoates (PHA) directly from lactose, two different strategies were initially taken into account. The first hypothesis was to select strains able to utilize lactose and trying to make them producing PHA. The second was to look for strong PHA producing strains and trying to make them able to use lactose as a substrate. This latter strategy was preferred considering the complexity of the gene cluster to be mobilized and expressed for PHA production. Therefore, *C. necator* (formerly *W. eutropha*) was selected for this work because of its ability to accumulate high amounts of polyhydroxyalkanoates, although it cannot utilize lactose as a carbon source. In order to make *C. necator* able to use lactose for PHA production

we constructed a recombinant strain by transferring to these bacteria the *E. coli lacZ*, *lacI* and *lacO* genes. With the aim to obtain a suitable stability the genes were introduced directly into the chromosome by knocking out a gene already present. The chosen target gene was *phaZ1*, one of the three PHA intracellular depolymerases of *C. necator*. Therefore, the objective was clearly to obtain a new mutant able to grow on lactose and at the same time less able to degrade the intracellular PHA accumulated during its growth. The recombinant strain was obtained by isolating a fragment of the *phaZ1*, by interrupting this gene with a cartridge (containing the *lacZ*, *lacI* and *lacO* genes and a synthetic promoter, constructed on the basis of the consensus of a number of naturally occurring promoters) and transferring the disrupted gene into *C. necator* by gene replacement as described in Section 2. The sequence of *phaZ1* was searched in data bank and suitable primers were designed to amplify a fragment of the gene (as indicated in Section 2). After conjugation, were a double crossing-over occurred and the *phaZ1* gene was replaced, some colonies of *C. necator* became Cm-sensitive and able to degrade lactose. The 16S rDNA sequence of these isolated strains were analyzed after PCR amplification to check their identity as *C. necator*. All colonies resulted to be the same strain and BLAST analysis of the amplified sequence indicated that they were effectively *C. necator* (data not shown). The selected strain was named *C. necator* mRePT. To test if the cartridge was inserted in the chromosome, PCR amplification of the *lacZ* gene was performed from the total DNA extracted from strain mRePT. A clear band revealed the presence of the gene in strain mRePT and not in the wild-type strain (data not shown).

3.2. Disruption of phaZ3 gene with gfp

The inactivation in *C. necator* of a second intracellular depolymerase gene could presumably further prevent the degradation of the accumulated PHB. With this aim *phaZ3* gene (a possible depolymerase) was disrupted by the insertion of the *gfp* gene in *C. necator* mRePT, the modified strain obtained and described above. The *gfp* gene was inserted inside *phaZ3* following the strategy already described in Section 2. The selected strain was named *C. necator* mRePT-*gfp3* and its 16S rDNA sequence was analyzed after PCR amplification to check its identity. BLAST analysis of the amplified sequence indicated that the strain was effectively *C. necator*. To test if the *gfp* gene was inserted in the chromosome PCR amplification of the gene was performed from the total DNA extracted from strain mRePT-*gfp3*. A clear band indicated the presence of the gene in strain mRePT-*gfp3* and not in the parental strain (data not shown).

3.3. Effect of lacZ, lacI and lacO genes introduction and phaZ1 and phaZ3 disruption in C. necator

Growth of recombinant *C. necator* mRePT was analyzed on DSMZ81 medium with lactose (20 g L^{-1}) as the sole carbon source. The strain could use lactose as carbon source, while the wild-type strain DSM 545 did not grow in the same media (Fig. 1). β-Galactosidase activity was also determined in strain mRePT when grown in lactose, confirming that *C. necator* acquired the possibility to degrade lactose and use it for growing (270 ± 45 β-galactosidase units min^{-1} mL^{-1}, while no activity was revealed for the wild-type strain).

With the aim to verify if the inactivation of *phaZ3* and *phaZ1* could give an advantage to *C. necator*, both modified and wild-type strains were first incubated in a carbon rich media with low nitrogen source to promote the accumulation of the polyhydroxybutyrate, and straight after their growth and possible accumulation the cultures were transferred to DSMZ81 medium without any carbon source. PHB content was then followed during a period of 76 h

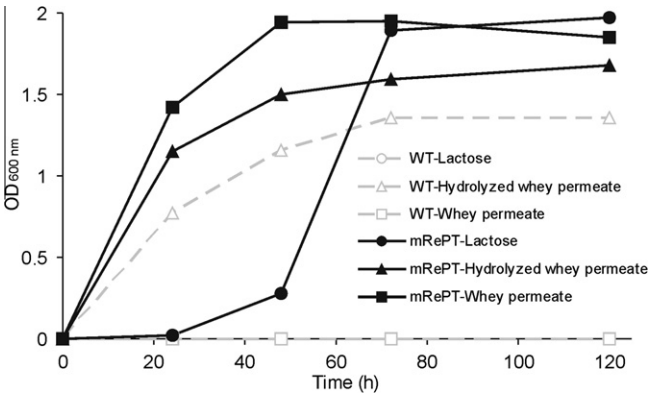

Fig. 1. Growth of *Cupriavidus necator* and its GM strain mRePT in DSMZ81 medium with lactose as sole carbon source (○ and ●), hydrolyzed whey permeate (△ and ▲) and not-hydrolyzed whey permeate (□ and ■). Dotted lines are referred to the wild-type strain and continuous lines to mRePT mutant. Data are the mean of three independent experiments. Sample standard deviations were within the size of symbols and thus were not shown in graph.

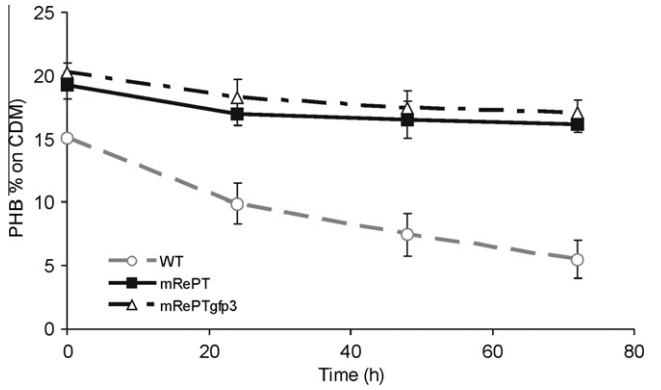

Fig. 2. Profile of polyhydroxybutyrate degradation by *Cupriavidus necator* and its GM strains mRePT and mRePT-*gfp3* in modified DSMZ81 medium without carbon source. PHB content is expressed as % of total cell dry mass (CDM), data are the mean of three independent experiments and the bars represent ± SD.

(Fig. 2). Results indicated that the inactivation of *phaZ1* gives a clear advantage in terms of reduced degradation of PHB as compared to the wild-type strain, but this benefit was not further emphasized when in the GM strain mRePT (*phaZ1*-minus) the other putative intracellular depolymerase *phaZ3* was knocked out by the insertion of the *gfp* gene (Fig. 2).

3.4. Growth of recombinant C. necator strain in lactose and modified cheese whey

The growth profiles of *C. necator* mRePT and its parental strain were analyzed in DSMZ81 medium with lactose (20 g L^{-1}) as the only carbon source and also directly in whey permeate. As shown above by Fig. 1 *C. necator* mRePT became able to grow on lactose. The performance of the strains in terms of sugar consumption in the presence of glucose plus galactose as carbon sources (Fig. 3a) and in hydrolyzed whey permeate (Fig. 3b) was also tested and compared to that of the wild-type strain. Both strains did not use galactose from the culture media although Fig. 1 indicated that the mutant strain mRePT was able to grow well with not-hydrolyzed whey permeate. However, the efficiency of the mutant strain in terms of sugar consumption in such not-hydrolyzed substrate indicated that *C. necator* mRePT can use lactose from the substrate, not releasing neither glucose nor galactose into the media (Fig. 3c). This clearly showed that the strain could effectively metabolize all

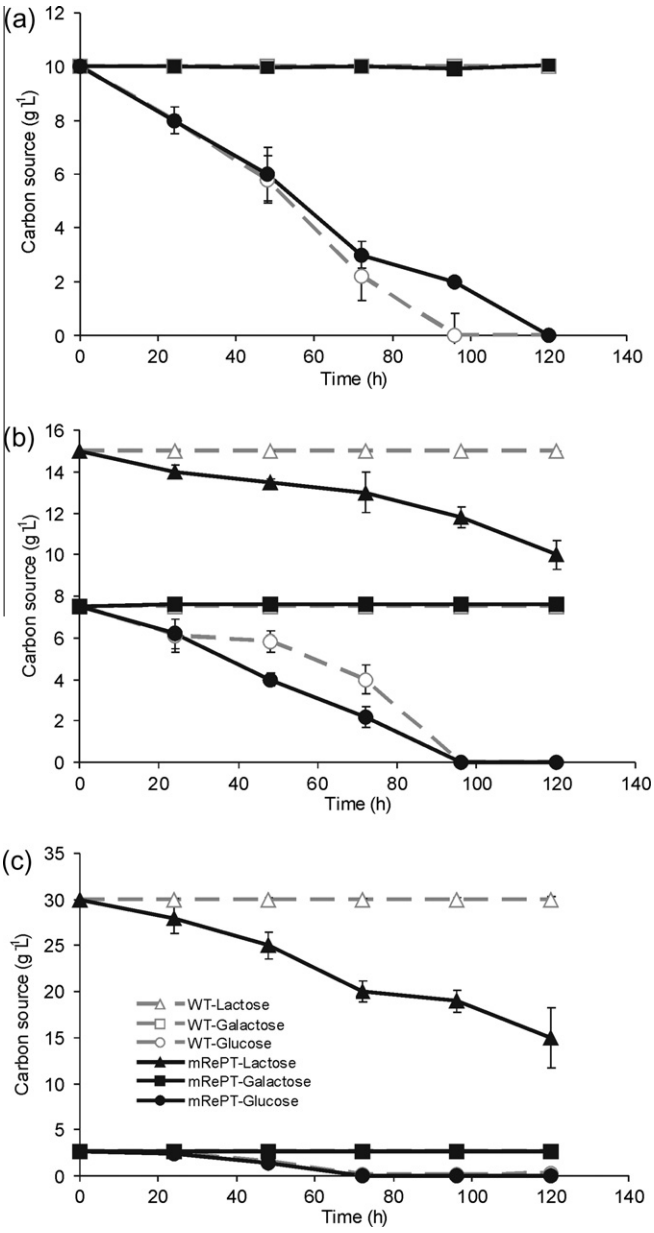

Fig. 3. Carbon source consumption by *Cupriavidus necator* and the GM strain mRePT in DSMZ 81 medium: (a) glucose and galactose; (b) hydrolyzed whey permeate diluted 10-fold; (c) not-hydrolyzed whey permeate diluted 10-fold. Dotted lines are referred to the wild-type strain and continuous lines to mRePT mutant. Symbols represent lactose (△ and ▲), galactose (□ and ■) and glucose (○ and ●). Data are the mean of three independent experiments and the bars represent ± SD.

these sugars, including galactose, once present inside the cell. Moreover, the mutant strain *C. necator* mRePT, able to degrade lactose, seems to use lactose much better when grown in the complex carbon source whey permeate rather than in a minimal medium containing lactose, indicating that some nutrients present in whey permeate can support or stimulate its growth (Fig. 1). The open question to be answered in the future is if the galactose, once in the cell, is used to accumulate the polymer or shifted to other metabolic pathways.

3.5. Production of PHB by recombinant C. necator on lactose, hydrolyzed whey permeate and whey permeate

The profiles of PHB accumulation were analyzed in *C. necator* mRePT and its parental strain grown in DSMZ81 with lactose

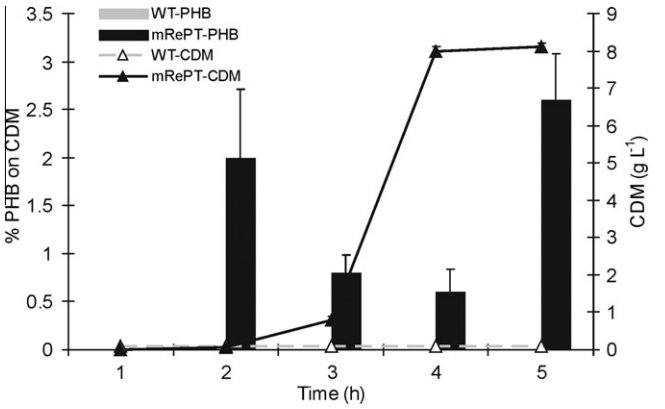

Fig. 4. Growth and PHB production by *Cupriavidus necator* and the GM strain mRePT in DSMZ81 medium with lactose as sole carbon source. Symbols (△) and (▲) represent total cell dry mass (CDM) expressed as g L^{-1} (dotted lines are referred to the wild-type strain and continuous lines to mRePT mutant); bars indicate the PHB content as % of CDM for wild-type strain (grey bars) and mutant strain mRePT (black bars). Data are the mean of three independent experiments and the bars represent ± SD.

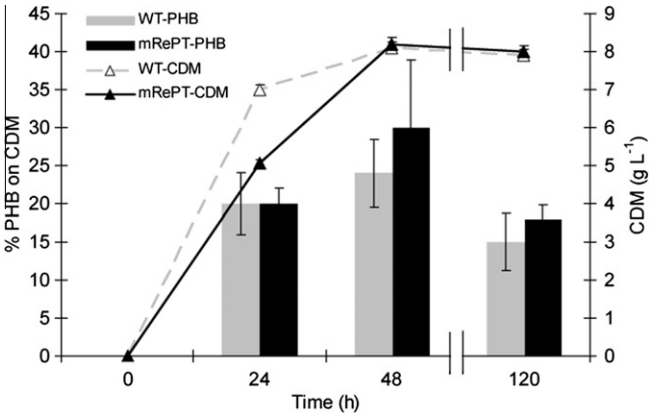

Fig. 5. Growth and PHB production by *Cupriavidus necator* and its GM strain mRePT in modified DSMZ81 medium with enzymatically hydrolyzed whey permeate as sole carbon source. Symbols (△) and (▲) represent total cell dry mass (CDM) expressed as g L^{-1} (dotted lines are referred to the wild-type strain and continuous lines to mRePT mutant); bars indicate the PHB content as % of CDM for wild-type strain (grey bars) and mutant strain mRePT (black bars). Data are the mean of three independent experiments and the bars represent ± SD.

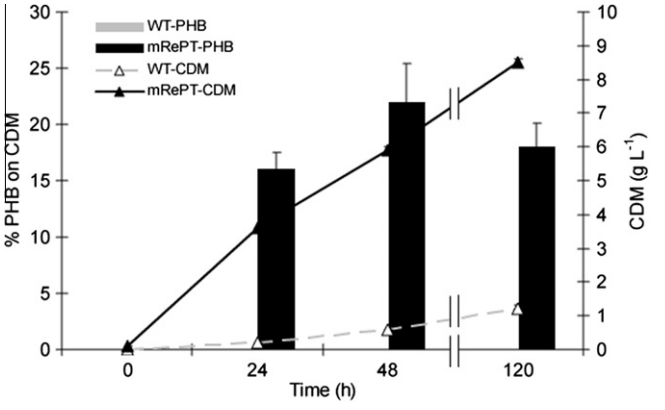

Fig. 6. Growth and PHB production by *Cupriavidus necator* and its GM strain mRePT in modified DSMZ81 medium with not-hydrolyzed whey permeate as sole carbon source. Symbols (△) and (▲) represent total cell dry mass (CDM) expressed as g L^{-1} (dotted lines are referred to the wild-type strain and continuous lines to mRePT mutant); bars indicate the PHB content as % of CDM for wild-type strain (grey bars) and mutant strain mRePT (black bars). Data are the mean of three independent experiments and the bars represent ± SD.

($20\ g\ L^{-1}$) as the sole carbon source. The medium was supplemented with high carbon and low nitrogen source, conditions known to support polyhydroxybutyrate accumulation. Although in low amounts, probably due to sub-optimal conditions obtainable by flasks cultivation, the recombinant strain mRePT produced PHB (Fig. 4). The same mutant strain mRePT could also produce PHB (30% of cell biomass after 48 h) when grown in hydrolyzed whey permeate, that is composed mainly of glucose and galactose (Fig. 5). When using not-hydrolyzed whey permeate the profiles of growth and polyester accumulation were higher (22% after 48 h growth) than those obtained in lactose (Fig. 6 compared to Fig. 4). The wild-type strain did not grow in this media (Fig. 6), confirming that only the modified strain carrying *lacZ* gene can use lactose.

3.6. Comparison of growth and polyhydroxybutyrate production of *C. necator* strains inactivated in *phaZ1* and *phaZ1–phaZ3* in cheese whey permeate

Growth and PHB production of the double mutant (strain mRePT-*gfp3*) inactivated in *phaZ1* and *phaZ3* were determined in DSMZ81 medium with diluted whey permeate as carbon source and compared to the performance of the single mutant strain mRePT. Almost no significant differences were observed between the two GM strains (data not shown). These results and the lack of differences observed between the two recombinant strains in a culture medium without any carbon source (see Fig. 2) suggest that *phaZ3* does not have a relevant role as PHA depolymerase.

4. Conclusions

This work indicates the possibility to produce, by "one-step process", a useful and completely biodegradable bioplastic such as PHA from whey permeate, a polluting by-product of dairy industry. A suitable strategy is to start from excellent PHA producing strains and try to confer them *lacZ* gene. The recombinant strains here obtained were able to produce the polymer directly from lactose and/or whey permeate. The insertion of the *lac* operon within *phaZ* gene may reduce the amount of PHA depolymerised by the cell, thus improving the final polymer yield.

Acknowledgements

We thank LATTERIE VICENTINE SCARL for providing whey permeate. We are grateful to Prof. A. Lante and S. Monni for carbohydrates measurements, F. Fontana for assistance in GC measurements. This work was supported by UNIPD 60% and partially by UE-WHEYPOL project. S.P. was supported by the special Grant "Assegno di Ricerca 2007-2008-2009" (University of Padova, Italy).

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